Diversity of microcystin-producing genotypes in Brazilian strains of *Microcystis* (Cyanobacteria)

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(With 1 figure)

Abstract

Microcystis Kützing ex Lemmermann is among the genera of cyanobacteria often associated to toxic blooms with the release of microcystins. A gene cluster codes for microcystin synthetases, which are involved in the biosynthesis of this toxin. The aim of the present study was to investigate the genetic diversity of the *mcy*B gene, specifically the B1 module, in Brazilian strains of *Microcystis* spp. and its microcystin variants. Broad genetic diversity was revealed in this region. From the phylogenetic analysis, three clusters were obtained that were not related to the geographic origin or morphospecies of the strains, nor with the variant of the microcystin produced. A group of strains that did not produce microcystins was found, despite the presence of the *mcy*B1 fragment. Eight microcystin isoforms were detected: MC-LR, [D-Asp^3]-MC-LR, [Asp^3]-MC-LR, MC-RR, [Dha^7]-MC-LR, MC-LF, MC-LW and [D-Asp^3, EtAdda^5]-MC-LH, the latter of which is described for the first time in Brazil. Moreover, five other variants were not identified and indicate being new.

*Keywords: mcy*B, microcystin, toxin, variants.

Diversidade de genótipos produtores de microcistinas em linhagens brasileiras de *Microcystis* (Cyanobacteria)

Resumo

Microcystis Kützing ex Lemmermann é dos gêneros de cianobactérias, frequentemente relacionados às florações tóxicas com a liberação de microcistinas. Um agrupamento de genes codifica as sintetases de microscistinas, as quais estão envolvidas na biossíntese desta toxina. O objetivo deste estudo foi investigar a diversidade genética do gene *mcy*B, especificamente o módulo B1, de linhagens brasileiras de *Microcystis* spp. e suas variantes de microcistinas. Uma ampla diversidade genética foi revelada nesta região. A partir da análise filogenética, três agrupamentos foram obtidos, os quais não se relacionaram com a origem geográfica ou com a morfoespécie das linhagens e nem tão pouco com a variante de microcistina produzida. Foi encontrado um grupo de linhagens que não produziu microcistinas, apesar da presença do fragmento *mcy*B1. Oito variantes de microcistinas foram detectadas: MC-LR, [D-Asp^3]-MC-LR, [Asp^3]-MC-LR, MC-RR, [Dha^7]-MC-LR, MC-LF e MC-LW e [D-Asp^3, EtAdda^5]-MC-LH, sendo esta última descrita pela primeira vez no Brasil. Além destas, cinco outras variantes não foram identificadas com indicativos de serem novas.

*Palavras-chave: mcy*B, microcistina, toxina, variante.

1. Introduction

Anthropogenic sources of pollution cause eutrophication of aquatic ecosystems, compromising the quality of water used for human consumption. The events most frequently associated with nutrient inputs are blooms of cyanobacteria, which produce toxins. *Microcystis* Kützing *ex* Lemmermann is a genus of cyanobacteria that is frequently related to toxic blooms. The species of this genus are prevalent and can produce microcystins, which are cyclic heptapeptides hepatotoxins that are highly toxic to mammals (Nishiwaki-Matsushima et al., 1992; Falconer and Humpage, 1996; Dinga et al., 1999).

At least 70 microcystin variants have been described (Babica et al., 2006). The degradation and removal of microcystins is difficult in aquatic ecosystems due to the solubility of the molecules. Thus, microcystins are a serious public health and environmental problem. High concentrations of cyanotoxins in the water supply have been responsible for the intoxication of humans and animals. The most severe case took place in the city of Caruaru, Brazil in 1996, when 76 patients in a dialysis unit died due to direct exposure to high concentrations of microcystins (Jochimsen et al., 1998).

The enzyme complex responsible for microcystin biosynthesis is encoded by the microcystin synthetase (*mcy*) gene cluster. Nishizawa et al. (1999, 2000) and Tillett et al. (2000) have sequenced this cluster in *Microcystis*, thereby offering new perspectives for the molecular study of non-ribosomal peptide biosynthesis, as well as the evolution of microcystin genotypes and factors that affect the production of toxins. These *mcy* gene clusters contain genes coding for non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS), hybrid NRPS-PKS enzymes and other enzymes. Cyanobacteria carrying the genes involved in microcystin biosynthesis are potential producers of this toxin (Ouellette and Wilhelm, 2003).

The first adenylation domain of *mcy*B in *M. aeruginosa* is thought to activate one of the various amino acids in the microcystin molecule in the *x* position (Tillett et al., 2000). Although some microcystin-producing strains are able to incorporate different L-amino acids in the *x* and *z* positions of the microcystin structure (thereby producing different microcystin variants), this process is not completely understood and further data from biochemical investigations are needed. According to Mikalsen et al. (2003), the relaxation of the adenylation domain and gene variants are responsible for microcystin variants.

The *mcy*B gene has been widely used in the investigation of the genetic diversity of genotypes in natural populations or strains isolated in the laboratory (Pan et al., 2002; Bittencourt-Oliveira, 2003; Kurmayer and Kutzenberger, 2003) and the diversity of microcystin genes (Kurmayer et al., 2002).

The aim of the present study was to investigate the genetic diversity in the adenylation domain of *mcy*B1 in Brazilian strains of *Microcystis* spp. and the microcystin variants produced.

2. Material and Methods

2.1. Strains and growth conditions

The 15 clonal, non-axenic strains of toxic and nontoxic *Microcystis* used in this study (Table 1) were taken from samples collected from different sites in Brazil. The strains were grown at 21 °C ± 1 ºC, 30 μmol.m^{-2}s^{-1} (Li-Cor 250 quantameter) under a 14/10-hour light/dark photoperiod in a liquid BG-11 culture medium (Rippka et al., 1979). All cultures belong to the Brazilian Cyanobacteria Collection of the University of São Paulo, Brazil. The unialgal strains NPLS-1, NPJB-1, NPLJ-4 and NPLJ-47 were obtained from the Cyanobacteria Collection of the Carlos Chagas Institute of the Federal University of Rio de Janeiro, Brazil (Lourenço and Vieira, 2004).

2.2. DNA extraction and PCR amplification

DNA was extracted from living cells during the exponential growth phase. Total genomic DNA was prepared using the commercial Gnome DNA kit (BIO 101, La Jolla, CA, USA), following the manufacturer's instructions. PCR amplifications were performed as described by Bittencourt-Oliveira (2003). As a positive control of DNA quality, all samples were tested through amplification reactions using oligonucleotide primers for the *cpc*BA (Neilan et al., 1995). Negative control reactions were carried out with the same reaction conditions and primers, but without the DNA template.

Amplification products were visualised by electrophoresis on 0.7% agarose gels stained with ethidium bromide (0.2 μg.mL^{-1}) in 1 X TBE running buffer (pH 8.0, 89 mM of Tris, 89 mM of boric acid and 2 mM of EDTA). PCR products were purified using a PCR purification kit (QIAquick, Qiagen, Valencia, CA, USA), following the manufacturer's instructions. The agarose gels were recorded and DNA concentrations were estimated by comparisons to standard DNA (Low DNA mass, Invitrogen, Carlsbad, CA, USA) using the EDAS 290 (Kodak, Japan).

2.3. Sequencing

The purified PCR product was cloned in the plasmid vector (pCR®2.1) using the TOPO-TA Cloning® Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's recommendations. Plasmid DNA was extracted using the S.N.A.P.™ MiniPrep Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The recombinant clones bearing the correct insert size were sequenced with the ABI Prism® Big Dye® Terminator Cycle Sequencing Ready Kit (Applied Biosystems, Foster City, CA, USA) and 3100 ABI sequencer (Applied Biosystems), following the manufacturer's instructions. To avoid errors caused by the PCR, at least five separate amplification reactions were pooled for sequencing. The nucleotide sequences described in this study have been deposited in the GenBank under accession numbers.

Table 1. *Microcystis* strains used in the present study and accession numbers of *mcy*B sequences obtained from this study and the GenBank for the analysis of phylogenetics, microcystin variants and phylogenetic clusters; from Brazil: BB: Barra Bonita Reservoir, SP (22º 32' 34.5" S and 48º 29' 26.4" W). CT: Cantareira Reservoir, SP (23º 19' 12" S and 46º 35' 18" W). GA: Garças Lagoon, SP (23º 39' S and 46º 37' W). JP: Jacarepaguá lagoon, RJ (22º 54' 10" S and 43º 12' 27" W). SL: Santa lagoon, MG (19º 38' 0" S and 43º 53' 0" W). TM: Três Marias Reservoir, MG (18º 12' 23" S and 45º 14' 30" W). BCCUSP: Brazilian Cyanobacteria Collection of the University of São Paulo. NP: Cyanobacteria Collection of the Carlos Chagas Institute- Federal University of Rio de Janeiro. NIES: National Institute for Environmental Studies. PCC: Pasteur Culture Collection.

Strain	Sample date	Morphospecies [a]	Locality	Accession number	Microcystin	*mcy*B region (size, nt)	Cluster
PCC7806		*M. aeruginosa*	The Netherlands	U97078	MC-LR, [Asp^3]-MC-LR[b]	-	I
NIES298		*M. aeruginosa*	Japan	AB092806	MC-LR[g]	-	I
N-C 31		*M. aeruginosa*	Canada	AJ492552	MC-LR[c]	-	I
N-C 118/2		*Microcystis* sp.	Norway	AJ492554	MC-LR, [Asp^3]-MC-LR[c]	-	I
N-C 161/1		*M. botrys*	Norway	AJ492556	MC-LR, MC-YR[c]	-	I
BCCUSP 18	Unknown	*Microcystis* sp.	TM	AY147796	[D-Asp^3,EtAdda^5]-MC-LH	759	I
NPJB1	1990	*M. aeruginosa*	GA	HQ852449	MC-LR, [D-Asp^3]-MC-LR^3	759	I
NPLJ4	1995	*Microcystis* sp.	JP	HQ852451	MC-RR	771	I
NPLJ47	1996	*Microcystis* sp.	JP	HQ852452	MC-LR	771	I
BCCUSP 100	Apr 2000	*M. panniformis*	BB	HQ852443	[Asp^3]-MC-LR, MC-LR[d] and one unidentified variant (MC-1)	759	I
N-C 169/7		*M. viridis*	Denmark	AJ492557	MC-LR, MC-RR[c]	-	II
N-C 264		*M. botrys*	Norway	AJ492559	[Dha^7]MC-RR[c]	-	II
N-C 324/1		*Microcystis* sp.	Norway	AJ492560	MC-LR, [Dha^7]-MC-LR, [Dha^7]-MC-RR, [Asp^3, Dha^7]-MC-RR[c]	-	II
NIES102		*M. viridis*	Japan	AB092807	MC-LR, MC-RR, MC-YR[g]	-	II
BCCUSP 232	Mar 1997	*M. aeruginosa*	GA	HQ852445	[D-Asp^3]-MC-LR	783	II
BCCUSP 236	Mar 1997	*M. aeruginosa*	GA	HQ852446	[D-Asp^3]-MC-LR	783	II

[a]Species designations are as determined by morphology; [b]Data from Fastner et al. (1999); [c]Data from Mikalsen et al. (2003). Microcystin content determined by MALDI-TOF mass spectrometry; [d]Data from Bittencourt-Oliveira et al. (2005); [e]Non-microcystin-producing strain; [f]Lost strain; and [g]Data from Yoshida et al. (2003).

Table 1. Continued...

Strain	Sample date	Morphospecies [a]	Locality	Accession number	Microcystin	*mcy*B region (size, nt)	Cluster
BCCUSP 262	Mar 1997	*M. aeruginosa*	GA	HQ852447	[Dha^7]-MC-LR, MC-LR, MC-LF, MC-LW	783	II
BCCUSP 299	Feb 1997	*M. aeruginosa*	GA	HQ852448	[D-Asp^3]-MC-LR, one unidentified variant (MC-2)	783	II
BCCUSP 298	May 1997	*M. aeruginosa*	GA	AY147795	[Dha^7]-MC-LR, one unidentified variant (MC-3)	783	II
BCCUSP 235	Apr 1997	*M. aeruginosa*	GA	HQ852440	[D-Asp^3]-MC-LR, MC-LR, one variant not detected (MC-4)	783	II
HUB 5-2/4		*M. aeruginosa*	Germany	AJ492561	MC-LR, MC-RR[b]	-	II
N-C 57		*M. aeruginosa*	Norway	AJ492553	[Asp^3, Dha^7]-MC-RR, [Dha^7]-MC-RR[c]	-	II
N-C 143		*M. aeruginosa*	Norway	AJ492555	__[c, e]	-	II
N-C 228/1		*M. aeruginosa*	Norway	AJ492558	[Dha^7]-MC-LR, [Dha^7]-MC-RR[c]	-	II
BCCUSP 155	Dec 1996	*M. aeruginosa*	GA	HQ852439	Unidentified variant (MC-5)	759	II
BCCUSP 255	Dec 1996	*M. aeruginosa*	GA	HQ852441	__[e]	822	III
NPLS1	1993	*Microcystis* sp.	SL	HQ852450	__[e]	822	III
BCCUSP 225	Mar 1997	*Microcystis* sp.	CT	HQ852442	__[f]	822	III

[a]Species designations are as determined by morphology; [b]Data from Fastner et al. (1999); [c]Data from Mikalsen et al. (2003). Microcystin content determined by MALDI-TOF mass spectrometry; [d]Data from Bittencourt-Oliveira et al. (2005); [e]Non-microcystin-producing strain; [f]Lost strain; and [g]Data from Yoshida et al. (2003).

2.4. Analyses of sequence data and phylogeny

The *mcy*B1 nucleotide sequences from each strain were initially compared with entries deposited in the GenBank database (available at http://www.ncbi.nml.nih.gov) (Altschul et al., 1997) in order to verify the taxonomic accuracy and identify homologue sequences. Sequences corresponding to the amplification primers, insertions/deletions (indels) and variable regions that could not be unambiguously aligned were removed. Phylogenetic inferences were carried out with the maximum-likelihood (ML), maximum-parsimony (MP) and neighbour-joining (NJ) methods, using the PAUP* 4.0 program (Swofford, 2000). Bootstrap analyses (Felsenstein, 1985) were performed with 1000 replicates of the heuristic search algorithm, except for ML, for which 100 replicates were performed. For all analyses, bootstrap values up to 70% were considered low; those from 70 to 90% were considered moderate; and those above 90% were considered high.

2.5. Extraction and determination of microcystins

The extraction of fresh cyanobacterial material was performed based on the method described by Anjos et al. (2006). Samples were injected into a high-performance liquid chromatography (HPLC) system equipped with a LC-10AD pump, PDA detector (SPD10AV) and SCL-10Avp System Controller (Shimadzu™, Japan). The HPLC column used was a Phenomenex™, Luna C_{18} (4.6 mm x 250 mm, particle: 5µm) eluted with a mixture of acetonitrile (ACN) and 20 mM of NH_4CH_3COO (27:73), pH 5, at a flow rate of 1 $mL.min^{-1}$(detected at 238 nm). UV spectra of the peaks were compared with the MC-LR standard in order to isolate possible microcystin analogues. Peaks with similar MC-LR spectra were collected and dried for further infusion in a triple-quadrupole ESI-MS/MS. The determination of microcystin by mass spectrometry was performed based on the method described by Frias et al. (2006).

3. Results

The *mcy*B (B1 module) was PCR amplified from fifteen Brazilian strains of *Microcystis,* among which the presence of microcystin was only detected in 12 strains using HPLC (Table 1). No microcystin was detected for the strains BCCUSP255 and NPLS1. The strain BCCUSP225 was lost during the course of the study. Eight microcystin variants were detected in the Brazilian strains: [D-Asp^3, $EtAdda^5$]-MC-LH, MC-LR, [D-Asp^3]-MC-LR, [Asp^3]-MC-LR, MC-RR, [Dha^7]-MC-LR, MC-LF and MC-LW, along with five other microcystin variants (named MC1-MC5) that were not identified due to low biomass (Table 1). This is the first occurrence in Brazil of the variant [D-Asp^3, $EtAdda^5$]-MC-LH produced by the BCCUSP18 strain.

The amplified region of *mcy*B1 (A3 and A5 regions) corresponded to a portion of the AMP-binding domain of the McyB protein. The PCR fragments amplified with the same set of primers ranged in size from 759 to 822 nucleotides among the samples (Table 1). The putative protein sequences inferred for these *mcy*B fragments exhibited a corresponding variation ranging from 252 to 273 amino acids. None of the sequences exhibited frameshifts or premature stop-codons; only amino acid indels and substitutions were present.

The phylogenetic trees obtained from the three different inference methods used for the *mcy*B matrix were very similar and only minor changes in topology were found in branches with low bootstrap values. Three major clusters (denominated Group I, II and III) were formed in all three methods used, all with high bootstrap support (Figure 1). Some sequences of the Brazilian strains and others from the GenBank database were grouped in Groups I and II, whereas Group III was only made up of three sequences from Brazilian strains. None of the clusters was associated to the geographic origin of the strains or variants of the microcystins produced. However, Group III included Brazilian strains that did not produce microcystin, despite the presence of a PCR fragment for *mcy*B. Moreover, strains from the same morphospecies were arranged in different clusters, such as *M. aeruginosa* and *M. botrys,* which were joined in both Groups I and II.

Considering all sequences for Group I (ours and sequences available from the GenBank), two fragment sizes were found in this region of *mcy*B, with 759 and 771 nucleotides, the difference in size corresponded to one indel of 12 nucleotides. Three sizes were detected in Group II: 783, 759 and 762 nucleotides long. The first (783 nucleotides) was found in the strains BCCUSP232, BCCUSP235, BCCUSP262 and BCCUSP299 due to one indel (close to the insertion site observed in sequences in Group I) with 24 nucleotides. The size of 759 nucleotides was found in all the other sequences, except for AJ492559, which had 762 nucleotides (insertion of 3 nucleotides). For Group III, all sequences were identical, both in size (822) and composition. The nucleotide sequence identities within the major phylogenetic branches ranged from 93.6 to 100% for Group I, from 89 to 100% for Group II and 100% for Group III.

In Group I, the strains NPLJ4 and NPLJ47 (collected at the same site) (Table 1) had identical *mcy*B sequences, but their microcystin variants differed (Table 1). The other strains in monophyletic Group I (BCCUSP18, BCCUSP100 and NPJB1) were highly bootstrap supported. Group II (Figure 1) included the strains BCCUSP235, BCCUSP236, BCCUSP262, BCCUSP298, and BCCUSP299 (collected at the same site) (Table 1), which had identical *mcy*B sequences, but with five identified and three unidentified variants of microcystins (Table 1). The strains AJ492561 (Germany) and AJ492558 (Norway) (Table 1) had identical *mcy*B sequences, but different microcystin variants. No microcystin was detected in the BCCUSP225, BCCUSP255 and NPLS1 strains, which had identical *mcy*B sequences (Group III, Fig. 1) and were collected at different sites (Table 1).

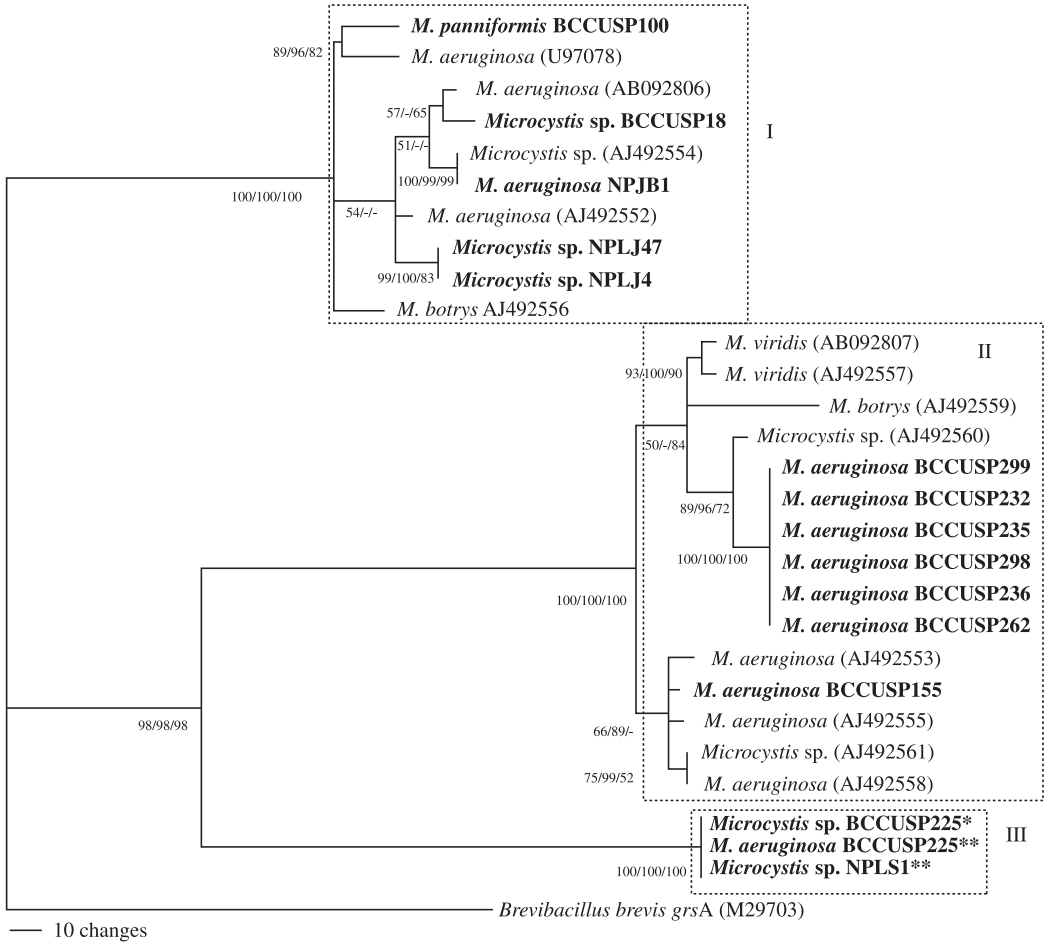

Figure 1. Maximum parsimony (MP) phylogenetic tree built from 852 bp of the *mcy*B1 showing the relationship of Brazilian strains of *Microcystis* (in bold) with sequences obtained from the GenBank (accession numbers in brackets). Bootstrap values are displayed at the internal nodes, when greater than 50%, for MP, NJ (Neighbor-joining) and ML (maximum-likelihood) analyses (MP/NJ/ML). The analyses were bootstrapped (n = 1000, except for ML, where n = 100). The corresponding region in *grs*A gramicidin coding gene from *Brevibacillus brevis* was used as the outgroup. The scale bar shows the branch length corresponding to 10 nucleotide substitutions per site. BCCUSP: Brazilian Cyanobacteria Collection-University of São Paulo. *Non-microcystin-producing genotypes.

4. Discussion

Several microcystin variants have been described for Brazil, such as MC-LR, MC-YR, MC-RR (Anjos et al., 2006), MC-hRhR (Frias et al., 2006), [Asp^3]-MC-LR (Bittencourt-Oliveira et al., 2005) and [D-Leu^1]-MC-LR (Matthiensen et al., 2000). In the present study, a new variant was found, and there are indications of others variants that will be described in subsequent studies. The Brazilian legislation establishes a maximal concentration of 1 μg.L^{-1} for microcystin in water for human consumption (Brasil, 2004). As chromatographic standards for the quantification of different microcystin variants are not always available on the market, the concentrations detected in drinking water reservoirs may be underestimated.

The HPLC technique did not detect microcystin in the BCCUSP255 and NPLS1 strains, which had a slightly larger *mcy*B PCR product than the other microcystin-producing strains. The occurrence of microcystin in the BCCUSP255 (= FCLA255) strain was previously detected by the enzyme-linked immunosorbent assay (ELISA) test (Bittencourt-Oliveira, 2003). The non-detection of the toxin in these genotypes by HPLC can be attributed to a) toxin levels below the resolution limit for the HPLC method; b) whether this gene is indeed expressed; c) whether the gene depends on transcriptional or posttranscriptional regulation; or d) losses of gene function due to mutations.

The amplification of *mcy*B by PCR with no concomitant toxin production has previously been reported for *Microcystis* (Nishizawa et al., 1999; Tillett et al., 2001; Mikalsen et al., 2003). Kaebernick et al. (2001) reported a spontaneous mutant of microcystin biosynthesis that had lost its capacity for toxin production. Kurmayer et al. (2004) found that a

few strains of *Planktothrix* in nature had no microcystin production, but contained all the genes for microcystin synthetase. The authors speculate that these strains had lost the ability to synthesise microcystin due to gene inactivation and that another small peptide could functionally substitute this toxin. In other studies, the presence of microcystin was correlated, almost without exception, to the presence of *mcy* genes (Kurmayer and Kutzenberger, 2003; Via-Ordorika et al., 2004; Dittmann and Börner, 2005). The occurrence of these inactive microcystin genotypes is rare and little understood, but this should not be a significant limitation to the applicability of molecular detection.

Toxicity may vary between different strains or even within a single strain under different laboratory conditions (Kaebernick et al., 2001). Kaebernick et al. (2000, 2002) demonstrated increment in the amount of *mcy*B and *mcy*D genes as the result of strong light intensity. Several environmental factors can affect microcystin production rates, such as light, temperature, nutrients (Watanabe and Oishi, 1985; Lukac and Aegerter, 1993; Utkilen and Gjølme, 1992), biological rhythm (Bittencourt-Oliveira et al., 2005) and production of allelopathic chemicals (Kardinaal et al., 2007; Schatz et al., 2007). However, the factors that govern microcystin production are largely unknown, as is the function of this peptide in the organism itself.

The same morphospecies isolated from the same body of water (NPLJ4 and NPLJ47) were able to perform the synthesis of different microcystins, which confirms findings described by Kurmayer et al. (2002) and Mikalsen et al. (2003) in the sense that there is no specific substrate activation during microcystin biosynthesis. Even strains isolated from a single population, such as BCCUSP236 and BCCUSP262, which were isolated from the same sample collected from the Garça Reservoir in March 1996, exhibited different microcystin variants, despite having identical sequences of *cpc*BA (Bittencourt-Oliveira et al., 2001) and *mcy*B. Microcystin diversity is not necessarily governed by genotype diversity, but may occur from substantial amino acid activation (Kurmayer et al., 2002).

From the analysis of the amplified region of the *mcy*B gene (Module 1), considerable genetic diversity was found in the sequences from the 14 Brazilian strains of *Microcystis* arranged in three distinct groups. No association was found between the eight microcystin variants produced and the clusters formed.

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