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Research Article

Co-occurrence of microcystin and microginin congeners in Brazilian strains of Microcystis sp.

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Abstract

Species of Microcystis are the most common bloom-forming cyanobacteria in several countries. Despite extensive studies regarding the production of bioactive cyanopeptides in this genus, there are limited data on isolated strains from Brazil. Three Microcystis sp. strains were isolated from the Salto Grande Reservoir (LTPNA01, 08 and 09) and investigated for the presence of mcy genes, microcystins and other cyanopeptides. Microcystin and microginin production was confirmed in two isolates using high-resolution tandem mass spectrometry after electrospray ionization (ESI-Q-TOF), and the structures of two new microginin congeners were proposed (MG756 Ahda-Val-Leu-Hty-Tyr and MG770 MeAhda-Val-Leu-Hty-Tyr). The biosynthesis profile of the identified cyanopeptides was evaluated at different growth phases via a newly developed HPLC-UV method. Results demonstrated no substantial differences in the production of microcystins and microginins after data normalization to cell quota, suggesting a constitutive biosynthesis. This study represents the first confirmed co-production of microginins and microcystins in Brazilian strains of Microcystis sp. and highlights the potential of Brazilian cyanobacteria as a source of natural compounds with pharmaceutical interest.

Introduction

Microcystis species are the most common bloom-forming cyanobacteria worldwide. They represent significant health concerns because of an increasing bloom frequency prediction and because of their ability to produce microcystins (Rouco et al., 2011). Microcystins are cyclic heptapeptides and comprise a diverse group containing around 90 structurally similar congeners (Pearson et al., 2010). These hepatotoxins are potent inhibitors of protein phosphatases from the serine/threonine family (types 1 and 2A) in eukaryotic organisms (Runnegar et al., 1995).

In addition to toxins, cyanobacteria species are able to synthesize numerous other bioactive compounds (Singh et al., 2005; Welker et al., 2006; Cardozo et al., 2007; Gademann & Portmann, 2008). Most of these secondary metabolites are oligopeptides containing unusual amino acids in their composition (Welker et al., 2006). These unique peptides are presumably assembled by non-ribosomal peptide synthetase (NRPS) or NRPS/polyketide synthetase (PKS) hybrid pathways and present a considerable array of biological activities (Welker & von Dohren, 2006). Among these activities, protease inhibition appears to be a common feature for several oligopeptides, such as the anabaenopeptins (cyclic hexapeptides) (Harada et al., 1995; Murakami et al., 1997, 1998, 2000) and the microginins (linear peptides) (Okino et al., 1993; Ishida et al., 1997, 1998, 2000).

The microginins are a peptide group consisting of more than 30 variants described in Microcystis strains or field samples. These linear peptides vary from four to six amino acids and usually present a decanoic acid derivative (Ahda; 3-amino-2-hydroxy-decanoic acid) at the N-terminus (Welker & von Dohren, 2006). Microginins are renowned inhibitors of the angiotensin-converting enzyme (ACE) (Okino et al., 1993; Neumann et al., 1997) and represent great interest as target compounds in the discovery of novel antihypertensive agents.

Several Brazilian strains of Microcystis have been investigated for the production of microcystins. However,
there have been few reports documenting the co-occurrence of other bioactive peptides. Carvalho et al. (2008) described a cyanobacterial bloom in a southern Brazilian reservoir with *Microcystis protocystis* and *Sphaerocystum cf. brasiliensis* as the dominant species. The authors confirmed the presence of MC-RR, MC-LR, anabaenopeptin B, and anabaenopeptin F. Recently, Silva-Stenico et al. (2011) screened Brazilian cyanobacterial isolates for the production of antimicrobial agents. The authors described putative microginin peptides in two *Microcystis* strains but further structure confirmation is required. In spite of these reports, the assessment of Brazilian cyanobacteria as a source of natural compounds with pharmaceutical potential is still interesting, especially when the substantial array of biological activities attributed to oligopeptides is considered.

In this study, the production of microcystin and cyanopeptides in three Brazilian strains of *Microcystis* was investigated. The co-occurrence of microcystins and microginins in cyanobacteria isolated from the Salto Grande Reservoir in southeast Brazil is demonstrated, and the structures of two new microginin congeners are proposed (MG756 Ahda-Val-Leu-Hty-Tyr and MG770 MeAhda-Val-Leu-Hty-Tyr). Additionally, the analysis of the cyanopeptide biosynthesis profiles at various stages of growth revealed a constitutive production, providing valuable information concerning the cell harvesting period when biomass production and isolation of active compounds are intended.

**Materials and methods**

**Isolation and strain selection**

The Salto Grande Reservoir is located in Americana City in the São Paulo State (22°45′40″ S and 47°09′15″ W). Water samples were collected in 2007, and microscopic observations revealed the presence of *Microcystis* spp. colonies (primarily *M. protocystis* and *Microcystis aeruginosa*) (Kőmárek & Anagnostidis, 1999). The samples were inoculated onto plates containing solidified ASM-1 medium in 1% agar (Gorham et al., 1964). Isolated colonies were transferred to liquid ASM-1 medium and incorporated into the culture collection of the Laboratory of Toxins and Natural Products of Algae (LPTNA-University of São Paulo). In addition to microscopic observations, the fact that the isolated strains never formed colonies in cultivating conditions supported their classification as *Microcystis* sp. The isolated strains LPTNA001, LPTNA008, and LPTNA009 were selected for use in this study.

**Maintenance and growth evaluation**

Strains were maintained in ASM-1 medium at 24 ± 2 °C, with a 14 : 10-h light/dark cycle and photon flux of 40 μmol photons m⁻² s⁻¹. Light was measured with a quanta sensor (QSL-2101 – Biospherical Instruments Inc.). To evaluate growth, a few milliliters of maintenance cultures from each strain (15 days of culture) were inoculated in Erlenmeyer flasks (2 L) containing 1.5 L of ASM-1 medium at an initial concentration of 10⁴ cells mL⁻¹. Each strain was cultured in triplicate. Several milliliters were harvested every 3 days under sterilized conditions to evaluate cell growth using a Fuchs-Rosenthal hemocytometer under light microscopy. To determine the exponential growth period, exponential regressions were applied to the cell count data vs. sampling time. Duration of the exponential growth phase was considered when data adherence was higher than 98% ($r^2 \geq 0.98$). Growth rates were calculated according to Reynolds (2006) using the exponential regression formula $N = N_0 e^{r_1 t}$, where $N$ is the number of cells at time $t$, $N_0$ is the initial number of cells, and $r_1$ is the growth rate.

**DNA extraction and PCR amplification**

Genomic DNA was extracted from 20 mL of fresh cyanobacterial cultures from each strain using the CTAB method described by Ausubel et al. (1990). All PCR were performed in a Techne TC-312 thermocycler (Duxford Cambridge®, UK) in a 30-μL reaction mixture containing 1 μL genomic DNA, 3 mL 1 × PCR buffer, 0.9 μL 1.5 mM MgCl₂, 1.5 μL 0.5 mM of dNTPs, 0.25 μL recombinant Taq DNA polymerase (Invitrogen®, Brazil), 1.5 μL 0.5 μM (each) primer and PCR quality water (Apiroflex®; Sanderson Laboratory S.A., Chile).

Three fragments of mcy gene clusters were amplified (mcyA-cd, mcyAB and mcyB) with the primers described in Table 1 (Valerio et al., 2010). Temperature cycling was performed at 94 °C for 5 min, 30 cycles at 94 °C for 1 min, annealing temperature at 50 °C for 1 min and 72 °C for 50 s, with a final extension step at 72 °C for 10 min. The aminotransferase domain of the mcyE (mcyE-AMT) was amplified (Table 1) with (GC-) HEPF and HEPR primers (Jungblut & Neilan, 2006). Preincubation was performed at 94 °C for 2 min, and a total of 35 cycles were performed at 94 °C for 50 s. Annealing temperatures of 52 °C for 1 min and 72 °C for 1 min were used. The temperature cycling was concluded with a final step at 72 °C for 10 min.

**Cyanopeptide identification**

Cellular material was obtained from cultures after centrifugation and lyophilization of the pellet. Fifty milligrams of dried cells from each strain were extracted with 5 mL aqueous 90% methanol in an ultrasonic bath (37 kHz) for 5 min. The samples were kept at room temperature.
Analyses were performed in a Shimadzu Prominence liquid chromatograph (Shimadzu, Kyoto, Japan) coupled with an electrospray source to a quadrupole time-of-flight instrument (MicroTOF-QII; Bruker Daltonics, MA). Separation was achieved in a Fusion-RP column of a Fusion-RP column equipped with a LC-20AT quaternary pump and a SPD-M20A photodiode array detector. Separation was achieved in a Luna C18(2) column (5 μm, 150 × 2.0 mm; Phenomenex, Torrance, CA) at 200 μL min⁻¹ using a linear gradient elution (10–60% B in 30 min). The mobile phases used were (A) water containing 5 mM ammonium formate and 0.1% formic acid, and (B) acetonitrile. The column effluent was split (1 : 5) before entering the mass spectrometer source, which was operated in the positive mode (3500 V). Nitrogen was used as nebulizing (35 psi) and drying gas (5 L min⁻¹ at 200 °C). Argon was employed as a collision gas (35% normalized flow rate). The equipment was calibrated with a commercial standard solution (Agilent TuneMix Low; Agilents). Survey scans were performed from m/z 100 to 2000 before selecting a precursor ion for collision-induced dissociation (CID). Product ion spectra were inspected to determine the amino acid sequences. All solvents used were HPLC grade or higher.

### Biosynthesis profiles of microcystins and microginins

Experimental cultures were sampled every 3 days in the middle of the day because of the reportedly higher production of cyanotoxins during this period (Bittencourt-Oliveira et al., 2005; Carneiro et al., 2009). Briefly, 200 mL of culture samples were filtered on glass fiber filters (Millipore) and the cell-containing filters stored at −80 °C until analyses. Extractions were performed as previously described by replacing the dried cellular material with the filters.

Considering that the classic method reported by Lawton et al. (1994) was unable to reliably separate microcystins and microginins in our samples, a new chromatographic method was implemented to adequately separate these peptides. Different gradient elution profiles were tested using phosphoric acid as mobile phase additive in water (A) at a final concentration of 0.08%. The best peak resolution was obtained with a linear gradient from 10% to 50% B (acetonitrile) in 40 min (Supporting Information, Table S1). Analyses were carried out in a Shimadzu Prominence system equipped with a LC-20AT quaternary pump and a SPD-M20A photodiode array detector. Separation was achieved in a Luna C18(2) column (5 μm, 250 × 4.6 mm; Phenomenex) at 1.0 mL min⁻¹. Microcystins were quantified by peak area at 238 nm using a calibration curve constructed with standard solutions of MC-RR and MC-LR (Sigma-Aldrich). Calculated detection limits were 15 ng to MC-RR (r² = 0.9997) and 31 ng to MC-LR (r² = 0.9995). Because of the lack of analytical standards, microginins were quantified as MC-LR equivalents by peak area at 225 nm using a calibration curve of MC-LR monitored at this wavelength (r² = 0.9991). As the absorption coefficients of microginins and microcystins at 225 nm are likely different, the quantitative results here obtained are estimates of the true microginin concentration. Nevertheless, these results are suitable to assess the relative changes in microginin production at different growth phases (Tonk et al., 2009). All data are corrected to cell count and hence presented as cell quotas (fg per cell).

### Statistical analysis

Data are expressed as the mean values ± standard deviation (SD). Data for each experimental variable were tested for normality, and differences between the standard deviations were determined using the Kolmogorov–Smirnov test and Barlett’s test, respectively. An ANOVA was performed using the Kruskal–Wallis (KW) test followed by Dunn’s post hoc test. All tests were performed with a significance of 95% (P < 0.05) using the STATISTICA for Windows (v9.0) application.

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**Table 1. PCR sequencing primers and amplification product size**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>Amplification product (pb)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mcy A</td>
<td>cd 1 F</td>
<td>5′-AAAATTAAAAAGCCTAAAAA-3′</td>
<td>300</td>
<td>Hisbergues et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>cd 1 R</td>
<td>5′-AAAAATTTTATTTAGCCGTCT-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mcy B</td>
<td>2156 F</td>
<td>5′-ATCACCTCATATCTAGACT-3′</td>
<td>955</td>
<td>Mikalsen et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>311 R</td>
<td>5′-AGTGTGCTGCTGAAAGAAA-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mcy a-b</td>
<td>135 F</td>
<td>5′-GACTTATAGCCATCTCCT-3′</td>
<td>541</td>
<td>Mikalsen et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>676 R</td>
<td>5′-TGACGCTCTTTTGTTAA-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mcy E</td>
<td>HEP F</td>
<td>5′-TTGGGCTATCTTGGGCAAGAC-3′</td>
<td>472</td>
<td>Jungblut &amp; Neilan (2006)</td>
</tr>
<tr>
<td></td>
<td>HEP R</td>
<td>5′-AATCTTGAAGCCTGAAATCGGGTTT-3′</td>
<td></td>
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</tr>
</tbody>
</table>
Oligopeptides in Brazilian cyanobacteria

Results

Growth evaluation

Microcystis sp. cells under test conditions grew exponentially until the sixth day of culture (LTPNA01, $r^2 = 0.9834 \pm 0.002$; LTPNA08, $r^2 = 0.9897 \pm 0.011$; LTPNA09, $r^2 = 0.9876 \pm 0.051$). The growth rate of LTPNA01 ($r_n = 0.9183 \pm 0.003$) was higher than the growth rates of LTPNA08 ($r_n = 0.8059 \pm 0.012$) and LTPNA09 ($r_n = 0.8111 \pm 0.031$) (Dunn test, $P < 0.001$) (Fig. 1).

Genetic analyses and peptide identification

The key marker genes ($mcyA$, $mcyB$, $mcy$ a-b, and $mcyE$) of the toxigenic $mcy$ cluster were amplified in the LTPNA08 and LTPNA09 strains but not in LTPNA01. These results indicate that LTPNA08 and LTPNA09 are able to synthesize microcystins, which was confirmed by mass spectrometry analyses.

A representative chromatogram of the LTPNA08 extract is depicted in Fig. 2, with the mass spectra of the major peptides identified. Mass spectra typical of microcystins were obtained for fractions 1 and 4. These compounds were identified as MC-RR ($m/z$ 519.8) and MC-LR ($m/z$ 995.5), respectively. In both spectra, the characteristic product ion at $m/z$ 135 is observed. This product ion originates from the alpha cleavage of the lateral chain of the Adda residue and is considered diagnostic of the general structure of microcystins. The identity of fractions 1 and 4 was further confirmed via co-chromatography with commercial standards of MC-RR and MC-LR. Low-intensity ions typical of MC-YR ($m/z$ 1045.5), MC-FR ($m/z$ 1029.5), and MC-WR ($m/z$ 1068.5) were also observed in the LTPNA08 and LTPNA09 strains (< 2% of total microcystins). Although the low intensity of these ions prevented the acquisition of adequate product ion spectra, in-source CID experiments showed ions at $m/z$ 135 and neutral losses of 134 Da. These results strongly suggest that other microcystin isoforms are synthesized at low levels besides the major congeners MC-RR and MC-LR.

Product ion spectra of $m/z$ 756.5 (fraction 2) and $m/z$ 770.5 (fraction 3) contain ions at $m/z$ 128 and $m/z$ 142, respectively (Fig. 2). These ions are characteristic to the general structure of microginins and originate from bond dissociation between C2 and C3 in the N-terminal residue 3-amino-2-hydroxydecanoic acid (Ahda) (Fig. 3). N-terminal methylation is a common occurrence and accounts for the product ion at $m/z$ 142 (Welker et al., 2006). Further ion assignment based on accurate mass measurements allowed the identification of the b and y ion series for both fractions (Fig. 3). As a result, we propose the sequences of two new microginin congeners: Ahda-Val-Leu-HTy-Tyr (MG756) and MeAhda-Val-Leu-HTy-Tyr (MG770). Interestingly, cell extracts from LTPNA09 and LTPNA08 provided similar chromatographic profiles, indicating that these isolates are clones of the same strain. As expected, no microcystin congeners could be identified in LTPNA01.

Microcystins and microginins biosynthesis profiles

Initial analyses of the extract from LTPNA08 using the method of Lawton et al. (1994) provided a nonconforming UV spectrum at the retention time corresponding to MC-LR (Fig. 4). The second absorption maximum observed at 278 nm suggests the co-elution of MC-LR with a microginin congener. Although different gradient profiles were tested using trifluoroacetic acid, reliable separation could not be achieved. However, the replacement of trifluoroacetic acid by phosphoric acid as the mobile phase additive in A (0.08% in ultrapure water) provided satisfactory separation. As shown in Fig. 5, a linear gradient from 10% to 50% acetonitrile in 40 min provided the best resolution between the peaks (Table S1).

The developed method was employed to monitor cyanopeptide production at different phases of growth. Microcystin total concentration varied from 30.2 to 53.9 fg per cell in LTPNA08 and from 20.9 to 30.8 fg per cell in LTPNA09 during the sampling period. As mentioned before, microginins were quantified as MC-LR equivalents at 225 nm. Total concentration of
these peptides ranged from 14.0 to 23.4 fg per cell in LTPNA08 and from 11.3 to 26.5 fg per cell in LTPNA09. As can be seen in Fig. 6, no significant variations are observed in total microcystins or microginins production during growth or between the two strains (KW test, \( P > 0.05 \)).

**Fig. 2.** LC-MS base peak chromatogram for the LTPNA08 strain. (1) Product ion spectrum of m/z 518.9 (MC-RR); (2) Product ion spectrum of m/z 756.5 (MG756); (3) Product ion spectrum of m/z 770.5 (MG770); (4) Product ion spectrum of m/z 995.5 (MC-LR). *Unknown peptide.
Microcystis spp. blooms have been reported frequently in several Brazilian environments as reviewed by Dörr et al. (2010). In the State of São Paulo, for example, the eutrophicated reservoirs Guarapiranga and Billings are recurrently affected by toxic outbreaks of *M. aeruginosa* and *Microcystis panniformis* (Sant’Anna & Azevedo, 2000; Anjos et al., 2006; Frias et al., 2006; Moschini-Carlos et al., 2009; Silva-Stenico et al., 2011). The three *Microcystis* spp. strains used in this study were isolated from the Salto Grande Reservoir, which is located in the city of Americana, São Paulo State. This eutrophicated reservoir is intensively used for recreational activities, and previous reports have documented the occurrence of toxic *Microcystis* spp. blooms (Bittencourt-Oliveira, 2003). Despite several Brazilian strains of *Microcystis* have been investigated for the production of microcystins, few studies have explored the production of other cyanopeptides. Congeners of cyanopeptolins, aeruginosins, micropeptins, anabaenopeptins, and aeruginosides from twelve strains were recently described as putative cyanopeptides (Silva-Stenico et al., 2011). The authors also described putative microginins from the Brazilian *Microcystis* strains NPCD-1 and SPC804. In agreement with these observations, the strains LTPNA08 and LTPNA09 were characterized as microginin and microcystin producers.

Microcystins are synthesized non-ribosomally by large multienzyme complexes that comprise different modules: NRPS, PKS, and mixed peptide/polyketide synthases (Jungblut & Neilan, 2006). The microcystin biosynthesis gene cluster (*mcy*) spans 55 kb and is arranged as 10 genes that form two cross-transcribed operons, *mcyA-C* and *mcyD-J* (Pearson & Neilan, 2008). This gene cluster has been sequenced and characterized in various cyanobacterial strains, including *Microcystis*, *Anabaena*, and *Planktothrix* (Pearson et al., 2010). In consequence, genetic analyses to detect the presence of *mcy* genes in environmental samples can provide rapid identification of a potentially toxic population of cyanobacteria. In general, nontoxic strains do not contain *mcy* genes and afford negative results, while toxic strains have different *mcy* copies and provide positive amplifications, especially for *mcyE* (Vaitomaa et al., 2003). However, caution should be used when interpreting results because there is no clear correlation between the presence of specific genes and the production of microcystins (Vaitomaa et al., 2003; Bittencourt-Oliveira et al., 2011). In our study, mass spectrometry experiments corroborated the genetic analyses results: microcystin congeners were detected only in strains LTPNA08 and LTPNA09.

Although the putative gene cluster for microginin synthesis has been sequenced (Welker et al., 2006; Rounge...
et al., 2009), a clear and concise method to evaluate the presence of microginin genes in *Microcystis* has not been described. Therefore, production of these compounds was investigated only through high accuracy mass spectrometry. Typical product ion spectra of microginins were observed infractions 2 and 3 (Fig. 1) for both LTPN08 and LTPNA09 strains. Interestingly, the mass-to-charge ratio of the precursor ion in fraction 3 (m/z 770.5; Fig. 2) corresponds to a compound with the same molecular formula (C_{41}H_{63}N_{5}O_{9}) as microginin 478, a congener already described by Ishida et al. (2000). Microginin 478 contains three N-methylated residues in its sequence MeAhda-Val-MeVal-MeTyr-Tyr. On the basis of this assumption, the downshift of 14 Da in the product ion spectra of the peptide in fraction 2 (Fig. 2) could lead to the identification of a demethylated congener of microginin 478 (m/z 756.5). In this case, MeAhda in the N-terminal position would be replaced by an Ahda residue. However, inspection of the CID spectra for both variants reveals prominent y_{1} and y_{2} ions at m/z 182 and m/z 359, respectively (Fig. 2). These features are incompatible with the classic model of low-energy bond dissociation for peptides containing N-methylated residues. It is well accepted that N-methyl residues at low-energy CID conditions prevent the formation of y ions through the oxazolone fragmentation pathway because the proton transfer step in the dissociating proton-bound dimer is not permitted (Paizs & Suhai, 2005). As a result, the fixed charge in the oxazolone derivative leads to the exclusive formation of b ions (Fig. S1). It is worth mentioning that direct amide bond dissociation is not favored in low-energy CID conditions, and the fragmentation pathways involve rearrangement-type reactions (Paizs & Suhai, 2005). Because of the observation of intense y_{1} and y_{2} ions, we believe that HTy (homotyrosine) and Leu are present in the peptide sequences, replacing MeTyr and MeVal, respectively, in microginin 478. Assignment of a Leu residue is supported by the observation of a weak d_{3} ion at m/z 342.3 in the product ion spectra of peptide 3 (Fig. 2). Discrimination of Leu and Ile residues via observation of d ions has been reported previously (Johnson et al., 1988; Baptista-Saidemberg et al., 2011). Additionally, the following characteristic immonium ions were observed: m/z 72.1 for Val, m/z 86.1 for Leu (or MeVal), and m/z 150.1 for HTy (or MeTyr). On the basics of these results, we propose the sequences of the two microginin congeners as Ahda-Val-Leu-HTy-Tyr (MG756) and MeAhda-Val-Leu-HTy-Tyr (MG770) (Fig. 3). To the best of our knowledge, these two identified microginin congeners have not been previously described (Table 2).
Microginins are interesting molecules because they are able to inhibit enzymes, such as leucine aminopeptidase M and the ACE (Ishida et al., 2000). ACE inhibitors are the most effective treatments for hypertension and congestive heart failure, making microginins interesting candidates for purification, and biological activity evaluation. In this study, initial chromatographic separation efforts using the method of Lawton et al. (1994) were unsuccessful (Fig. 4). Therefore, to adequately separate these peptides, a new chromatographic method based on the gradient elution with phosphoric acid as the mobile phase additive was implemented (Fig. 5).

The newly developed method was further employed to assess the biosynthesis profile of microcystins and microginins in strains LTPNA08 and LTPNA09. The evaluation of the peptide content per cell at different stages of growth allows the determination of the most appropriate harvesting period when purification is intended. Our results demonstrate that microcystin and microginin are constitutively produced. In this sense, biomass harvesting can be performed at any time during the growth period evaluated.

Associations between growth and MC production in *Microcystis* strains are common in the literature. During different phases of growth, the MC content can be quite variable (Wiedner et al., 2003). Changes in the production of different MC variants were verified at different growth phases in *M. aeruginosa* under batch culture conditions (Lyck, 2004). However, changes in microginin production during the growth phases of *Microcystis* strains have not been well studied. In *Anabaena*, production of anabaenopeptins and anabaenopeptilides varied according to the age of the cultures. The highest MC concentrations were observed in the middle of the growth phase, whereas the highest anabaenopeptin and anabaenopeptilide concentrations were observed at the stationary phase (Repka et al., 2004). In this study, microcystin and microginin production by the LTPNA08 and LTPNA09 strains under test conditions did not change during the different growth phases and did not differ between the two lineages (Dunn test, \( P > 0.05 \)).

The cell harvesting for these analyses was performed always at the middle of the day. As previously reported, microcystins can vary threefold at the middle of the day in comparison with dark periods (Bittencourt-Oliveira et al., 2005). Other cyanotoxins as paralytic shellfish poisoning (PSP) toxins can vary from 2- to 4.5-fold from the first hours of light to the middle of the day (Carneiro et al., 2009).

Most of the knowledge regarding the function of cyanotoxins has focused on microcystins. The original hypothesis was that microcystins may have an ecological function that protects cyanobacteria against grazing and suppression of light competitors (Rohrlack et al., 1999; Babica et al., 2007). Zilliges et al. (2011) recently showed that microcystins bind to a number of proteins involved in basal metabolism and that the binding is strongly enhanced under high light and oxidative stress conditions. These observations led to the hypotheses that MC-protein interactions are responsible for the increased fitness of *Microcystis* in high light conditions.

In addition to the hepatotoxic microcystins, several other families of peptides have been identified from *Microcystis* and other genera of cyanobacteria. Because the biosynthesis of secondary metabolites, such as microcystins, aeruginosin, and cyanopeptolin, primarily occurs during the light period of the day, these metabolites may interact with molecules related to diurnal central metabolism (Straub et al., 2011). Microginins may have a similar role and function. However, the relevance to the co-production of toxic and nontoxic peptides in cyanobacteria requires further investigation.
In summary, this study represents the first confirmed co-production of microginins and microcystins in strains from the Salto Grande Reservoir. The structures of two new microginin variants, MG756 (Ahda-Val-Leu-HTy-Tyr) and MG770 (MeAhda-Val-Leu-HTy-Tyr), are also proposed. The occurrence of microginins with microcystins in the strains LTPNA08 and LTPNA09 highlights the potential of Brazilian cyanobacteria as a source of natural compounds with pharmaceutical interest.

Acknowledgements

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Authors’ contribution

R.L.C. and F.A.D. contributed equally to this paper.

References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Scheme depicting the fragmentation pathways leading to b and y ions for linear peptides.

**Table S1.** Resolution (Rs) analyses of peaks of microcystins and microginins from the two toxic strains of *Microcystis* sp. (LTPNA 08 and LTPNA 09).

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