A novel rhythm of microcystin biosynthesis is described in the cyanobacterium Microcystis panniformis Komárek et al.

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Received 12 November 2004
Available online 2 December 2004

Abstract

The presence of microcystins (MCY) in the cyanobacteria Microcystis panniformis Komárek et al. is reported for the first time. This strain of cyanobacterium has been isolated from Barra Bonita, an eutrophicated water reservoir in São Paulo state, Brazil. The identification of M. panniformis was confirmed by both traditional morphological analysis and the phycocyanin intergenic spacer sequences. MCY-LR and [Asp³]-MCY-LR were identified in this strain after HPLC purification and extensive ESI-MS/MS analysis. Their levels in this strain were determined by HPLC and ranged from 0.25 to 2.75 and 0.08 to 0.75 fmol/cell, respectively. Analyzing the levels of MCY-LR and [Asp³]-MCY-LR in different times during the light:dark (L:D) cycle, it was found that levels of MCYs per cell were at least threefold as high during the day-phase than during the night-phase. This may be associated to the biological clock since prokaryotic cyanobacteria express robust circadian (daily) rhythms under the control of a timing mechanism that is independent of the cell division cycle. Our findings also showed the same pattern under light:light (L:L) cycle.

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Keywords: Diurnal rhythm; Microcystin; Cyanobacteria; Microcystis panniformis; HPLC; ESI-MS/MS

Some cyanobacterial blooms of the genus Microcystis (Chroococcales, Cyanobacteria) can be a serious ecological and public health concern due to their ability to dominate the planktonic environment and produce cyclic heptapeptide toxins, named microcystins (MCYs) [1]. MCYs inhibit protein phosphatases, especially types 1 and 2A, in a similar way to the action of okadaic acid [2].

Seasonal changes of Microcystis species and production of MCYs and aeruginopeptins have been reported in the Lake Suwa, Japan [3]. Photosynthetically active radiation (PAR) can also interfere in some biochemical process in cyanobacteria. The psbA2 gene has exhibited light-dependent and rhythmic expression in Microcystis aeruginosa K-81 [4] and Microcystis strain PCC 7806 showed a positive effect of PAR on microcystin production and content [5].

It has been shown that several physiological and biochemical processes are controlled or at least are influenced by the biological clock in dinoflagellates and cyanobacteria [6–11]. For example, the cyanobacteria Cyanothecce strain CGD temporally separates nitrogen fixation and photosynthetic activity to protect...
oxygen-sensitive nitrogenase [12], three cyanobacteria, *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7942, and *Cyanothecce* RF-1, clearly exhibited circadian photosynthetic rhythm by employing a dissolved-oxygen meter to monitor the photosynthesis [13] and the cyanobacterial *KaiB* and *KaiC* proteins (encoded by the gene cluster *kaiABC*) are robustly rhythmic, whereas the *KaiA* abundance undergoes little if any circadian oscillation in constant light [11,14].

These findings strongly implicate a circadian regulatory mechanism operating on these metabolic processes are evidence for the importance of circadian rhythms in global metabolic regulation in some cyanobacteria species. For this reason, some natural peptides, including MCYs and other bioactive oligopeptides, synthesized by cyanobacteria may be influenced by the biological clock [15].

*Microcystis panniformis* strain BCCUSP 100 presented the gene *mcyB* that encodes a MCY synthetase [16]; however, the toxin itself has not previously been detected in this cyanobacterium species.

In this report, we isolated and identified for the first time by ESI-MS/MS two MCYs in this species. The identification of *M. panniformis* strain BCCUSP 100 was confirmed by traditional morphological criteria and the phylectic relationships and genetic diversity of the Brazilian strains of *M. panniformis* with other closely related species of *Microcystis* were investigated. HPLC analyses with *M. panniformis* samples collected during L:D and L:L cycles indicated that the MCY-LR and [Asp]³-MCY-LR levels were higher during the day. As supported by our data, the biosynthesis of MCYS is controlled by the biological clock. These findings are important to elucidate the mechanisms involved in MCYs biosynthesis as well as their isolation and characterization.

**Materials and methods**

**Reagents.** All reagents were obtained as ultrapure grade. Peptide toxins MCY-LR and [Asp]³-MCY-LR were purified from the cyanobacterium *M. panniformis* strain BCCUSP 100 grown in our laboratory [16] as described below.

**Study area and field sampling.** Water samples were collected from blooms in Barra Bonita reservoir (22° 32’ 34.5’’ S, 48° 29’ 26.4’’ W) in April 2000 using a 25 m plankton net. This reservoir is located at Barra Bonita city, Sao Paulo state, Brazil (total area: 308 km², water volume: 2,566 x 10⁶ m³, that has been used as hydroelectric plant and for recreation). *Microcystis*, *Cylindrospermopsis*, and *Raphidiopsis* blooms have been occurring often in this reservoir.

**Morphology.** Field colonies were identified according to the morphological criteria [17,18], as: colony shape, cell diameter, and mucilage aspect. Photomicrographs of colonies were taken with a microscope (Nikon E200, Melville, NY, USA) equipped with a video camera system Samsung S833 using the software ImageLab (Softium, Brazil). One drop of diluted nankeen was used on the lamina to make the mucilage evident. During the cultivation in the laboratory, the strain underwent morphological variation and became unicellular.

Measurements of cell diameter from *M. panniformis* strains were performed at random during the logarithmic phase of growth (*n* = 50) for each strain. The averages of cell diameter and standard deviation were statistically analyzed using SAS software (version 8.0) [19].

**Strains and growth conditions.** The non-axenic *M. panniformis* BCCUSP 100 strain was isolated in modified BG-11 medium [20] in glass tubes containing 10 ml culture medium. One individual colony was removed by micromanipulation techniques using a microscope (Nikon E200, Melville, NY, USA) at magnification of 100x to 400x. The isolated colony was washed by transferring it to several drops of water until all other microorganisms were removed and subsequently transferred to liquid medium. Some Brazilian *Microcystis* strains were isolated in our previous study [16] and maintained at the Brazilian Cyanobacteria Collection of University of Sao Paulo, Brazil (ex FCLA).

**Daily variation.** Prior to the beginning of the experiments, a pre-culture of 150 ml was grown on a 12:12 h (light:dark) photoperiod, at 23 ± 0.5 °C, light intensity was 75 ± 2 µmol photons m⁻² s⁻¹ to obtain inocula in an appropriate physiological condition. Light intensity was measured using a spherical quantum photometer (LI-COR mod. LI-250 Lincoln, NE, USA) placed inside a culture flask with the medium. The pre-culture (15 days, exponential growth phase) was divided into amounts of 50 mL and inoculated in 3 identical flasks with 2,2 L of new medium without aeration. The cultures were initiated with 2.6 x 10⁶ cells ml⁻¹ and maintained as previously described. The samples were collected in the middle of the exponential growth phase (around 10⁵ cells mL⁻¹). Two experiments were carried out: (i) light-dark cycle (12 h in the light and 12 h dark); samples were collected at 2 h intervals, 50 mL to toxin analysis and cell counting (1 mL) and (ii) light:light cycle (24 h with light): the same procedure was used as in (i). In both experiments, cells were grown as previously described. Fifty milliliters of culture was placed into Falcon tubes and centrifuged at 4000 rpm for 15 min at 23 °C. Cell pellets were immediately frozen in liquid nitrogen and stored at −86 °C until analysis. Cell densities were estimated by means of microscopic counts of cell samples, stained with Lugol’s 4% solution in Fuchs Rotenthal hemocytometer. It was established that a minimum of 400 cells needed to be counted to obtain an error of approximately 10% to a confidence level of 95%.

**DNA extraction.** Total genomic DNA was prepared using the commercial kit Gnome DNA (BIO 101, Vista, CA, USA) and DNA sample was cleaned and purified through QiaQuick columns (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions.

DNA concentrations were estimated directly from ethidium bromide fluorescence in agarose gel images against standard quantities of DNA (Low DNA mass, Invitrogen, Carlsbad, CA, USA) either by using a Kodak gel documentation system (Kodak, Melville, NY, USA) and associated software Kodak Digital Science 1D.

**Amplification of the cpcB-cpcA intergenic spacer sequencing.** The cpcB-cpcA phycocyanin intergenic spacer (PC-IGS) and flanking coding region were amplified by PCR as described by Bolch et al. [21]. The amplified fragments were directly sequenced using the forward and reverse primers (PC-F: 5’-GGGTGCTTTTGATACCCGACA-3’; PC-R: 5’-CGGAGTCGCTCCATACAACTA-3’) with ABI Prism Big Dye Terminator Cycle Sequencing Ready reaction Kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and 3100 ABI sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions.

To avoid errors by PCR, at least 3 separate amplification reactions were pooled for sequencing. The PCR products were sequenced on both strands at least four times. The cpcBA-IGS nucleotide sequence from strain BCCUSP 100 was compared to entries deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) to verify taxonomic accuracy. Automated base calls for both strands were checked by manual inspection and ambiguous calls and conflicts were resolved by alignment and comparison using Sequencer program (version 3.0) to establish a consensus sequence for the strain. The BCCUSP 100 consensus sequence and reference *Microcystis* sequences were assembled manually, using ESEE
Doubtful bands of low resolution were disregarded. Phenogram construction using the software NTSYS version 1.70. Jaccard Digital Science 1D. The amplified fragments were used to generate a 1.5% agarose gel and detected by staining with ethidium bromide by a 5 min. Amplification products were visualized by electrophoresis in a Kodak gel documentation system and associated software Kodak Digital Science 1D. The amplified fragments were used to generate a binary data matrix from which similar matrices were obtained using Jaccard's coefficient. The UPGMA algorithm [25] was used for the phenogram construction using the software NTSYS version 1.70. Doubtful bands of low resolution were disregarded.

Microcystin extraction and HPLC semipreparative analyses. MCYs were extracted with MeOH/H₂O 3:1 from freeze-dried samples (around 500 mg) and submitted to an ultrasound bath for 10 min. The extract was centrifuged (10,000 rpm, 15 min) and the supernatant collected. The pellet obtained was re-extracted according to the procedure described above. The supernatants were combined and dried in a rotaevaporator (bath at 40 °C). The dried material was resuspended in 3 mL DCM and applied to a silica column (20 × 5 cm, Silica Gel Keigel 60, Merck). The column was equilibrated with MeCN and the elution steps were: 30 mL DCM, 30 mL DCM:MeOH, 30 mL MeOH, and 30 mL MeOH/H₂O. Fractions were concentrated in a rotaevaporator (same as described above). Toxins were found to be in the last elution step. The last dried fraction was resuspended with 1 mL of mobile phase and then repurified in a HPLC system equipped with a pump LC-10AD, a PDA detector (SPD10AV), and a SCL-10Avp System Controller (Shimadzu, Kyoto, Japan). About 500 μL of the sample was injected in the system and chromatographed in a semipreparative HPLC column (Phenomenex, Luna C18, 5 μm, 250 × 10 mm) eluted with a mixture of MeCN and 0.05 mol/L, pH 3, NH₄CH₃COO (1:3) at a flow rate of 4.7 mL min⁻¹. The detection was set at 238 nm. Peaks 1 and 2 (Fig. 1) were further identified as MCY-LR and [Asp⁷]-MCY-LR.

Fig. 1. Typical chromatogram obtained with the extracts of Microcystis panniformis strain BCCUSP 100 (Phenomenex Luna column C₁₈, 256 × 4.6 mm, 5 μm, ACN:NH₄CH₃COO buffer, pH 3, flow rate = 1 mL min⁻¹ and λ = 238 nm), 1, microcystin-LR and 2, [Asp⁷]-microcystin-LR.

Mass spectrometric analysis. ESI-MS/MS spectra were obtained on a Quattro Micro tandem mass spectrometer (Waters Micromass, Manchester, UK). Samples (peaks 1 and 2) in MeCN:formic acid 0.1% (1:1, v/v) were introduced into the electrospray ion source by direct infusion using the integrated syringe pump at flow rates ranging from 2 to 10 μL min⁻¹. The mass spectra were acquired in the positive ion mode with the source and analyzer parameters were optimized for the protonated molecular ion. Tandem MS spectra (daughter scans) were acquired using Ar as a collision gas (4 × 10⁻³ mbar) at different collision energies (5–60 eV).

HPLC analysis for the daily variation experiments. MCYs were extracted with 3 mL MeOH/H₂O 3:1 from freeze-dried samples, around 10 mg, and submitted to an ultrasound bath for 10 min. The extract was centrifuged (10,000 rpm, 15 min) and the supernatant was evaporated (SpeedVac, Savant, City, US state). The precipitate was resuspended in 1 mL MeOH and injected into a Sep-Pak cartridge (C₁₈, Waters, Milford, MA). The preconditioning step included washing with MeOH (2 mL) and H₂O (2 mL) and the elution steps were: (i) 1 mL H₂O, (ii) 1 mL MeOH/H₂O 1:1, and (iii) 1 mL MeOH. MCYs were found to be in the last eluate. This fraction was also evaporated (SpeedVac) and the precipitate resuspended with 200 μL HPLC mobile phase and analyzed in the HPLC system equipped with a C₁₈ Luna column (5 μm, 0.46 × 25 cm) eluted with MeCN/0.05 mol/L NH₄CH₃COO buffer (pH 3) 1:3 at 1 mL min⁻¹. Detection was performed at 238 nm with a SPD10AV PDA detector. HPLC analyses were performed with a Shimadzu HPLC system. The calibration curve was obtained with the MCY LR and [Asp⁷]-MCY-LR isolated from M. panniformis.

Statistical analyses. The data were expressed as mean values ± standard deviation (SD). The data for each experimental variable were tested for the basic premises of analysis of variance (ANOVA) model, using the Bartlett test for homogeneity of variances and the χ² test for normality [26]. A single-factor ANOVA was applied to the mean values obtained for each experimental time in order to detect significant differences. Whenever the null hypothesis of ANOVA was rejected, the Tukey test of multiple comparisons [27,28] was employed and the statistically significant differences (P < 0.05) between each pair of mean values were discriminated.

Results and discussion

Microcystis panniformis identification

Previously, the strains BCCUSP 03, BCCUSP 30, BCCUSP 158, BCCUSP 200, and BCCUSP 310 were denominated as belonging to the “Microcystis aeruginosa complex” [29]. In this paper, it was denominated as M. panniformis following the suggestion of Dr. Jiri Komárek ([30] see editorial remark). However, the diameter of the cells of the same strain was larger than the original descriptions, 3.9–6.4 μm diam. (Table 1), results that corroborated with White et al. [31].

The morphospecies M. panniformis showed broad diversification (Fig. 2). Description of population and their life cycle were also described in the literature [16,29,30]. The M. panniformis populations found in the environmental samples from Barra Bonita reservoir showed elongate and flat colonies with densely arranged cells in (see Figs. 2A–C) agree with Komárek et al. [16,18].

Similar groupings emerged in the parsimony, neighbor-joining, and maximum-likelihood. Thus, only the
The phylogenetic tree was constructed from the 465 nucleotides of the *cpc*BA-IGS and flanking regions. The 21 strains analyzed were separated in five major clusters (I–IV) divisible into taxonomically relevant groups. Two Brazilian *M. aeruginosa* strains were composed of the Cluster I and they did not group together with Cluster II composed of eight *M. aeruginosa*, *Microcystis viridis*, and *M. panniformis*.
and Microcystis flos-aquae strains from Brazil, Japan, Canada, and Spain. Three Spanish strains formed the Cluster III: two Microcystis novacekii and M. aeruginosa. Finally, the Cluster IV showed three Microcystis wesenbergii strains from Japan and Brazil shared 99.98 to 100% similarity. The M. panniformis BCCUSP 100 was isolated from the others.

On the other hand, the Hip1CA primer generated PCR products and agarose gel electrophoresis banding patterns (Fig. 4) that did not distinguish the M. panniformis, M. wesenbergii, and Microcystis spp. Three distinguished clusters and one strain that did not group were observed (Fig. 5). The three clusters had low similarity (A = 0.38, B = 0.49, and C = 0.40). M. panniformis strains were present among clusters A (BCCUSP 158, BCCUSP 03, BCCUSP 30, and BCCUSP 310), B (BCCUSP 200), and C (BCCUSP 100).

**Microcystin isolation and identification by ESI-MS/MS**

We found a very effective and simple method for microcystin purification combining extraction by MeOH/H2O [3:1], silica column chromatography, and one-step semipreparative HPLC. Due to microcystin’s high polarity, it could be dissolved in DCM and eluted from silica gel using MeOH/H2O [1:1]. Two suspected compounds were found in the HPLC profile, named as Peaks 1 and 2. A typical chromatogram can be seen in
Fig. 1. These compounds were analyzed by ESI-MS; producing protonated molecular ions at \( m/z \) 995.4 and \( m/z \) 981.4, respectively. As suggested by their molecular weights and chromatographic behavior, we suspected they could be toxins [Asp\(^3\)]-MCY-LR and MCY-LR. In order to confirm this hypothesis we analyzed both compounds by tandem MS.

Product ion spectra of 995.4 and 981.4 (Figs. 6A and B) showed typical fragments from MCYS such as \( m/z \) 135 that had been attributed to a fragment originated from \( \alpha \)-cleavage of the methoxy group of the Adda [32]. Ion \( m/z \) 213 had been assigned to the dipeptide Glu-Mdha and ion \( m/z \) was proposed to be produced by the loss of the fragment 135 from the tetrapeptide Adda-Glu-Mdha-Ala [32,33]. The ion \( m/z \) 599 is also present in both spectra and could be assigned to the sequence Arg-Adda-Glu.

The identities of 1 and 2 as being MCY-LR and [Asp\(^3\)]-MCY-LR are supported by a series of ions that include MeAsp or Asp and consequently presented a mass difference of 14 Da between spectra. Thus, it was found \( m/z = 967 \) [cyclo(Ala-Leu-MeAsp-Arg-Adda-Glu-Mdha)-CO] and \( m/z = 953 \) [cyclo(Ala-Leu-MeAsp-Adda-Glu-Mdha)-CO]; \( m/z = 553 \) [Mdha-Ala-Leu-MeAsp-Arg] and \( m/z = 539 \) [Mdha-Ala-Leu-MeAsp-Arg]; \( m/z = 470 \) [Ala-Leu-MeAsp-Arg] and \( m/z = 456 \) [Ala-Leu-Asp-Arg]; \( m/z = 397 \) [Mdha-Ala-Leu-MeAsp] and \( m/z = 383 \) [Mdha-Ala-Leu-Asp]; and \( m/z = 286 \) [MeAsp-Arg] and \( m/z = 272 \) [Asp-Arg].

Daily variation of microcystins

It has been demonstrated that PAR, pH, and nutrients (iron, phosphorus, and nitrogen) influence the
growth and MCY content of *Microcystis* spp. ([5,34–39], respectively). However, to our knowledge, the circadian variation of MCYs levels in *Microcystis* spp. has never been reported.

In the studies presented, the levels of MCY-LR and [Asp³]-MCY-LR in *M. panniformis* under L:D and L:L cycles showed a peak at the middle of the day, around 12–14 h (Fig. 7). Also, in both L:D and L:L cycles, MCY-LR is almost fourfold more abundant around 12 h than during the dark phase. [Asp³]-MCY-LR, in the L:D experiment, shows the same pattern as that of MCY-LR. Nevertheless, [Asp³]-MCY-LR contents with the L:L treatment are twice as high in comparison with the L:D experiment.

These results may be associated to the biological clock since in cyanobacteria, circadian rhythms have been found for photosynthesis, nitrogen fixation, some protein synthesis, and cell division [6–10].

**Conclusion**

The identification of the *M. panniformis* strain BCCUSP 100 was confirmed by morphological profiles and based on sequence homology of the intergenic spacer region between the cpcA and cpcB-phycocyanin subunits with database records and two MCYs were isolated and identified in this species, MYC-LR and [Asp³]-MYC-LR. We conclude from our results that the biological clock controls the production of MCYs and their content in *M. panniformis*, since their production was observed to peak at the middle of the day phase, in both L:D and L:L experiments. This shows the importance of circadian regulation in this cyanobacterium.

Also, this strain can be readily utilized as a reference for MYC-LR and [Asp³]-MYC-LR production and to follow MCYs biosynthesis under many culture conditions.

**Acknowledgments**

The authors thank Dr. Paul Gates (Organic and Biological Chemistry Section, School of Chemistry, University of Bristol, UK) for helpful comments on the mass spectrometry analysis and also for the English language revision. This research was supported by grants from FAPESP (2003/06443-0 and 2003/05773-6), CNPq (302439/2002-1), and CAPES.

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