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Active release of microcystins controlled by an endogenous rhythm in the cyanobacterium *Microcystis aeruginosa*

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SUMMARY

The active release of microcystins in cyanobacterium *Microcystis aeruginosa* (Kützing) Kützing, strain BCCUSP232 was confirmed. The microcystin release is controlled by an endogenous rhythm, pointing to a biosynthetic pattern of toxins in cyanobacteria. Proofing tests for this active release were carried out by experiments at two independent 24 h cycles, light : dark and continuous light (12:12 h) along the exponential growing phase. Cultivation samples at light, temperature and photoperiod controlled conditions were collected in 2-h intervals. Microcystin concentrations from the pellet aliquots (intracellular microcystin per cell-quota –IMC) and supernatant (extracellular microcystin per equivalent cell-quota – EMC) were quantified with enzyme linked immunosorbent assay. The IMC concentrations showed increases and decreases in both cycles. Decreases of IMC clearly demonstrate that the toxin was actively released to the surrounding medium and not by cell lysis. The total microcystins concentrations (IMC and EMC) between the light : dark and continuous light cycles presented similar variations between the same hours.

Key words: circadian rhythm, extracellular microcystins, intracellular microcystins, *Microcystis aeruginosa*.

INTRODUCTION

Cyanobacteria are photosynthesizer prokaryotes which produce an enormous variety of secondary metabolites, among them the cyanotoxins, responsible for the poisoning of wild and domestic animals, and fishes (Sivonen & Jones 1999; Carmichael *et al*. 2001). Occurrence of human contamination was also extensively observed (Jochimsen *et al*. 1998).

Microcystin (MC) is one of the most studied cyanotoxins. In fact, it is commonly found in water bodies all over the world, presenting a wide structural variation constituted by more than 80 variants (Meriluoto & Spoof 2008). These metabolites are hepatotoxic, inhibiting the protein phosphatases 1 and 2A with cytoskeleton loss of integrity leading, as a consequence, to apoptosis of hepatocytes and other cells (Mackintosh *et al*. 1990; Toivola & Eriksson 1999; Gehringer *et al*. 2004).

It is known that release of microcystin to the external medium takes place during cell lysis and death (Sivonen & Jones 1999; White *et al*. 2005). The presence of an enzymatic complex (MycH) acting as a possible microcystin conveyor outside the cell provided indications about the possibility for an alternative process of MC release (Pearson *et al*. 2004). Oscillations (ups and downs) were verified in concentrations of two intracellular MC variants in short time intervals without the expected cell lysis, implying a passive release of the toxin (Bittencourt-Oliveira *et al*. 2005).

Biosynthesis of microcystins and saxitoxins was related to the existence an endogenous control by a circadian rhythm in *M. panniformis* Komárek *et al*. and *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya & Subba Raju, respectively (Bittencourt-Oliveira *et al*. 2005; Carneiro *et al*. 2009). High light intensities increase transcription of the genes *mcy*B and *mcy*D related to the biosynthesis of microcystins (Kaebernick *et al*. 2000).

Studies with cyanobacteria have proved the existence of circadian rhythms acting in many other processes as nitrogen fixation (Grobbelaar *et al*. 1986; Mulholland & Capone 2000; Staal *et al*. 2007), cell division (Sweeney & Borgese 1989; Lorne *et al*. 2000), photosynthesis (Schneegurt *et al*. 1994; Yen *et al*. 2004), aminoacids capture and carbohydrates synthesis (Iwasaki & Kondo 2004). These circadian rhythms could be controlled by a genetic grouping (cluster) (*kai*ABC), where expressed proteins (KaiA, KaiB and KaiC) would work in several functions exhibiting daily cycles (Johnson & Golden 1999; Lorne *et al*. 2000).

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The goal of the present study is the investigation of microcystin active release controlled by an endogenous rhythm in *Microcystis aeruginosa* (Kützing) Kützing, a potentially MC-producing species that is distributed worldwide.

MATERIAL AND METHODS

Strain, cultivation conditions and experiment delineation

A clonal non-axenic, microcystin-producing strain *M. aeruginosa* (BCCUSP232; Bittencourt-Oliveira *et al*. 2011), isolated from a water body southeast of Brazil, was used. Determination of the exponential phase was accomplished from growing curves, in triplicate, and at the same conditions of the experiment with a 12:12 h LD (light : dark) photoperiod.

Two different experiments were carried out: (i) 12:12 h LD cycle; and (ii) 24 h continuous light, both starting with 10^6 cells mL^{-1} and kept in climate chambers under controlled conditions of temperature (23 ± 0.5°C), photoperiod (12:12 h LD or 24 h continuous light) and light intensity (40 μmol m^{-2} s^{-1}) measured by a photometer (LI-COR, mod. LI-250) with a spherical and underwater sensor inserted into cultivation flasks with medium. Before the start of the continuous light cycle the cultivations were acclimatized under uninterrupted illumination for 48 h. Cultivations were performed in 1-L Erlenmeyer flasks containing 800 mL of liquid medium BG-11, pH 7.4 (Rippka *et al*. 1979) modified by Bittencourt-Oliveira (2000).

Samples were collected in 2-h intervals under axenic conditions for the analysis of intracellular microcystin by cell quota (IMC) and extracellular microcystin by equivalent cell quota (EMC) (2 mL) and cell counting (cell density) (2 mL). The cycles were initiated at the 10^{th} (LD) and 13^{th} (continuous light) days, both in the exponential phases. All experiments were performed in triplicate, as well as the analysis of IMC and EMC and cell density.

Samples for cell counting were processed at once in a Fuchs Rosenthal hemocytometer by means of a binocular optical microscope (Nikon E200, Melville, NY, USA). A minimum of 1600 cells per sample were counted in order to achieve a 5% error level (Lund *et al*. 1958).

For the analysis of IMC and EMC the samples were centrifuged for 3 min for the separation of the MC pellet (intracellular MC) from the supernatant (extracellular MC). The supernatant was removed by means of an automatic pipette and accommodated in new microtubes. Pellet and supernatant were immediately frozen and stored at –80°C up to the microcystin analysis.

Analysis of IMC and EMC in the 12:12 h LD and continuous light cycles

For the IMC analysis the pellets were placed in polyethylene microtubes, dried in speedvac (model Eppendorf AG 22331, Hamburg, Germany) and resuspended in 3-mL volumes. The cells were lysed with a sonicator (Ultrasonic Cell Disruptor XL – Misonix), in an iced bath, and immediately analyzed.

Intracellular microcystin and EMC quantifications were carried out using a commercial enzyme linked immunosorbent assay (ELISA) kit (Beacon Analytical Systems, Portland, ME, USA), following the manufacturer's protocols. The detection limit for MC by ELISA was 0.10 to 2.0 ppb (ng mL^{-1}). The absorbance was measured using an ELISA plate reader (Asys Expert Plus, Cambs, UK) at a wavelength of 450 nm. The negative and positive controls of ELISA analysis are included. Analyses were carried out in triplicate.

The IMC was calculated from a relationship between microcystin concentration (ng mL^{-1}) and cell density (cell mL^{-1}). The EMC, on the other hand, was directly measured from the supernatant and, afterward, related to the sample cell density in the analyzed volume.

Statistical analysis

Intracellular microcystin and EMC data were expressed as average ± standard deviation (SD). The normality was analyzed for each experimental variable by the Kolmogorov-Smirnov test (Massey 1951). The data for each experimental variable were tested for the basic premises of the variance analysis (one way ANOVA). In all cases where the variance null hypothesis was rejected, the Tukey test was used, and the statistically significant differences were discriminated ($P < 0.05$) between each pair of average values (Neter *et al*. 1985). The analysis was performed with the SAS Institut 9.2 program.

RESULTS

Active release of microcystins

The IMC concentrations showed increases and decreases in both cycles (Table 1 and Fig. 1). Decreases of IMC clearly demonstrate that the toxin was actively released to the surrounding medium and not by cell lysis. It is noticeable that during the LD cycle, between the times of 8 and 10 pm, the average IMC difference was 0.80 fg $cell^{-1}$, that is, a variation of approximately 17.8% (Table 1). A similar decrease of IMC concentrations also took place between 10 am and 12 pm with a 16.49% difference.

The EMC concentrations, however, had increases of 20.3% and 22.8% between 10 am and 12 pm hours, and between 8 pm and 12 am hours, respectively.

Table 1. Intracellular microcystins concentrations per cell-quota (IMC) (fg cell^{-1}) and extracellular microcystins per equivalent cell-quota (EMC) (fg cell^{-1} equivalent) of the *Microcystis aeruginosa* BCCUSP232 strain in the 12:12 LD (light : dark) and continuous light cycles during the exponential phases. Data are presented as average ± standard deviation ($n = 3$).

Time (hours)	Light : dark		Continuous light	
	IMC	EMC	IMC	EMC
2 am	4.79 ± 0.32	12.43 ± 0.56	4.44 ± 0.79	11.25 ± 1.13
4 am	4.56 ± 0.65	11.79 ± 2.45	5.06 ± 0.50	9.33 ± 0.91
6 am	4.73 ± 0.46	11.30 ± 0.88	5.43 ± 0.86	9.28 ± 0.74
8 am	4.31 ± 1.29	18.63 ± 1.35	3.84 ± 0.66	12.25 ± 0.42
10 am	6.15 ± 0.96	11.56 ± 1.76	4.58 ± 0.11	11.60 ± 1.33
12 pm	5.14 ± 1.03	14.57 ± 0.91	4.71 ± 0.20	10.85 ± 1.15
2 pm	5.49 ± 0.65	16.82 ± 3.55	4.27 ± 0.28	10.87 ± 1.40
4 pm	4.67 ± 0.73	15.20 ± 1.88	4.37 ± 0.32	8.43 ± 0.50
6 pm	4.52 ± 0.98	15.35 ± 1.67	4.86 ± 0.77	10.30 ± 0.78
8 pm	4.50 ± 0.54	12.07 ± 0.99	4.18 ± 0.46	7.93 ± 0.80
10 pm	3.70 ± 0.67	15.64 ± 2.16	4.35 ± 0.30	11.87 ± 2.64
12 am	5.43 ± 0.45	10.75 ± 1.46	4.69 ± 0.56	11.24 ± 0.24

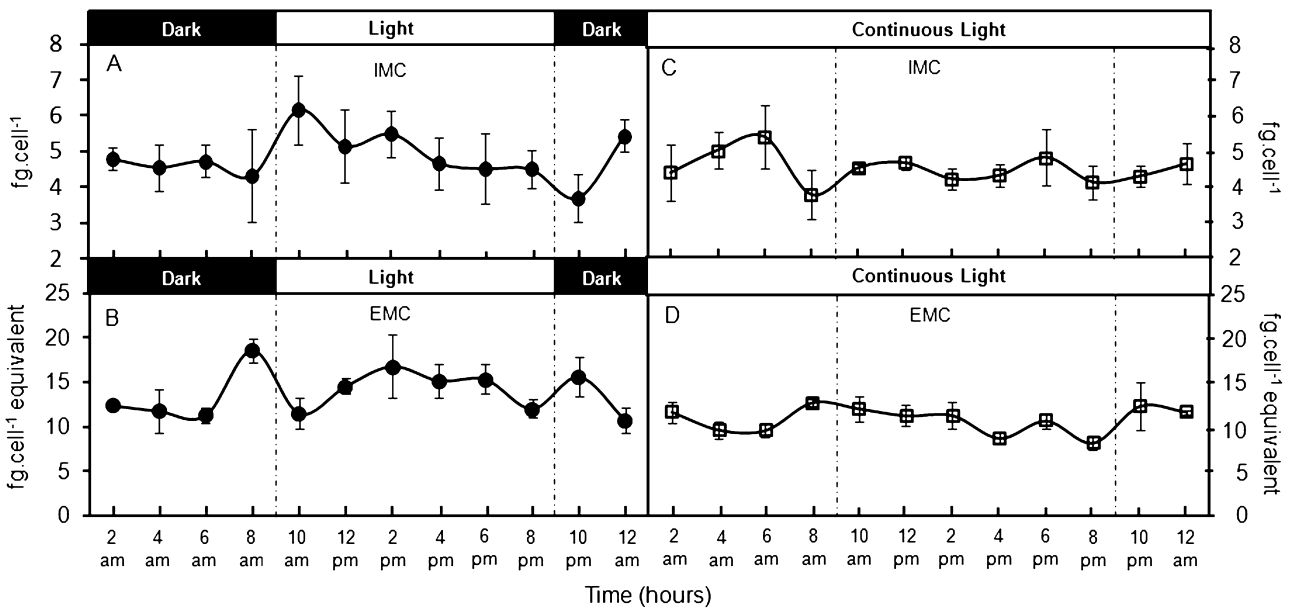

Fig. 1. *Microcystis aeruginosa* BCCUSP232 strain: variation of microcystin concentrations at 12:12 LD (light : dark) cycles during the growing exponential phase. (A) Intracellular microcystins per cell-quota (IMC) (fg cell^{-1}) in the LD cycle. (B) Extracellular microcystins per equivalent cell-quota (EMC) (fg cell^{-1} equivalent) in the LD cycle. (C) Intracellular microcystins per cell-quota (IMC) (fg cell^{-1}) in the continuous light cycle. (D) Extracellular microcystins per equivalent cell-quota (EMC) (fg cell^{-1} equivalent) in the continuous light cycle. Bars represent standard deviation ($n = 3$).

The presence of an endogenous rhythm controlling microcystins production

We observed that IMC showed a controlled endogenous rhythm as function of time in a 24-h period. Most of the time IMC and EMC were inversely proportional to the LD and continuous light cycles (Fig. 1a–d).

No statistically significant differences were observed among the IMC variations between the LD and continuous light cycles ($F = 3.87$; $P > 0.05$). However, IMC values in the LD cycle were slightly higher than in continuous light, except at 4 and 6 am hours and 6 and 10 pm hours (Table 1). The highest IMC peak occurred in the LD cycle at 10 am hours (6.15 fg cell^{-1}), but

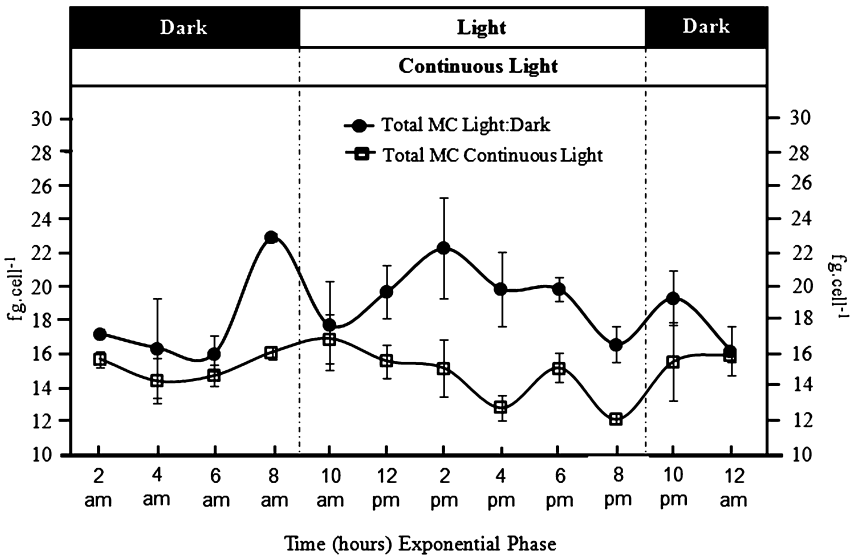

Fig. 2. *Microcystis aeruginosa* BCCUSP232 strain: variation of the total microcystin concentrations (MC) (IMC and EMC) (fg cell^{-1}) in the LD (light : dark) and continuous light cycles during the exponential growing phase. Bars represent standard deviation ($n = 3$).

such an occurrence was verified in the continuous light cycle at 6 am hours (5.43 fg cell^{-1}) (Fig. 1a,c; Table 1). On the other hand, EMC concentrations exhibited statistically significant differences ($F = 17.15$; $P < 0.05$) between the LD and continuous light cycles, although the simultaneous increase/decrease patterns, among the corresponding hours, were similar (Fig. 1b,d; Table 1).

The total microcystins concentrations (IMC and EMC) (fg cell^{-1}) between the LD and continuous light cycles presented similar variations between the same hours, but a statistically significant difference have occurred ($F = 21.55$; $P < 0.05$) (Fig. 2). Higher release of MC to the extracellular medium has occurred under continuous light.

The continuous light cycle presented the lowest total microcystins concentrations in all hours (Fig. 2), but with the highest EMC concentrations (Fig. 1b,d; Table 1).

DISCUSSION

The IMC variations along 24 h in the cycles (LD and continuous light) characterized an endogenous control in *M. aeruginosa* microcystins biosynthesis. Similar results were found for two other cyanobacteria species, *M. panniformis* and *C. raciborski*, (Bittencourt-Oliveira *et al*. 2005; Carneiro *et al*. 2009) which could be considered a pattern in cyanotoxins biosynthesis.

The highest measured concentrations were from EMC either in the LD or in the continuous light cycles (Fig. 1b,d; Table 1). The main factor responsible for the release of cyanotoxins to the extracellular medium is attributed to the cell lysis and death (Sivonen & Jones 1999). When variations in the intra and extracellular concentrations of microcystins are verified in cultivation, it is suggested that they are related to the cell lysis caused by the proximity of the middle of stationary phase (White *et al*. 2005).

Although significant statistical difference did not happen between cycles, the IMC and EMC concentrations were lower in the continuous light cycle and in most of the hours, which could be related to the oxidative stress originated by the intense exposure to light. Light is an external stimulus influencing circadian rhythms in cyanobacteria by means of a bacteriophytochrome-like protein CikA. The CikA protein takes part in photosynthesis transmitting external environmental stimuli to this process, like light, which further participates in the regulation of the circadian rhythm (Wood *et al*. 2010).

On the other hand, the LD cycle favored toxins production, because the biosynthesis of the genes of microcystins and other secondary metabolites occur mostly during the light period (Straub *et al*. 2011), suggesting that these substances could directly interact with light in the endogenous controlling put in action by the *kai* genes. The *kai* genes controls circadian rhythms, either to express a protein or to perform physiologic functions (Kageyama *et al*. 2003; Johnson *et al*. 2008). According to Kaebernick *et al*. (2000), the lack of correlation between the increase of genes transcription responsible for the biosynthesis of microcystins, *mcy*B and *mcy*D, with the content of the intracellular toxin suggests its release to the medium.

The microcystins show stability of their molecules in natural environments. However, the role of oxidizing agents, the high incidence of ultraviolet light and the action of heterotrophic bacteria used by their molecules as carbon supply, could degrade them quickly (Sivonen & Jones 1999, Park *et al*. 2001; Imanishi *et al*. 2005). Moreover, photosynthetically active radia-

tion could also affect the concentrations of extracellular microcystins (Wiedner *et al*. 2003). In non-axenic conditions, the MC were not cumulative in the cultivation medium, indicating a possible influence of non-evaluated factors, as the bacteria present in the experiment.

CONCLUSION

On the basis of these arguments and reasoning, we conclude that active release of microcystins controlled by an endogenous rhythm in *M. aeruginosa* has occurred, pointing to a biosynthetic pattern of toxins in cyanobacteria.

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