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Profiles of toxic and non-toxic oligopeptides of *Radiocystis fernandoii* (Cyanobacteria) exposed to three different light intensities

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ABSTRACT

Cyanobacteria produce a high variety of bioactive oligopeptides, which function, ecological, physiological roles and responses to environmental changes are still unclear. The influence of light intensity on the cell quota and the diversity of oligopeptides of two strains of the cyanobacterium *Radiocystis fernandoii* were experimentally tested. The peptides were quantified by HPLC and identified by a MALDI-TOF–TOF. Microcystins (MC) were generally more abundant in the treatment with low light. A compensatory mechanism was observed for the different variants of microcystin, whereby MC-RR responses were contrary to those observed for the other three variants and showed higher concentration in the treatment with intermediate light. Two microviridins were also produced at higher amounts at intermediate irradiance. For cyanopeptolins and a third microviridin no significant difference among treatments was found. The absence of a similar response for all peptides suggests that these compounds may have unique cellular functions, which better understanding could help explaining changes in toxicity. Finally, we observed that each chemical profile reflected in physiological differences between strains, strengthening the idea that chemotypes may act as distinct ecotypes in nature.

1. Introduction

Cyanobacteria are an important component of the phytoplankton community, but their relative importance and dominance in aquatic systems has augmented in the last years, as a consequence of increasing eutrophication and nutrient input. They are morphologically simple but chemically diverse, and are acknowledged to produce several bioactive peptides, such as aeruginosins (Murakami et al. 1994), cyanopeptolins (Martin et al. 2003), microginins (Okino et al. 1993), microviridins (Ishitsuka et al. 1990), anabaenopeptins (Harada et al. 1995) and the well studied microcystins (Carmichael 1992) known for their toxic effects on mammals and humans that cause public health concerns around the world. According to Welker and von Döhren (2006), at least 600 peptides have been already described in cyanobacteria. The high variability of compounds found in these organisms can be explained by their biosynthethic pathways, which are independent from ribosomes and RNA and consist of multifunctional enzyme complexes composed of non-ribosomal peptide synthetases (NRPSs) and polyketide synthase (PKS) modules (Börner and Dittmann 2005; Welker and von Döhren 2006).

Despite their ubiquity and variety, the ecological and physiological functions of these peptides are still not well understood. Some studies have suggested allelopathic effects (Pflugmacher 2002; Schatz et al. 2005; Smith and Doan 1999), others have proposed a bacterial quorum-sensing hypothesis (Kaebernick et al. 2000) or a possible relation with internal metabolism (Lyck 2004). A few peptides were shown to be inhibitors of proteases of the cladoceran *Daphnia* (Agrawal et al. 2001, 2005; Czarnecki et al. 2006; Rohrlack et al. 2003; Von Elert et al. 2004), suggesting protection against grazing. Additionally, natural populations of cyanobacteria are composed of producers and non-producers strains, for each peptide or peptide class (Fastner et al. 2001; Rohrlack et al. 2001; Welker et al. 2004), and up to now, non-producing strains have not been shown to have any clear advantage or disadvantage when compared with producing strains (Hesse et al. 2001; Kaebernick et al. 2001).

An understanding of the factors affecting the production of the cyanobacterial peptides may contribute to the comprehension of the functions of these compounds and their relationship with the environment. It can also be an important tool for the assessment of the cyanotoxin risk and water treatment policies. To date, several studies have investigated the effect of light, temperature, phosphorus and nitrogen on cyanobacterial peptides production (Oh et al. 2000; Repka et al. 2004; Rohrlack and Utkilen 2007; Sivonen 1990; Tonk et al. 2005; Tonk et al. 2009; Utkilen and Gjolme 1995; Wiedner et al. 2003; Zilliges et al. 2011). Because of its toxicity to

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humans, microcystin has been, in general, the main focus in these studies and just few have considered other peptides. However, understanding the interaction among several peptides could help bringing new knowledge about the function of these compounds.

There are indications that that MC may be involved in photosynthetic light-related processes (Long et al. 2001; Young et al. 2005). Some studies showed that toxin production and growth can be stimulated over a certain range of light intensities, but at saturating light intensities, toxin production decreased (Hesse and Kohl 2001; Utkilen and Gjolme 1992; Wiedner et al. 2003), even when no inhibitory effect was observed on growth rates (Wiedner et al. 2003).

Many cyanobacteria prefer growing at lower irradiance ($30\ \mu mol\ m^{-2}\ s^{-1}$) and can be sensitive to photoinhibition (Walsh et al. 1997). They can adapt to varying irradiance by changing the proportion of light-harvesting complexes in the thylakoid membrane and the pigment ratios. Fluctuations in light intensity may also have a significant impact in species interactions (Lichtman et al. 2004), slowing rates of competitive exclusion or reversing the identity of the superior competitor, and finally altering the structure of phytoplanktonic communities.

In this study, we tested the influence of light intensity in the production of the oligopeptides cyanopeptolins, microviridins and microcystins. Two strains of the cyanobacterium *Radiocystis fernandoii* (Komárek and Komárková-Legenerová, 1993) isolated from two Brazilian reservoirs were used in the experiments. This genus is morphologically similar to *Microcystis* except for its characteristic radially oriented cell disposition and by the crosswise cell division in one plane. *Radiocystis* is found in lakes in the northern hemisphere, but also in tropical waters. Komárek described the species *R. fernandoi* as occurring in tropical regions of central America (Komárek 2003). In Brazil, it is quite common in tropical and subtropical regions where it is one of the main genera responsible for toxin production (Sant'Anna et al. 2008). Borges et al. (2008) observed blooms in subtropical Brazil where this species represented up to 70% of the total biomass. Anjos et al. (2006) and Fonseca et al. (2011) observed the presence of toxic genes (mcy B and mcy A, respectively) in natural blooms of *R. fernandoi*. The level of toxicity and MC-LR production can be quite elevated in this species (Vieira et al. 2003), nevertheless still not much information concerning its ecology, physiology and toxicity is available. A recent study reported the presence in this species of MC-LR, one micropeptin and two other compounds identified as belonging to the aeruginosin class (Lombardo et al. 2006). Our research however is the first one, to our knowledge, describing the presence and behaviour of different oligopeptides in two strains of *R. fernandoii*, when submitted to different growth conditions. These two strains were selected from our cultures initially because of their fast growth rates, which allowed easier experiments' development. Second, we also wished to compare strains with different peptide profiles, looking for possible peptide effects on the strain fitness. The individuality of strain responses can eventually turn to be important in the management of toxic blooms.

2. Material and methods

2.1. Strains

The *R. fernandoii* 28 strain was isolated from Furnas reservoir (20°40′S; 46°19′W), located in the south-eastern region of Brazil. Furnas is a large oligo- to mesotrophic reservoir that receives inputs of nutrients from agricultural activities and domestic sewages and frequently presents cyanobacterial blooms in the upper reaches. The *R. fernandoii* 86 strain was isolated from Pampulha reservoir (19°55′S; 43°56′W), which is an eutrophic urban reservoir located in the city of Belo Horizonte, Brazil, that suffers heavy impact of pollution from domestic and industrial sewages and shows permanent cyanobacterial blooms. Both strains are maintained in the culture collection of the Phycology Laboratory of the Botany Department in the Federal University of Minas Gerais.

2.2. Experiments

The experiments were performed in triplicates, in batch cultures of 500 ml of WC medium (Guillard and Lorenzen 1972). Growth conditions were a 12 h light: 12 h dark photoperiod at 20 °C. Experiments were carried out at three different irradiances: $25\ \mu mol\ m^{-2}\ s^{-1}$, $65\ \mu mol\ m^{-2}\ s^{-1}$ and $95\ \mu mol\ m^{-2}\ s^{-1}$. These light intensities were selected as low, medium and high, based on literature data (see for example Hesse et al. 2001; Tonk et al. 2005; Preussel et al. 2009). In all treatments, experiments were interrupted when cultures growth was still exponential and always before reaching the stationary phase (approximately 7–10 days). At the end of the experimental period, the content of the entire flask of each replicate was freeze dried and the dry material was accurately weighed and then used for further biochemical analyses.

During the growing period, cell growth was daily assessed by measuring culture's optical density (OD) at 750 nm and growth rates were calculated. At the end of each experiment, chlorophyll content was estimated from spectrophotometric measurements after extraction in 90% hot ethanol (Nusch 1980). In the same samples, cell numbers were estimated by counting a minimum of 400 cells in a Fuchs-Rosenthal hemocytometer.

2.3. Oligopeptide analysis

The dry material was extracted three times using methanol 75% (v/v), by sonication on ice followed by centrifugation, and the final extract was applied to SPE C18 cartridges (Waters, Sep-Pak Vac 3cc – 500 mg) for purification by reverse phase chromatography, as described by Lawton and Edwards (2001). The dry extracts were resuspended in methanol 75% (v/v) and analysed by HPLC (Waters Alliance 2695) with a photodiode array detector (Waters 2996) at 225 nm and a Waters symmetry C18 Column (4,6 × 250 mm I.D., 5 μm ODS). Mobile phase A was acetonitrile, containing 0.1% (v/v) trifluoroacetic acid (TFA), and mobile phase B was water, containing 0.1% (v/v) trifluoroacetic acid (TFA). The chromatographic run consisted of a linear gradient from 30% A to 34% in 33.5 min then 40% for 6.5 min. The flow-rate was 1 ml/min. Because of the lack of standards for most peptides, their quantification was done by dividing the peak area of each compound by the dry weight of the culture, obtaining a measurement of the relative change in the peptide concentration. For the identification of the peptides, fractions were collected and analysed in a MALDI-TOF–TOF Autoflex III mass spectrometer (Bruker Daltonics, Billerica, USA). The products were mixed with α-cyano-4-hydroxycinnamic acid matrix solution (1:1, v/v) and left to dry at room temperature in a MALDI target plate Anchorchip 600 (Bruker Daltonics, Billerica, USA). The peptide masses were obtained using a reflector mode and compared with known cyanobacterial metabolites. Known and unknown peptides were then fragmented using the LIFT fragmentation mode (MS/MS), and the fragment patterns were analysed according to Welker et al., 2006. Chromatographic profiles of both *Radiocystis* strains and the MS/MS spectra of three identified and fragmented fractions, corresponding to each one of the observed classes of peptides, can be seen in Appendix A.

We used dry weight to standardize the measurement of peptides, since it is a parameter that has a high correlation with cell number and cellular biovolume. This relationship was tested