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Production of cyanopeptolins, anabaenopeptins, and microcystins by the harmful cyanobacteria *Anabaena* 90 and *Microcystis* PCC 7806

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ABSTRACT

This study investigated the effects of light intensity, temperature, and phosphorus limitation on the peptide production of the cyanobacteria *Microcystis* PCC 7806 and *Anabaena* 90. *Microcystis* PCC 7806 produced two microcystin variants and three cyanopeptolins, whereas *Anabaena* 90 produced four microcystin variants, three anabaenopeptins, and two anabaenopeptilides. Microcystin and cyanopeptolin contents varied by a factor 2–3, whereas the anabaenopeptins and anabaenopeptilides of *Anabaena* varied more strongly. Under phosphorus limitation, peptide production rates increased with the specific growth rate. The response of peptide production to light intensity and temperature was more complex: in many cases peptide production decreased with specific growth rate. We observed compensatory changes of different peptide variants: decreased cyanopeptolin A and C contents were accompanied by increased cyanopeptolin 970 contents, and decreased anabaenopeptin A and C contents were accompanied by increased anabaenopeptilide 90B contents. Compensatory dynamics in peptide production may enable cyanobacteria to sustain stable peptide levels in a variable environment.

1. Introduction

Several cyanobacterial toxins have been studied extensively, particularly the microcystins, nodularins, and anatoxins. During the past 15 years, however, many other bioactive peptide groups have been discovered in cyanobacteria, such as aeruginosins (Murakami et al., 1994), microginins (Okino et al., 1993), cyanopeptolins (Martin et al., 1993), anabaenopeptilides (Rouhiainen et al., 2000), microviridins (Ishitsuka et al., 1990), anabaenopeptins (Harada et al., 1995), and nostophycins (Fujii et al., 1999). These peptides are frequently found in cyanobacterial blooms, along with numerous other not yet identified peptides (Fastner et al., 2001; Welker et al., 2006). A better understanding of the production and toxicity of these additionally discovered peptides is essential to assess the environmental controls on peptide production and to guide cyanotoxin risk assessment.

The striking variability in peptide production of cyanobacteria can probably be attributed to the process of oligopeptide biosynthesis. Most of these oligopeptides are synthesized by large multifunctional enzyme complexes consisting of non-ribosomal peptide synthetases (NRPSs) and polyketide synthase (PKS) modules (Börner and Dittmann, 2005; Welker and von Döhren, 2006). For instance, *Anabaena* 90 harbors at least three peptide synthetase gene clusters for synthesis of microcystins, anabaenopeptilides, and anabaenopeptins (Fujii et al., 2002). The relationships between multiple gene clusters within a single strain are still unknown, but they seem to operate largely independently of each other (Fujii et al., 2002). Disruption of genes within the *mcy* cluster of *Microcystis* blocked microcystin production without affecting the production of cyanopeptolins (Dittmann et al., 1997). This observation suggests that the production of cyanopeptolins may respond differently to environmental factors than the production of microcystins.

To investigate this hypothesis, we focused on the effects of different environmental factors on the peptide production of two widespread freshwater cyanobacteria: *Microcystis* and *Anabaena*. Both species produce multiple toxic and non-toxic peptides and are known to form dense surface blooms in eutrophic lakes (Reynolds, 2006). In this study, we investigate how light, phosphorus availability, and temperature affect the peptide content and peptide production of *Microcystis aeruginosa* PCC 7806 and *Anabaena* 90. We set out to explore whether peptides are continuously produced and whether variation in peptide production is found between and within peptide classes. Additionally, we investigate whether peptide contents are related to the growth rate of *Microcystis* and *Anabaena*.

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2. Material and methods

2.1. Strains

M. aeruginosa strain PCC 7806 was provided by the Pasteur Institute. The strain was originally isolated from the Braakman Reservoir, The Netherlands, in 1972 and grows as single cells. *Anabaena* 90 was provided by the University of Helsinki, and originally isolated from Lake Vesijärvi, Finland, in 1986 (Sivonen et al., 1992). Both *Microcystis* and *Anabaena* have relatively high phosphorus requirements (De Nobel et al., 1997; Fujimoto et al., 1997). *M. aeruginosa* PCC 7806 is a single-celled cyanobacterium with optimal growth at relatively high temperatures and light intensities (Van der Westhuizen and Eloff, 1985; Wiedner et al., 2003). *Anabaena* 90 is a filamentous heterocyst-forming species capable of nitrogen fixation, with optimal growth at relatively low temperature and light intensity (Rapala et al., 1997) compared to *Microcystis*.

2.2. Light experiments

Microcystis PCC 7806 and *Anabaena* 90 were grown in semi-continuous cultures consisting of flat plastic vessels (400 ml) aerated by a continuous airflow and provided with a nutrient-rich mineral medium (O2 medium; Van Liere and Mur, 1978) to avoid nutrient limitation. The phosphorus concentration in O2 medium was 144 μM. Temperature was kept constant at 20 ± 2 °C (by leading the compressed air used for aeration through a temperature-controlled water bath (Colora thermocryostat)). A ventilator was used to diffuse the warmth emitted by the light source. Light was provided by white fluorescent tubes (Philips PL-L 24W/840/4P), directed towards the front surface of the culture vessel. Incident irradiance (I_{in}) and outgoing irradiance (I_{out}) were measured with a LI-COR LI-250 quantum photometer at seven points on the front surface and back surface of the culture vessel, respectively. The average photon irradiance inside the culture vessel, I_{avg}, was calculated as follows (Huisman et al., 2002):

$$I_{avg} = \frac{I_{in} - I_{out}}{\ln I_{in} - \ln I_{out}} \quad (1)$$

Microcystis and *Anabaena* cultures were placed at two different average light intensities: a light-saturated photon irradiance (*Microcystis* PCC 7806: I_{in} = 175 μmol m^{-2} s^{-1}, I_{avg} = 150 μmol m^{-2} s^{-1}; *Anabaena* 90: I_{in} = 75 μmol m^{-2} s^{-1}, I_{avg} = 50 μmol m^{-2} s^{-1}) and a light-limited photon irradiance (*Microcystis* PCC 7806: I_{in} = 58 μmol m^{-2} s^{-1}, I_{avg} = 40 μmol m^{-2} s^{-1}; *Anabaena* 90: I_{in} = 30 μmol m^{-2} s^{-1}, I_{avg} = 22 μmol m^{-2} s^{-1}). The experiments were performed in triplicate at both light intensities. A day–night cycle of 12-h light/12-h dark was imposed. The optical densities (at 750 nm) of the semi-continuous cultures were kept constant between 0.1 cm^{-1} and 0.2 cm^{-1} by diluting the culture material every other day with mineral medium. After a period of acclimation, for at least 2 weeks, each culture was sampled four times during 1 week. The data were averaged over these four samples, and these average values per culture were used in the statistical analysis.

2.3. Phosphate experiments

Microcystis PCC 7806 and *Anabaena* 90 were grown in continuous cultures under phosphorus-limited conditions. The flat culture vessels were based on the design of Huisman et al. (2002), with a working volume of 1.85 l and a continuous supply of phosphorus-limited O2 medium containing 6 μM phosphate. Temperature was kept constant at 22 ± 1 °C by a water jacket placed between the culture vessel and the light source. The light source consisted of four white fluorescent tubes (Philips PL-L/24W/840/4P) directed towards the front of the culture vessel. The average photon irradiance was calculated according to Eq. (1). Average photon irradiance was I_{avg} = 100 μmol m^{-2} s^{-1} (I_{in} = 150 μmol m^{-2} s^{-1}) for the *Microcystis* cultures, and I_{avg} = 22.5 μmol m^{-2} s^{-1} (I_{in} = 44 μmol m^{-2} s^{-1}) for the *Anabaena* cultures. A day–night cycle of 12-h light/12-h dark was imposed. The cultures were aerated with a continuous airflow to ensure homogeneous mixing and to provide sufficient amounts of carbon dioxide for photosynthesis. The dilution rates of two continuous cultures with *Microcystis* were set at D = 0.21 d^{-1} and 0.35 d^{-1}, respectively, while the dilution rates of two continuous cultures with *Anabaena* were set at D = 0.20 d^{-1} and 0.26 d^{-1}. A higher dilution rate implies that, at steady state, phosphate concentrations in the culture vessel are typically slightly higher. Once the cultures reached steady state, for at least 2 weeks, each culture was sampled four times during 1 week. The data were averaged. Phosphate concentrations were analyzed according to Murphy and Riley (1962).

2.4. Temperature experiments

The effect of temperature on the peptide production of *Microcystis* PCC 7806 and *Anabaena* 90 was investigated in batch cultures at 20 ± 1 °C and 30 ± 0.5 °C, respectively. Temperature was kept constant by performing the experiment in an orbital incubator (Gallenkamp). The batch cultures were run in triplicate, and were inoculated with *Microcystis* and *Anabaena* at an optical density of OD_{750} = 0.025 cm^{-1}. The batch cultures were sampled during the exponential growth phase, at an optical density of OD_{750} = 0.1 cm^{-1}. Standard O2 medium was used. A day–night cycle of 12-h light/12-h dark was imposed, with an incident irradiance of I_{in} = 40 μmol m^{-2} s^{-1} during the day period.

2.5. Growth rates and toxin production rates

To quantify cyanobacterial biomass in the experiments, biovolumes were measured using an automated cell counter (Casy 1 TTC, Schärfe System), with a 60-μm capillary for *Microcystis* cells and a 150-μm capillary for *Anabaena* filaments. The specific growth rate, μ, was calculated according to the following equation:

$$\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} + D \quad (2)$$

where x_1 and x_2 are the biovolumes measured at times t_1 and t_2, respectively, and D is the dilution rate.

The peptide production rate was calculated as the product of peptide content and specific growth rate.

2.6. Peptide analysis

For analyses of cell-bound peptides, 15 ml of culture suspension was filtered in triplicate using Whatman GF/C filters (pore size ~1.2 μm). Filters were freeze dried and stored at −20 °C. Peptides were extracted in 50% methanol (three extraction rounds) as described by Fastner et al. (1998) with an extra step for grinding of the filters in a Mini Beadbeater (Biospec products) with 0.5 mm silica beads (Tonk et al., 2005). Dried extracts were stored at −20 °C and dissolved in 50% MeOH for analysis of peptide contents using high performance liquid chromatography (HPLC) with photodiode array detection (Kontron instruments). The *Microcystis* PCC 7806 extracts were separated using a 30–70% acetonitrile gradient, and the *Anabaena* 90 extracts were separated using a 20–60% acetonitrile gradient, both with 0.05% (v/v) trifluoroacetic acid at a