Available online at www.sciencedirect.com
SCIENCE DIRECT®
Harmful Algae 4 (2005) 1044–1052
HARMFUL ALGAE
www.elsevier.com/locate/hal

Molecular characterization of *Cylindrospermopsis raciborskii* strains isolated from Portuguese freshwaters

E. Valério [a], P. Pereira [b], M.L. Saker [c], S. Franca [b], R. Tenreiro [a,*]

[a] *Universidade de Lisboa, Faculdade de Ciências, Centro de Genética e Biologia Molecular, and Instituto de Ciência Aplicada e Tecnologia, Edifício ICAT, Campus da FCUL, Campo Grande, 1749-016 Lisboa, Portugal*
[b] *Instituto Nacional de Saúde Dr. Ricardo Jorge, Av. Padre Cruz, 1649-016 Lisboa, Portugal*
[c] *Centro de Investigação Marinha e Ambiental, Rua dos Bragas 289, 4050-123 Porto, Portugal*

Received 5 August 2004; received in revised form 28 February 2005; accepted 10 March 2005

Abstract

Cylindrospermopsis raciborskii is a toxic bloom forming cyanobacteria that is a common component of the phytoplankton assemblage in temperate freshwaters, as well as in temperate climates. This species is of major concern in public health, due to its known ability to produce toxins, including cylindrospermopsin and paralytic shellfish poisoning toxin (PSP).

In this study, M13 PCR fingerprinting, ERIC PCR fingerprinting and amplification of the internal transcribed spacer (ITS) region were used to characterize nine cultured strains of *C. raciborskii*, sourced from several freshwater lakes and rivers in Portugal, and two other Australian. Strains belonging to other taxa including *Microcystis aeruginosa*, *Aphanizomenon* spp., *Planktothrix agardhii* and *Oscillatoria neglecta* were also analysed to evaluate the taxonomical potential of the fingerprinting methods.

Data obtained from genomic fingerprinting were used to perform hierarchical cluster analysis and demonstrated ability to differentiate strains at intra-specific level. However, the high level of variability prevents their use as an identification tool. ITS amplification displayed intra-specific polymorphism both in number and length of the obtained amplicons, but revealed itself as a good method for strain clustering. The unsuccessful amplification of peptide synthetase (PS) and polyketide synthase (PKS) genes pointed to the inability of Portuguese *C. raciborskii* strains to produce cylindrospermopsin. HPLC analysis further confirmed this lack of toxicity, since negative results were obtained for cylindrospermopsin and PSP toxins.

Keywords: *Cylindrospermopsis raciborskii*; ERIC PCR fingerprinting; ITS; M13 PCR fingerprinting; *PS* and *PKS* genes; PSP toxins

1. Introduction

Cylindrospermopsis raciborskii is a well-known toxic bloom-forming cyanobacteria, originally described as a species of tropical origin (Woloszynska, 1912), but increasingly found across a wide range of

* Corresponding author. Tel.: +351 21 750 00 06; fax: +351 21 750 01 72.
E-mail address: rptenreiro@fc.ul.pt (R. Tenreiro).

1568-9883/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.hal.2005.03.002

latitudes. This species is of major concern from a water quality and public health perspective, due to its known ability to produce toxins, including the potent hepatotoxic alkaloid cylindrospermopsin (Hawkins et al., 1985; Li et al., 2001; Saker and Neilan, 2001; Fastner et al., 2003), and the highly toxic paralytic shellfish poisons (PSP) (Lagos et al., 1999). Suspected causation of human sickness by cylindrospermopsin (Hayman, 1992) and cattle mortality associated with *C. raciborskii* (Saker et al., 1999) has, so far, been restricted to Australia. However, the recent reports of *C. raciborskii* from many temperate countries have highlighted the invasive nature of this species, spreading worldwide.

Saker et al. (2003) recently reported the occurrence of *C. raciborskii* in several Portuguese water bodies used for potable and recreational purposes. Although tests for the presence of cylindrospermopsin and PSP were negative, a number of strains grown in pure culture showed atypical toxicity by mouse bioassay, suggesting that a new type of toxin should be present. While the chemical structure of the causative compound(s) was not determined, this report drew attention to the need for a comprehensive research on the genetic variability of Portuguese *C. raciborskii* and pointed to the need for an adequate monitoring of this cyanobacterium in freshwater reservoirs.

So far, several molecular methods have been used to characterize *C. raciborskii*, including the genetic analysis of the 16S rRNA (Chonudomkul et al., 2004; Saker and Neilan, 2001) and *rpoC1* (Wilson et al., 2000) genes, which display a considerable consensus within the species. On the contrary, other genomic targets such as *Hip1* (Saker and Neilan, 2001; Smith et al., 1998), STRR sequences (Fergusson et al., 2000; Wilson et al., 2000), *nifH* (Dyble et al., 2002) and PC-IGS (Baker et al., 2001; Dyble et al., 2002) showed significant variations among different strains of *C. raciborskii*.

The M13 PCR fingerprinting has never been used in cyanobacteria, although its use in other bacteria (Grif et al., 1998) and yeasts (Valério et al., 2002) pointed to its potential as an identification tool. On the other hand, techniques based on highly repetitive sequences such as enterobacterial repetitive intergenic consensus (ERIC) have already been used for differentiation of some cyanobacterial genera showing different profiles for all the strains tested (Lyra et al., 2001). The amplification of the internal transcribed spacers (ITS) of rDNA operons from planktonic heterocystous cyanobacteria (Iteman et al., 2002) also showed that these regions displayed size variability.

In the search for molecular tools able to differentiate the toxic from non-toxic strains of *C. raciborskii*, nine cultured strains of this species, sourced from several Portuguese freshwater lakes and rivers, were characterized by using M13 and ERIC fingerprinting and amplification of ITS region, techniques that have not been applied yet to this particular species. The Portuguese isolates were compared with two Australian ones and strains belonging to other taxa, including *Microcystis aeruginosa*, *Aphanizomenon* spp., *Planktothrix agardhii* and *Oscillatoria neglecta*, were also analysed in order to evaluate the taxonomical potential of these molecular methods.

The ability of *C. raciborskii* to produce cylindrospermopsin was evaluated by the amplification of the polyketide synthase (PKS) and peptide synthetase (PS) genes. Detection of cylindrospermopsin and PSP toxins was performed by HPLC analysis.

2. Materials and methods

2.1. Strains

The strains used in this study are listed in Table 1, including their code, origin and references to toxicity assessment. Lyophilised samples of the isolates were obtained as described by Saker et al. (2003). All strains were identified morphologically at species level. All *C. raciborskii* strains shared common morphological traits and presented straight trichome morphology, as described for this species.

2.2. DNA extraction

Genomic DNA of cyanobacterial strains was extracted following the method described by Pitcher et al. (1989), with some modifications. Briefly, an aliquot of 100 μl of lyophilised culture was suspended in a lysis buffer containing 50 mM Tris, 250 mM NaCl, 50 mM EDTA, 0.3% SDS, pH 8 and mechanically broken with glass beads (400–600 μm) by vortex shaking for 2 min. The suspension was incubated for 1 h at 65 °C. After new vortex shaking for 2 min, 1000 μl of GES (5 M guanidium thiocyanate, 100 mM