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Toxicity phenotype does not correlate with phylogeny of *Cylindrospermopsis raciborskii* strains

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Abstract

Cylindrospermopsis raciborskii is a species of freshwater, bloom-forming cyanobacterium. *C. raciborskii* produces toxins, including cylindrospermopsin (hepatotoxin) and saxitoxin (neurotoxin), although non toxin-producing strains are also observed. In spite of differences in toxicity, *C. raciborskii* strains comprise a monophyletic group, based upon 16S rRNA gene sequence identities (greater than 99%). We performed phylogenetic analyses; 16S rRNA gene and 16S-23S rRNA gene internally transcribed spacer (ITS-1) sequence comparisons, and genomic DNA restriction fragment length polymorphism (RFLP), resolved by pulsed-field gel electrophoresis (PFGE), of strains of *C. raciborskii*, obtained mainly from the Australian phylogeographic cluster. Our results showed no correlation between toxic phenotype and phylogenetic association in the Australian strains. Analyses of the 16S rRNA gene and the respective ITS-1 sequences (long L, and short S) showed an independent evolution of each ribosomal operon. The genes putatively involved in the cylindrospermopsin biosynthetic pathway were present in one locus and only in the hepatotoxic strains, demonstrating a common genomic organization for these genes and the absence of mutated or inactivated biosynthetic genes in the non toxic strains. In summary, our results support the hypothesis that the genes involved in toxicity may have been transferred as an island by processes of gene lateral transfer, rather than convergent evolution.

Keywords: Phylogeny; Cylindrospermopsis raciborskii; PFGE; ITS; lateral gene transfer; 16S Rdna

Introduction

Cylindrospermopsis raciborskii Seenayya *et* Subba Raju [37] is defined as a planktonic, filamentous, nitrogen-fixing, terminal heterocystous, freshwater, bloom-forming cyanobacterium. Strains of *C. raciborskii* present two morphologies, with straight or coiled

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trichomes (Table 1), and produce hepatotoxin cylindrospermopsin (CYL), a potent protein synthesis inhibitor [13], paralytic shellfish poisoning (PSP) toxins [23], or do not produce toxins. Since its first appearance in the tropical island of Java (Indonesia) [37], blooms of *C. raciborskii* have been reported in diverse countries of tropical, as well as temperate conditions [30]. Besides this temperature dependence, *C. raciborskii* can grow under different nutrient conditions, with urea, nitrate or ammonium as well as atmospheric nitrogen [12,33] and with low phosphate concentrations [19], making water supplies favorable places for the growth of this cyanobacterium. The occurrence of a bloom of *C. raciborskii* in a water supply in Palm Island, Australia was associated with the first case of CYL human intoxication [6], which caused concern among health authorities. Despite the differences in morphology and growth conditions, toxicity and geographical distribution, the 16S rRNA gene sequences of strains of *C. raciborskii* are homogeneous. Neilan et al. [28] studied the genotypic variation between strains isolated from several geographic regions, finding 99.1% sequence identity in the 16S rRNA genes. Similarly, studies of strains isolated in Australia exhibited not less than 99.8% sequence identity, demonstrating that *C. raciborskii* is a well-defined species [28]. The phylogenetic relationships observed between *C. raciborskii* strains analyzed so far have revealed a phylogeographic relationship, although correlations between phylogenetic association (using markers from complete genome sequences) and toxigenic phenotypes had not been properly explored.

Pulsed-field gel electrophoresis (PFGE) is a genomic tool widely used in epidemiological analyses of pathogenic bacteria, due to its high discriminatory power [5]. PFGE restriction patterns may be used for inferring phylogenetic relationships between closely related strains. In the case of cyanobacteria, PFGE has been useful for discerning relevant information on genome size and for constructing physical and genetic maps of cyanobacterial chromosomes [9]. Nevertheless, to date, no PFGE studies have been performed on *C. raciborskii*. We investigated the genetic variability and the genome organization in *C. raciborskii* strains, mainly in Australian phylogeographic groups, and analyzed whether the toxic phenotype is correlated with the phylogenetic relationships.

The goal of this study was to determine whether the presence of toxicity genes in *C. raciborskii* is the result of convergent evolution or lateral gene transfer (LGT). The first step corresponded to a phylogenetic analysis based on 16S rDNA plus the 16S-23S rRNA gene internally transcribed spacer (ITS-1) sequence and the restriction fragment length polymorphism (RFLP) of the complete genome resolved by PFGE. The second step was to explore, by Southern blot hybridization, the number and the organization of 16S rRNA genes and detect in toxic and non toxic strains the presence of three genes: *aoaA*; *aoaB;* and *aoaC* genes; encoding an amidinotransferase, a hybrid non-ribosomal peptide synthetase (NRPS)/polyketide synthase (PKS) and a PKS, respectively, putatively involved in the CYL biosynthetic pathway [21].

Materials and methods

Strains and culture conditions

Non-axenic cultures of *C. raciborskii* and *Anabaena circinalis* were obtained from the culture collection of the Commonwealth Scientific and Industrial Research Organisation (CSIRO) (Table 1). *C. raciborskii* strain D9 corresponds to a clonal culture isolated from the mixed culture SPC-338, obtained from the Billings water reservoir, Sao Paulo, Brazil. The strain D9 is genetically indistinguishable of strain C10, previously described by Castro et al. [8]. More information, including strain source, morphology and toxicity of strains are detailed in Table 1. Cyanobacteria were cultured in 250 ml flasks containing 100 ml of MLA medium [8], without aeration, at 25–28 °C, under fluorescent light with a photon flux density of 40 μmol m^{-1} s and a 12:12-h light/dark cycle.

DNA extraction and PCR amplification

Genomic DNA was extracted from 20 ml of fresh cyanobacterial culture, using the CTAB method described by Ausubel et al. [3]. PCR-amplifications of 16S rRNA genes and 16S-23S rRNA gene ITS-1 regions were performed with primers 27F and 340R (Table 2). The PCR reagents for each amplification were: 0.25 U Taq DNA polymerase (Invitrogen®); 3 μl 10X PCR buffer (Invitrogen®); 2.5 mM MgCl (Invitrogen®); 0.4 μM primers; and 0.93 μM of each deoxynucleotide. Thermal cycling was performed in an Eppendorf Mastercycler, under the following conditions: initial DNA denaturation for 3 min at 95 °C; primer annealing for 6 min at 58 °C; primer-extension for 1.5 min at 72 °C; a second cycle of 1.5 min at 95 °C; 3 min at 58 °C; 1.5 min at 72 °C; followed by 29 cycles of 1.5 min at 95 °C; 1.5 min at 58 °C; and 1.5 min at 72 °C; with a final elongation of 10 min at 72 °C. PCR products were checked by electrophoresis in 8% polyacrylamide gels stained with silver nitrate [4].

The PCR reaction for the analyses of putative CYL genes was performed with primers previously described by Kellmann et al. [21], in 30 μl reaction volumes containing: 1X *Taq* polymerase buffer; 2.5 mM $MgCl_2$; 0.46 μM of each dNTP; 1 pmol forward and reverse