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Cyanotoxin production and phylogeny of benthic cyanobacterial strains isolated from the northeast of Brazil

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ABSTRACT

Most of the knowledge about cyanobacteria toxin production is traditionally associated with planktonic cyanobacterial blooms. However, some studies have been showing that benthic cyanobacteria can produce cyanotoxins. According to this, we aimed to evaluate the production of microcystins and saxitoxins in benthic cyanobacteria isolated from aquatic ecosystems in the Northeast of Brazil and to use a polyphasic approach for their identification. Forty-five clonal strains were isolated from rivers and water supply reservoirs, and identified using morphological and molecular phylogenetic characteristics. In order to evaluate the toxins production, the strains were screened for genes involved in the biosynthesis of microcystins and saxitoxins, positive results were confirmed and cyanotoxins quantified using HPLC. Eight species were identified belonging to the Phormidiaceae, Pseudanabaenaceae and Nostocaceae families. This is the first study in Brazil that shows that strains from the *Geitlerinema* genus correspond to at least three phylogenetic lineages, which possibly correspond to three distinct species to be subsequently reclassified. The strains that showed one of the genes involved in the cyanotoxins production were analyzed by HPLC and *Geitlerinema amphibium*, *Geitlerinema lemmermannii*, *Cylindrospermum stagnale* and *Phormidium uncinatum* were identified as producing one or more saxitoxins variants. Thus, this is the first report of saxitoxins production for those first three species and the first report in Brazil for *P. uncinatum*.

1. Introduction

Among approximately 150 described genera of cyanobacteria, 40 are known to produce toxic compounds that can impact terrestrial and water-based organism (Van Apeldoorn et al., 2007). These toxins can cause dermatitis, gastroenteritis, others have antimicrobial and cytotoxic activities and some inhibit microalgae and macrophytes growth (Codd et al., 2005). A subset of these toxins, known as cyanotoxins, has attracted significant scientific and management attention due to their toxicity to humans and animals. Cyanotoxins include: neurotoxins (saxitoxins, anatoxin-a, and anatoxin-a(s)) and hepatotoxins (microcystins, cylindrospermopsin and nodularins). The biological function and ecological role of these toxins for cyanobacteria are still unclear (Chorus and Bartrum, 1999); however, some recent studies on microcystins suggest protection against reactive oxygen species (Zilliges et al., 2011).

Globally planktonic cyanobacterial blooms, which occur generally in lentic or semilotic water bodies, have been considered a risk to the health of humans and animals for many decades (Chorus and Bartrum, 1999). In Brazil, this perception was intensified after the fatal poisoning caused by microcystins in at least 65 renal patients in a hemodialysis clinic in Caruaru, Pernambuco, Brazil (Carmichael et al., 2001). As a result of this

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tragedy, monitoring of planktonic species of cyanobacteria and cyanotoxins in drinking water supply reservoirs is mandatory in Brazil (BRASIL, 2011). Ordinance 2.914/11 requires that when cyanobacterial cells concentrations exceed 20,000 cells per milliliter, analysis of microcystins and saxitoxins must be undertaken in tap water and it also recommends analysis of anatoxin-a(s) and cylindrospermopsin.

However, little is known about the extent and frequency of toxin production by benthic cyanobacteria in Brazil despite records of deaths and poisonings of various animals around the world caused by benthic species (Edwards et al., 1992; Mez et al., 1997; Hamill, 2001; Gugger et al., 2005; Izaguirre et al., 2007; Quiblier et al., 2013). Benthic cyanobacteria are now known to produce most of the cyanotoxins produced by planktonic species, including: anatoxin-a and homoanatoxin-a (Edwards et al., 1992; Hamill, 2001; Gugger et al., 2005; Wood et al., 2007), saxitoxins (Onodera et al., 1997; Teneva et al., 2005; Smith et al., 2011) cylindrospermopsins (Seifert et al., 2007), microcystins (Mez et al., 1997; Mohamed et al., 2006; Izaguirre et al., 2007; Mohamed and Al Shehri, 2010) and nodularins (Wood et al., 2012).

Baker et al. (2001) reported a case in which pieces of benthic *Phormidium* mats detached from the sediment and entered in the public water supply. Bioassays conducted on mice found that the samples with *Phormidium* were producing toxins, which could not be identified. Wood and Young (2011) recently used an *in situ* technique known as solid phase adsorption toxin tracking technology (SPATT) and showed that cyanotoxins are released into the water from benthic cyanobacterial mats, highlighting the risk they may pose when present in drinking water supplies.

Correct identification of benthic cyanobacteria would help to identify potential toxin producers species, however, the identification is challenging when based solely on morphological features, particularly the Oscillatoriales order. Many benthic species are from this order and recent researches have demonstrated the benefits of using a polyphasic approach for their identifications (Komárek, 2003; Willame et al., 2006; Heath et al., 2010).

In Brazil few studies have been undertaken on benthic species. The only studies published so far were those of Fiore et al. (2009), who reported the production of microcystin-LR by a strain of *Fischerella* sp. isolated from a small reservoir supply in Piracicaba, São Paulo State, and Genuário et al. (2010), who reported the production of microcystin-YR by a strain of *Nostoc* sp. also isolated from a reservoir in Piracicaba. Thus, the aim of the present study was to isolate benthic cyanobacteria from the Mundaú river basin, Pernambuco State, and the Environmental Protection Area of Araripe, Ceará State to verify the presence of microcystins and saxitoxins, the main toxins found in Brazil, in the isolates and use a polyphasic approach for their identification.

2. Materials and methods

2.1. Sites and sample collection

Samples were collected on May 2011 from seven sampling sites distributed in rivers and drinking water reservoirs in the Mundaú River basin in the Pernambuco semi-arid region and three sites in the Environmental Protection Area (EPA) of Araripe, Ceará, Brazil (Table 1). At each site, sediment samples or benthic scrapings from rocks, macrophytes or the walls of the dams were collected using sterile spatulas. Samples were placed in sterile plastic bags (NASCO, 100 mL) and transported to laboratory in coolers at ambient temperature. In the laboratory, aliquots of samples were transferred to 15 mL borosilicate glass tubes containing 5 mL of ASM-1 medium for subsequent isolation, also, aliquot of each sample was fixed in acetic Lugol (5%) for later species identification.

2.2. Culture and strains isolation

Tubes containing the field samples were vortexed for 1 min to separate the aggregated trichomes. Aliquots were placed on glass slides and observed under an optical microscope (Zeiss AxioScope.A1) at 200× magnification. Free floating trichomes were transferred successively using a Pasteur pipette to drops of ASM-1 medium (Gorham et al., 1964) until only one trichome was left in the drop. Each single trichome was transferred to a 5 mL borosilicate glass tubes containing 5 mL of ASM-1 medium and cultured in chambers (Marconi MA402) at $25 \pm 0.1\ ^{\circ}C$, 80 μmol photons $m^{-2}\ s^{-1}$ – 12 h light:dark.

Additionally, aliquots of the previous isolated samples were spread on Petri plates containing ASM-1 solid medium (1% agar) aiming to remove any planktonic contamination (*e.g.* planktonic algae). The plates were observed under an inverted microscope (Zeiss Axiovert 40 CFL) and solely trichomes were removed from the plates using a sterile platinum loop and transferred to glass tubes containing 5 mL of ASM-1 medium and incubated using the conditions described above. The isolation process of the 45 strains took six months.

Successfully isolated strains were cultured for approximately ten days in 2 liter flasks containing 1.6 L of ASM-1 medium at $26 \pm 2\ ^{\circ}C$, 80 μmol photons $m^{-2}\ s^{-1}$, 12 h dark:light and aeration to obtain sufficient biomass for toxins analysis and DNA extraction. At the end of the exponential growth phase or early stationary phase, an aliquot of the material was removed for DNA extraction and the remainder centrifuged (18,000 × *g*, 15 min; Thermo Scientific Evolution RC) and the samples frozen (−18 °C) for subsequent lyophilization (Terroni, LD1500) and toxins extraction.

2.3. Taxonomic identification

Morphological features including trichome and filament sizes, cells width and length, species motility and, when present, heterocytes and akinetes were used for species identification according to the specific literature. At least 50 cells and 30 trichomes dimensions were measured for each strain. Whenever possible, natural population samples were also used to confirm species identification. Photos and measurements were taken using Axion Vision 40 software, V. 4.8.2.0.

2.4. Extraction, amplification and DNA sequencing

DNA was extracted from the 45 isolated strains using the Cetyl Trimethyl Ammonium Bromide (CTAB) method described in Rogers and Bendich (1985). PCR amplifications were performed in Eppendorf Mastercycler® Pro thermocycler, using Invitrogen Supermix® following manufacturer's recommendations. The 16S rDNA was amplified using the primers 27F1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and 809R (5′-GCTTCGGCACGGCTCGGGTCGATA-3′) (Jungblut et al., 2005). All primers were purchased from Integrated DNA Technologies, Inc. PCR amplicons used for sequencing were purified using PureLink commercial kit (Invitrogen, Carlsbad, CA, USA).

2.5. Sequences analysis and phylogenetic

The 16S rDNA region of the strains was sequenced bidirectionally (Macrogen Inc., Seoul, Korea). Sequences were aligned using ClustalW and manually inspected in the BioEdit program (Hall, 1999) and their similarity compared using the BLAST (Basic Local Alignment Search Tool) with the other sequences in GenBank. Phylogenetic trees were constructed using Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods, with the PAUP version 4.02b (Swofford, 1998), and Bayesian Inference (BI), using MrBayes