Cylindrospermopsin and Saxitoxin Synthetase Genes in Cylindrospermopsis raciborskii Strains from Brazilian Freshwater

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Abstract

The Cylindrospermopsis raciborskii population from Brazilian freshwater is known to produce saxitoxin derivatives (STX), while cylindrospermopsin (CYP), which is commonly detected in isolates from Australia and Asia continents, has thus far not been detected in South American strains. However, during the investigation for the presence of cyrA, cyrB, cyrC and cyrJ CYN synthetase genes in the genomes of four laboratory-cultured C. raciborskii Brazilian strains, the almost complete cyrA gene sequences were obtained for all strains, while cyrB and cyrC gene fragments were observed in two strains. These nucleotide sequences were translated into amino acids, and the predicted protein functions and domains confirmed their identity as CYN synthetase genes. Attempts to PCR amplify cyrJ gene fragments from the four strains were unsuccessful. Phylogenetic analysis grouped the nucleotide sequences together with their homologues found in known CYN synthetase clusters of C. raciborskii strains with high bootstrap support. In addition, fragments of sxtA, sxtB and sxtI genes involved in STX production were also obtained. Extensive LC-MS analyses were unable to detect CYN in the cultured strains, whereas the production of STX and its analogues was confirmed in CENA302, CENA305 and T3. To our knowledge, this is the first study reporting the presence of cyr genes in South American strains of C. raciborskii and the presence of sxt and cyr genes in a single C. raciborskii strain. This discovery suggests a shift in the type of cyanotoxin production over time of South American strains of C. raciborskii and contributes to the reconstruction of the evolutionary history and diversification of cyanobacterial toxins.

Introduction

The cyanobacterial genus Cylindrospermopsis (Woloszynska) Scenanya and Subba Raju [1] belongs to the order Nostocales, family Nostocaceae [2,3]. To date, 10 species have been described, and all of them have been found in the phytoplankton community of freshwater environments [3]. C. raciborskii has been designated as the type species and the C. raciborskii strain AWT205, which was isolated from an ornamental lake (Oatley Pond) in Sydney, Australia [4,5], as the recognized type strain. C. raciborskii has received attention in the last decade due to its frequent dominance in freshwater blooms and its ability to synthesize cyanotoxins. The production of cylindrospermopsin (CYN) and saxitoxin (STX) by this cyanobacterial species has been reported, but in distinct strains [6,7]. Both toxins are synthesized on large modular non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) enzyme complexes [8]. CYN is a cyclic sulfated guanidine alkaloid that inhibits glutathione, cytochrome P450 and protein synthesis, causing injury and cell necrosis mainly in liver, kidneys, thymus and heart of vertebrates [9–11]. The gene cluster for the biosynthesis of cylindrospermopsin (cyr) in C. raciborskii strains spans 42-kb encoding 15 open-reading frames (cyrA-O) [12]. STX is also an alkaloid but acts as a neurotoxin that blocks neuronal sodium, potassium and calcium channels, affecting the propagation of nerve impulses resulting in neuromuscular paralysis [13,14]. Thereby, these two cyanotoxins found in aquatic environments are a potential risk for human health.

Various strains of C. raciborskii are found colonizing eutrophic reservoirs in tropical, subtropical and temperate regions [15–19]. In Brazil, C. raciborskii is the main species found in freshwater bodies [7,16,20–31], although the occurrence of C. philippinensis, C. catenata and C. acuminato-crispa has also been reported [30,32,33]. The most studied Brazilian strain of Cylindrospermopsis is C. raciborskii T3, isolated in 1997 from the Taquacetuba branch of the Billings reservoir in São Paulo municipality [34]. Surprisingly, this strain was found to produce the neurotoxin saxitoxin and some of its derivatives [7]. Unlike the South American isolates, the C. raciborskii strains originated from Australia and Asia were found to synthesize the alkaloid cylindrospermopsin [6,35–39]. The elucidation of the saxitoxin biosynthesis gene cluster (sxt) was based on the C. raciborskii T3 genomic DNA and comprises approximately 35-kb encoding for 31 open reading frames [40]. Chemical analyses conducted on other C. raciborskii Brazilian isolates also
confirmed the production of saxitoxin and its analogues [7,41,42]. Although there are reports on the occurrence of CYN in environmental samples [43,44], the production of this toxin by Brazilian C. raciborskii isolates has not been confirmed. In order to better understand this, four C. raciborskii strains were investigated for the presence of genes associated with the biosynthesis of CYN and SXT analogues. In this way, the cyrA, cyrB, cyrC and cyrJ genes, considered as being exclusives of CYN producers [12,45,46], were assessed, as well as sxtA, sxtB and sxtC genes. Both cyanotoxin groups were also evaluated by LC-MS analysis, and all strains were identified by both morphological and molecular analyses.

**Methods**

**Cyanobacterial strains and morphological identification**

The Brazilian cyanobacterial strains used in this study are shown in Table 1. The strains CENA302, CENA303 and CENA305 were isolated from water samples by transferring single *Cylindrospermopsis* trichomes using a sterilized Pasteur pipette to sterile test tubes containing 9 mL of ASM-1 [47] liquid medium. The trichomes were repeatedly transferred into new ASM-1 medium until a pure culture was established. Cycloheximide to a final concentration of 75 mg L<sup>-1</sup> was added to inhibit eukaryotic cell growth. The cells were grown under a 14:10 light-dark regime, with continuous aeration in a thermostat controlled incubator at 25 ± 1°C. Moreover, fluorescence microscopy studies (AxioSkop 2, Carl Zeiss, Jena, Germany, equipped with digital camera AxioCamMR3, AxioVision program, Rel. 4.6 software) of cyanobacterial cultures were also performed to confirm the absence of other cyanobacteria. Morphology was evaluated using reference literature [3,48].

The *C. raciborskii* strain T3 was isolated in 1997 by Dr. Pedro A. Zagatto (CTESB, São Paulo, Brazil) from Billings reservoir but in the Taquacetuba branch, São Paulo, São Paulo State, and was deposited in the culture collection of the Botanic Institute of São Paulo (CCIBt), Brazil. This strain was originally obtained from the CCIBt on June 4, 2003 and has since been maintained in culture in our lab. The Brazilian *C. raciborskii* strain CYP011K, originally isolated by Dr. Peter Baker from the Julius Lake, Mount Isa, Queensland, was obtained from the laboratory of Prof. Sandra M.F.O. Azevedo (Federal University of Rio de Janeiro, RJ, Brazil). This strain is known as a CYN producer and therefore was considered as a reference sample.

**DNA extraction, PCR amplification, sequencing and phylogeny**

A total of 4.5 mL of cyanobacterial liquid culture was collected at the final exponential growth phase and concentrated by centrifugation (5 min at 13,000 × g). Total genomic DNA was extracted from the pellet using a modified CTAB (cetyl-trimethyl-ammonium bromide)-based extraction method adapted for cyanobacteria [49]. The presence of genes involved in the biosynthesis of *Cylindrospermopsis* and saxitoxin were investigated using the specific primer sets (Table S1). All of the PCR reactions were performed in a 25 µL reaction volume containing 1X PCR buffer, 1.5 U Platinum<sup>®</sup> Taq DNA polymerase (Life Technologies, Carlsbad, CA, USA), 3.0 mM MgCl<sub>2</sub>, 200 µM dNTP, 0.2 µM of each primer and 10 ng of genomic DNA. Thermal cycling was performed in a Techne TC-412 Thermal Cycler (Bibby Scientific Limited, Stone, Staffordshire, England) using the designed primers (for sxt genes) under the following conditions: sxtA (C-terminal domain), 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 61 °C for 30 s, 72 °C for 30 s, and final extension of 72 °C for 7 min; sxtB, 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 61 °C for 30 s, 72 °C for 90 s, and final extension of 72 °C for 7 min; sxtC, 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 53 °C for 1 min, 72 °C for 1 min, and a final extension of 72 °C for 7 min. Thermal cycling conditions for cyrJ gene amplification were 94 °C for 3 min, followed by 30 cycles of 94 °C for 20 s, 53 °C for 1 min, 72 °C for 1 min, and a final extension of 72 °C for 7 min, adapted from Mazmouz et al., [50]. The cyrA, cyrB and cyrC fragments were amplified using the conditions previously described by the authors [51], with the exception of annealing temperature that was 57 °C.

The amplified gene fragments were cloned using pGEM<sup>®</sup>-T Easy Vector Systems (Promega, Madison, WI, USA). Competent *Escherichia coli* DH5α cells were transformed, and recombinant plasmids were purified from white colonies by the alkaline lysis method [52]. The cloned PCR products were sequenced using the DYEnamic ET Terminator Cycle Sequencing (GE Healthcare, Little Chalfont, Buckinghamshire, England) using the T7 and SP6 primer sites of the vector. The cycle sequencing reaction was performed with a Techne TC-412 (Bibby Scientific Limited) for 25 cycles of 95°C for 20 s, 52°C for 15 s, and 60°C for 1 min. After the completion of the reaction, a 75% isopropanol wash followed by a 70% ethanol wash was performed to remove residual dye terminators. The purified reaction mixtures were reconstituted in HiDi formamide (Applied Biosystems/Life Technologies, Foster City, CA, USA), and the samples were analyzed in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems/Life Technologies).

The 16S rRNA genes were PCR amplified using the specific primers listed in Table S1 and PCR amplification and sequencing were performed as described previously [53].

The partial nucleotide sequences of the *cyr* and *sxt* genes obtained in this study and reference sequences retrieved from GenBank were aligned, refined, and used to generate phylogenetic trees. Trees were reconstructed with maximum-likelihood (ML)

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**Table 1. Brazilian isolates of *Cylindrospermopsis raciborskii* used in this study.**

| Cyanobacterial strains | Origin | Date of sample collections |
|------------------------|--------|--------------------------|
| *C. raciborskii* CENA302 | Riacho Grande branch, Billings reservoir, São Bernardo do Campo, SP | May 26, 2008 |
| *C. raciborskii* CENA303 | Theobaldo Dick lake, Lajeado, RS | July 3, 2009 |
| *C. raciborskii* CENA305 | Riacho Grande branch, Billings reservoir, São Bernardo do Campo SP | May 26, 2008 |
| *C. raciborskii* T3 | Taquacetuba branch, Billings reservoir, São Paulo, SP | May 7, 1997 |

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and neighbor-joining (NJ) algorithms implemented by the MEGA version 5.0 program package [54] using the Tamura-Nei and p-distance models, respectively. The stability of the phylogenetic relationships was assessed by bootstrapping (1,000 replicates). Moreover, the gene sequences were analyzed by BLASTX (NCBI GenBank database) to compare each of the nucleotide sequence with its translated proteins in all possible reading frames. After identifying the correct reading frame, the nucleotide sequence was translated into protein using the translate tool from ExPASy Proteomic Server (Swiss Institute of Bioinformatics).

Nucleotide sequences generated in this study have been deposited in the NCBI GenBank database under the following accession numbers: JX175238 to JX175243 and KC894581 to KC894586 for the cyr genes; JX175232 to JX175237 and KC894587 to KC894589 for sxt genes; JQ707291 to JQ707296 for 16S rRNA genes.

Chromatographic analyses

Culture samples of the *C. raciborskii* strains CENA302, CENA303, CENA305 T3 and CYP011K (20 days old) were filtered through Millipore glass fiber filters (Millipore, Milford, MA, USA) to harvest cells. Filters were frozen and later extracted with 0.05 M acetic acid by vortexing and cell disruption by sonication in a water bath for 15 min (2 cycles). After centrifugation (10,000 × g, 10 min), supernatants were recovered and filtered (0.45 μm, PVDF, Millipore) into appropriate vials. To assess the intra- and extracellular content of CYN and STX, 30 mL of homogeneous whole cultures were frozen and freeze-dried. After reconstitution and extraction in 1 mL 0.05 M acetic acid, samples were centrifuged (10,000 × g for 10 min) and filtered into appropriate vials.

Chromatographic analyses were performed with a Shimadzu Prominence (Kyoto, Japan) liquid chromatography system equipped with a diode array detector (SPD-M20A) and coupled to an ion trap mass spectrometer (Esquire HCT, Bruker Daltonics, Billerica, MA, USA). The identity of STX derivatives was confirmed by the fragmentation behavior analyzed according to Doerr et al. [55]. Because a standard solution of 7-epi-CYN was not available, efforts were not undertaken to separate this isomer, and therefore the single chromatographic band at 6.7 min for *m/z* 416 was assumed as CYN. A commercially available standard of CYN (Abraxis, Warminster, PA, USA) was also used for identity confirmation.

The saxitoxin analogues were investigated by three complementary analytical methods: two post-column oxidation methods with fluorescence detection (HPLC-FD), according to Diener et al. [56,57], and a method based on hydrophilic interaction liquid chromatography coupled to mass spectrometry (HILIC-MS) following the recommendations of Soto-Liebe et al. [58]. Briefly, the compounds were separated on a Shimadzu Prominance (Kyoto, Japan) liquid chromatography system equipped with a post-column reaction oven and a fluorescence detector (RF-10AX). For HILIC-MS analyses, the chromatography system was coupled to an ion trap mass spectrometer (Esquire HCT, Bruker Daltonics, Billerica, MA, USA) through an electrospray ionization source. The identity of STX derivatives was confirmed by the fragmentation behavior of the [M+H]+ as well as the [M-H]− ions [59]. Commercially available standards of STX derivatives (National Research Council/Institute for Marine Biosciences, Halifax, NS, Canada) were employed in HPLC-FD experiments for compound identification.

Enzyme-linked immunosorbent assay (ELISA)

The presence of STX and CYN were tested with the saxitoxin and cylindrospermopsin ELISA kit (Abraxis LLC, PA, USA). All analyses were performed in accordance with the manufacturer’s instructions.

| Table 2. Size of cylindrospermopsin and saxitoxin gene sequences obtained from the *Cylindrospermopsis raciborskii* strains. |
|---------------------------------------------------------------|
| **Cyanobacterial strains** | **Cylindrospermopsis genes (bp)** | **Saxitoxin genes (bp)** |
| **cyrA** | **cyrB** | **cyrC** | **cyrJ** | **sxtA** | **sxtB** | **sxtL** |
| C. raciborskii CENA302 | 1,106 | 470 | 515 | – | 201 | 336 | 904 |
| C. raciborskii CENA303 | 1,106 | 470 | 515 | – | 200 | 270 | 589 |
| C. raciborskii CENA305 | 1,106 | – | 413* | – | 202 | 305 | 925 |
| C. raciborskii T3 | 1,104 | 521* | 413* | – | 3,738** | 957** | 1,839** |
| C. raciborskii CYP011K | 1,106 | 470 | 515 | 556 | – | – | – |

* no gene detected; * nonspecific sequences; ** complete sequences obtained from GenBank.

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A

sxtA4: 8-amino-7-oxononanoate synthase

B

sxtB: cytidine deaminase

C

sxtf: O-carbamoyl-transferase
Cylindrospermopsin (cyr) and saxitoxin (sxt) genes

The specific primer set (CYLAT-R/CYLAT-F), targeting the gene encoding the amidinotransferase enzyme involved in the biosynthesis of CYN, successfully amplified cyrA genes from the genomes of four Brazilian C. raciborskii strains (CENA302, CENA303, CENA305 and T3) and also from the Australian strain C. raciborskii CYP011K. These PCR products were sequenced and the almost entire cyrA gene sequences were obtained from the four Brazilian C. raciborskii strains (Table 2). However, the cyrB sequence of the T3 strain showed two nucleotide deletions in the position 525 and 1054 (Figure S1), causing a frameshift mutation. As a result, stop codons are replacing amino acid codons and hence the deduced protein sequence was truncated. The identities of the four cyrA sequences with homologous sequences from other CYN-producing closely related strains (C. raciborskii AW7205, C. raciborskii CS505, C. raciborskii CYP011K, R. curvata CHAB1150) were high and were larger than 98.8% (Table S2). However, lower identities were observed with Aphanothece halophila (varying 95.5 – 95.8%) and Oscillatoria sp. strain PC-635 (Figure S1) and PR-1, and in the predicted protein functions and domains agreed with proteins from C. raciborskii CS505 and CS505 (Figure S2), further supporting the identity of the sequences obtained as a CYN synthetase gene. The phylogenetic analysis grouped the cyrA sequences of C. raciborskii Brazilian strains together with other homologous nucleotide sequences from CYN-producing cyanobacteria with highly supported bootstrap value (100% ML and NJ) (Figure 1A). The amidinotransferase enzymes from other organisms grouped separately according to their biosynthetic pathway. Thus, sequences of the sxtG gene, that also encodes an amidinotransferase but is involved in the STX biosynthesis pathway, formed a fully supported clade distantly related to cyrA clade.

Three Brazilian strains (CENA302, CENA303 and T3) and also the Australian CYP011K strain showed positive results for the presence of a region of cyrB gene that encodes an adenylation domain of a NRPS. However, after sequencing the PCR products, the cyrB gene fragment obtained from the C. raciborskii T3 showed no identity with sequences deposited in the GenBank, an indicative of non-specific PCR amplification. The percentage of identity between partial cyrB adenylation domain of CENA302 and CENA303 was 99.4%, and ranged from 84.6 to 99.6% with other cyrB sequences retrieved from GenBank (Table S2). The phylogenetic analysis grouped the cyrB sequences of the two C. raciborskii Brazilian strains together with other homologous nucleotide sequences from CYN-producing cyanobacteria with highly supported bootstrap value (99% ML and NJ) (Figure 1B).

PCR products of a region of the cyrC gene that encodes a ketosynthase domain of a PKS were obtained from the genomes of the four C. raciborskii Brazilian strains as well as from the Australian CYP011K strain. However, after sequencing the amplicons, the sequences of the strains CENA302 and T3, with 99.0% identity between them, showed the highest identity (76.3% and 76.8%, respectively) with the phosphopantothenoylcysteine decarboxylase/phosphopantothenate-cysteine ligase of the Anabaena sp. 90. In the phylogenetic reconstruction of the partial cyrC sequences of the C. raciborskii Brazilian strains CENA302 and CENA303 a highly supported clade (bootstrap value of 99% ML and NJ) was formed with other homologous nucleotide sequences from CYN-producing cyanobacteria (Figure 1C).

Several attempts to amplify fragments of the cyrF gene from all four Brazilian C. raciborskii strains were unsuccessful. Only in the Australian strain C. raciborskii CYP011K (CYN producer used as reference) partial cyrF gene was successfully amplified.

The three sxt gene fragments (sxtA4, sxtB and sxtI) investigated were PCR amplified and sequenced from the genomes of almost all studied cyanobacteria, with the exception of the Australian CYN producer strain CYP011K, in which none of STX genes were detected (Table 2). BLAST analysis showed that the gene sequences of the sxtA4 (8-aminoo-7-oxanonoate synthase - AONS), sxtB (cytidine deaminase) and sxtI (6-carbamoyltransferase) from C. raciborskii CENA302, CENA303 and 305 strains had high similarity with the corresponding sequences from the STX-producing Brazilian strains C. raciborskii T3 and Raphidiopsis brookii D9 (Table S3). In the phylogenetic analysis, the sxt sequences clustered with other sequences of STX-producing cyanobacterial strains with supported bootstrap value (100% ML and NJ) for sxtA4, (99% ML and NJ) for sxtB and sxtI, (Figure 2). The sxt gene sequences from T3 were identified previously [40]. The sxt genes were also compared with sequences that encode for homologous enzymes from other organisms and demonstrate phylogenetic differences according to its biosynthetic pathway. The predicted SxtA4, SxtB and SxtI protein functions and domains agreed with those homologous proteins from T3 and D9 (Table S3).

Chemical and Enzyme Immunoassay analyses of CYN and STX

Despite several attempts to detect CYN and 7-deoxo-CYN derivatives in Brazilian strains, neither compound could be identified. As expected, both compounds were detected in the Australian CYP011K strain in the intra- and extracellular fractions (Figure 3). ELISA immunoassays were unable to detect CYN in the Brazilian strains of C. raciborskii, but showed positive results for Australian strain CYP011K. The lack of cyrF amplification prompted the search for possible CYN derivatives missing the sulfate group at C-12, but all efforts failed to detect such a compound in the Brazilian isolates.

Different chromatographic methods were employed to evaluate the production of saxitoxin analogues in the isolated strains. The post-column oxidation methods with fluorescence detection allowed the identification of the epimers GTX2/GTX3, STX and deSTX in CENA302 (Figure 4A), STX, deSTX, NEO and deNEO in CENA305 (Figure 4B), as well as NEO, STX and deSTX in strain T3 (Figure 4D). These results were further confirmed by HILIC-MS analyses. ELISA immunoassay provided positive results for STX in the strains CENA302, CENA303 and T3. None of the employed methods was able to detected STX analogues in the strains CENA302 and CYP011K.

Morphological and 16S rRNA gene phylogenetic analyses

Morphological analysis of the isolated strains obtained in this study showed that they belong to the order Nostocales, genus Cylindrospermopsis. The filamentous strains presented typical characteristics of the Cylindrospermopsis raciborskii species (Figure 5) such as: subsymmetrical trichomes with cylindrical cells but attenuated and pointed at the ends of trichomes; pale blue-green or yellowish
Figure 3. LC-UV-MS analysis of a freeze-dried culture sample of *C. raciborskii* CYP011K. (A) UV trace at 262 nm and extracted ion chromatograms for CYN (m/z 418) and 7-deoxy-CYN (m/z 400). Product ion spectra and UV absorption spectra are depicted for CYN (B and C) and 7-deoxy-CYN (D and E).

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color; presence of aerotopes; conical terminal heterocytes developed after asymmetrical division of the end cells; and elongated oval akinetes situated adjacent to the heterocyte or the terminal vegetative cell [48,3].

The nearly complete 16S rRNA gene sequences of *C. raciborskii* strains showed high similarity with sequences of *C. raciborskii* from GenBank (Table S4). The identities between the sequences of the four Brazilian *C. raciborskii* strains obtained in this study ranged from 99.1 to 100%. Moreover, these four 16S rRNA sequences displayed similarities varying between 99.2 to 99.6% with the sequence of the type strain *C. raciborskii* AWT205. The 16S rRNA sequences of the CENA302, CENA305 and T3 strains isolated from the Billings reservoir also showed high similarity (99.3%, 99.7% and 99.9%, respectively) with that of the *R. boschii* D9 found in the same reservoir. The 16 rRNA gene sequence of the *C. raciborskii* CYP01K showed 99.6 to 99.8% similarities with other sequences of *C. raciborskii* Australian strains (QHSS/NR/CYL/05, 05E and 23B).

In the phylogenetic tree, the 16S rRNA gene sequences of the *C. raciborskii* strains fall within a highly supported (bootstrap values of 99 and 100%) for ML and NJ algorithms, respectively) major clade containing sequences of planktonic members of *C. raciborskii* isolated from several countries and an internal separated clade with members of *Raphidocapsa* genus (Figure 6). Within this major clade, the Brazilian and Australian strains formed distinct clades according to their origin but with low supported bootstrap. Strains of North American, African, European and Asian origin were mixed in other internal clades. The evolutionary relatedness of the four cyanobacterial strains (*C. raciborskii* AWT205, *C. raciborskii* CS-505, *Aphanizomenon* sp. 10E6 and *Oscillatoria* sp. PCC 6506) with the *cyr* clusters already described can be visualized in the phylogenetic tree. The phylogeny of the polyphyletic genus *Aphanizomenon* that possess several CYN and STX producer strains was also given in the phylogenetic tree. After revision of several genera of cyanobacteria, new designation for some of them was adopted and it is also shown in the phylogenetic tree.

**Discussion**

In this study, cylindrospermopsin synthetase (*cyr*) genes were PCR amplified and sequenced for the first time from the genomes of four non-CYN-producing *C. raciborskii* strains isolated from Brazilian freshwater environments. Of the four *cyr* genes investigated and considered essential for the synthesis of CYN [12,45,46], three were amplified and sequenced from the genomes of two *C. raciborskii* Brazilian strains (CENA302 and CENA305). In other two Brazilian strains (CENA305 and T3) only the almost complete *cyrA* gene sequence was found. In addition, the four *cyr* genes were sequenced from the CYN-producing Australian strain *C. raciborskii* CYP011K. This Australian strain produces two CYN analogues, therefore must encode at least 11 genes (*cyrA, B, C, D, E, F, G, H, I, J, k* and *r*), that appear to be directly implicated in the synthesis of CYN according to the four *cyr* clusters already found in different cyanobacterial CYN-producing genera [60].

The almost complete *cyr* sequences obtained from the four non-CYN-producing Brazilian *C. raciborskii* strains (CENA302, CENA303, CENA305 and T3) resemble those identified in the *C. raciborskii* strains AWT205 and CS-505 [12,61]. The *cyrA* amplification product of T3 strain was achieved using more stringent annealing temperature than that recommended [51]. However, the truncated *cyrA* sequence of the T3 with the absence of two nucleotides indicated that this gene underwent reduction and was inactivated. The *cyrA* gene identified in the type strain *C. raciborskii* AWT205 is 1,176 bp long and encodes an L-arginine:glycine amidotransferase enzyme responsible for the formation of guanidinoacetate and ornithine from L-arginine and glycine, the first step in the CYN biosynthesis [12,46]. Amidotransferases are a monophyletic group of enzymes distributed among vertebrates, plants and prokaryotes [46]. Furthermore, these enzymes are involved in biosynthesis of the neurotoxin saxitoxin in cyanobacteria (SxtG, L-arginine/L-lysine amidotransferase) and dinoflagellates [40,62]. Despite a high level of conservation with regard to residues involved in catalysis and substrate binding, these enzymes have different substrate specificities and kinetic mechanisms [63]. The CyxA amidotransferase was shown to be unique to the metabolic pathway for biosynthesis of CYN [12,46]. In this manner, the *cyrA* gene sequences obtained in this study formed a fully supported clade in phylogenetic analysis, together with other *cyrA* sequences of CYN-producing cyanobacterial strains retrieved from GenBank, but distantly related to the also fully supported clade containing *sxtG* sequences. This topology is in agreement with *cyrA* and *sxtG* phylogenetic reconstructions performed by Orr *et al.*, [62], who defined the cluster containing the *cyrA* sequences as amidotransferase 2 and that with *sxtG* sequences as amidotransferase 1.

The CyB enzyme (mixed NRPS-PKS) catalyzes the second reaction in the proposed biosynthetic pathway, incorporating an acetate unit into guanidinoacetate [12]. In the present study, the primer set used for detection of *cyrB* gene targeted the NRPS adenylation domain, which is responsible for amino acid recognition and activation. Therefore, this adenylation domain of CyB uses the guanidinoacetate as a substrate for subsequent polyketide extensions. The two partial sequences of *cyrB* adenylation domain found in the genomes of two non-CYN-producing *C. raciborskii* Brazilian strains (CENA302 and CENA305) had high identities to other *C. raciborskii* *cyrB* adenylation domains and formed a fully supported clade in phylogenetic analysis. The partial *cyrB* amplification product of T3 strains was also achieved using more stringent annealing temperature than that recommended [51]. Nevertheless, the partial *cyrB* sequence obtained for T3 showed to be nonspecific amplification that led to a false-positive result. It is worth noting that a study applying neutrality test to the adenylation domain sequences of uncultured *A. ovalisporum*-like *cyrB* obtained from environmental samples indicated that it is under purifying selection. Purifying selection makes sure that deleterious mutations cannot take over a population and that any improved structures, once fixed in a population, are maintained as long as they are needed [64]. Furthermore, the *cyrB* gene of the CYN-producing *Oscillatoria* sp. PCC 6506 showed moderate identity (79%) to the one characterized in the toxic *A. ovalisporum* [50]. This *Oscillatoria* sp. PCC 6506 gene possesses a 150-bp-long GC rich fragment containing repeated sequences which encodes for a proline rich motif (PLLP) repetition that might function as a linker between the ketoreductase and methyltransferase domains of this NRPS-PKS hybrid enzyme. It is thus very likely that the *cyrB* from distinct cyanobacterial genera is evolutionarily related but that it substantially diverged from a common ancestor. The presence of *cyrB* sequence homologous to *cyrB* of the CYN producers was reported before for the non-toxic
strain *C. raciborskii* Hung1 (Hungarian), however, *cyrA* and *cyrC* genes were not detected [51].

The *CyrC*, a polyketide synthase, is the subsequent enzyme proposed in the biosynthesis of CYN. It is responsible for the incorporation of a further acetate unit into guanidinoacetate, while a subsequent keto reduction provides the next intermediate [12]. The primer set used in this study targeted a region of the ketosynthase domain of the *CyrC*, which was highly similar to other *C. raciborskii cyrC* ketosynthase domain and formed a fully supported clade in phylogenetic analysis.

The *cyrJ* gene (780 bp long in the *C. raciborskii* AWT205) encodes a sulfotransferase, a tailoring enzyme responsible for the sulfation of the CYN [12]. The three structural variants of CYN described so far (CYN, 7-epi-CYN and 7-deoxy-CYN) are sulfated [65-67], therefore, the CYN-producing cyanobacteria must have the *cyrJ* gene. The lack of *cyrJ* gene amplification in all four Brazilian *C. raciborskii* strains indicates absence of this gene in the genomes of these cyanobacteria and supports the negative results of chemical analyses. These data also corroborated other studies indicating that *cyrJ* gene is only present in the genome of CYN-producing cyanobacteria [12,50,68]. It remains to be shown if *cyrJ* variants produced by Brazilian *C. raciborskii* strains are replacing a possible cellular function of CYN.

The selective pressures in Brazilian environments that favored STX-producing *C. raciborskii* over CYN-producing strains are unknown. The physiological role of these secondary metabolites is still unclear, despite their effects on animal cells being relatively well understood. Recently, Soto-Liebe et al., [73] suggested STX analogues as protective compounds against elevated salt concentration in the environment. It remains to be demonstrated if STX variants produced by Brazilian *C. raciborskii* strains are replacing a possible cellular function of CYN.

Although the existence of CYN in Brazilian freshwater blooms has been known for several years, *C. raciborskii* strains that synthesize CYN have not been isolated so far. Thus, it is likely that other cyanobacterial genera are responsible for CYN production in Brazilian environments. In a similar way, no CYN-producing strains of *C. raciborskii* have been found in North America and Europe [74–76]. So far, in North America only *Aphanizomenon* strains were found to produce CYN [76,77], while in Europe strains of *Aphanizomenon* [78,79] and *Anabaena* [80] were identified as CYN-producers.

The presence of partial *sxt* gene sequences (*sxtA4, sxtB* and *sxtI*) in the CENA302, CENA303 and CENA305 strains with high similarities to those of other STX-producing strains already
described, as well as the detection of some congeners by chromatographic analyses in the strains CENA302 and CENA305, corroborate previous findings that the Brazilian C. raciborskii strains produce these neurotoxins [7,41,42]. Moustafa and collaborators [70] showed that SxtA is comprised of two distinct regions and resulted from the fusion of two proteins acquired from different bacterial sources. The C-terminal region encodes an enzyme that presents significant identity to class I and II aminotransferases from actinobacteria. This region includes the SxtA4 catalytic domain and presents high similarity to AONS [40,70]. Our phylogenetic analyses demonstrated that sxtA4 gene sequences from cyanobacteria strains are highly conserved and different from the actinobacteria Frankia sp. suggesting that AONS from cyanobacteria is involved only in the STX biosynthesis.

The strain CENA302 was found to produce the epimers GTX2/GTX3, STX and dcSTX while CENA305 produces...
NEO, STX and dcSTX. Low levels of dcNEO (decarbamoyl neosaxitoxin) congener were detected only by HILIC-MS because co-elution hampered its identification in the HPLC-FD methods. A similar toxin profile was reported for *C. raciborskii* strains T2 and T3 [7]. Coincidentally, these four strains were isolated from the Billings reservoir in southeastern Brazil (subtropical climate) but at different locations. Our results of the STX toxin profile of *C. raciborskii* T3 are comparable to those obtained by Soto-Liebe et al., [58]. As noted by these authors, available literature on the toxin profile of T3 has been confusing given that several groups have identified different saxitoxin derivatives. In this study, the production of NEO, STX and dcSTX was confirmed by three complementary analytical techniques while dcNEO was detected only by HILIC-MS.

Previous phylogenetic analysis studies using nucleotide sequences of the fast-evolving 16S-23S internal transcribed spacer, mfiI and gbdH-IGS have shown a separation of *C. raciborskii* strains according to their geographic origin [81–83]. In our study, a phylogenetic tree based on the low-evolving sequences of 16S rRNA gene also showed a geographic separation of Brazilian and Australian strains according to their origin, but with low supported bootstrap. However, strains of North American, African, European and Asian origin were not geographically separated. If geographic isolation, rather than environmental selection, drives diversity, location-specific lineages would arise in different provinces regardless of microhabitat [84]. Nevertheless, studies on global biogeography of the cyanobacterial genera *Chroococcidiopsis* [84] and *Microcystis* [85] concluded that environmental selection drives diversification. Although some studies have shown geographic distribution of *C. raciborskii* strains, a larger number of nucleotide sequences from a variety of regions of different continents are needed to better understand the distribution and evolution of this cyanobacterial species.

**Conclusion**

In this study, we identified and sequenced partial *cyr* and *sxt* synthetase genes of four Brazilian planktonic *C. raciborskii* strains, CENA302, CENA303, CENA305 and T3. Although the occurrence of both neurotoxins (anatoxin-a and homoanatoxin-a) and *cyr* genes has already been documented in a single *Oscillatoria* sp. strain [50], this is the first report to our knowledge of the presence of *sxt* and *cyr* genes in *C. raciborskii* strains. The results obtained here provide the first insight of the presence of CYN genes in *C. raciborskii* strains from an American country and contribute to the reconstruction of the evolutionary history and diversification of cyanobacterial toxins.

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**Author Contributions**

Conceived and designed the experiments: MFF CHR. Performed the experiments: CHR FAD PDCS. Analyzed the data: CHR FAD PDCS EP VRW. Wrote the paper: CHR MFF VRW. Contributed reagents/materials/analysis tools: MFF EP VRW.

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**Supporting Information**

**Figure S1** Alignment of partial *cyr*A nucleotide sequenc-
es showing the two nucleotide deletions in the position 525 and 1054 (red marks) of the *Cylindrospermopsis raciborskii* T3.

**Figure S2** Maximum likelihood phylogenetic trees of *Cyr* (A) and *Sxt* (B) amino acids sequences. The *C. raciborskii* strains used in this study are shown in bold. Bootstrap test (1,000 resamplings) was performed and values >50% for ML and NJ analyses are shown over the nodes.

**Table S1** PCR primer sequences used in this study. (DOCX)

**Table S2** Percentage of identities of *cyr* nucleotide sequences of CYN-non-producing Brazilian strains of *C. raciborskii* with other sequences from CYN-producing cyanobacterial strains. (DOCX)

**Table S3** Percentage of identities of *sxt* nucleotide sequences of *C. raciborskii* Brazilian strains with other sequences from STX-producing cyanobacterial strains. (DOCX)

**Table S4** The 16S rRNA gene sequences identities of the forms described under the genus *Anabaonopsis*. Toxicon 35: 341–346.

**Table S5** The 16S rRNA gene sequences identities among the Brazilian *C. raciborskii* strains and other sequences of related cyanobacterial strains. (DOCX)

**Table S6** The 16S rRNA gene sequences identities among the Brazilian *C. raciborskii* strains and other sequences of related cyanobacterial strains. (DOCX)
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