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Revealing Toxin Signatures in Cyanobacteria: Report of Genes Involved in Cylindrospermopsin Synthesis from Saxitoxin-Producing *Cylindrospermopsis raciborskii*

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ABSTRACT

Cyanotoxins are distinctive molecules in Cyanobacteria whose evolutionary origin, radiation and ecological role are still controversial. The cyanobacterium *Cylindrospermopsis raciborskii* is alternately capable of producing two types of potent toxins, cylindrospermopsin (CYN) or saxitoxin and analogues (SAX). It has been proposed that this species spread to all continents early in its evolutionary history and biogeographical differences in toxin production are found between populations. Most reports indicate that American strains are able to produce SAX but not CYN, while Australian strains are described to produce CYN but not SAX. Here we describe the presence of three genes belonging to the cylindrospermopsin cluster (*cyr*), *cyrA*, *cyrB* and *cyrC*, in two SAX producing South American *C. raciborskii* strains, MVCC14 and MVCC19, which due to their differences in morphology, growth preferences, SAX production and genetic context are defined as different ecotypes. No CYN production was detected in either strain (by ELISA) after growth under nitrogen replete or nitrogen-free nutrient conditions. Phylogenetic analyses of *cyrA*, *cyrB* and *cyrC* partial sequences from both strains showed high similarity (>99%) with CYN genes belonging to *C. raciborskii* strains from Australia and Germany and to *Aphanizomenon* strains. This is the first report of the presence of *cyr* genes in strains known to produce only SAX.

Keywords: Cylindrospermopsin; *Cyr*; American Ecotypes; Cyanotoxins; Blooms

1. Introduction

Secondary metabolites in microorganisms are particularly interesting in the case of cyanotoxins [1] because they can reach high concentrations in cyanobacterial blooms posing a serious threat to human and animal health [2]. Cyanobacterial toxins are produced by strains of different species. The evolutionary spreading of these molecules respond to horizontal or vertical genetic transfer mechanisms depending on the kind of toxin and the ability to produce toxins has been lost repeatedly in different lineages of cyanobacteria [3]. The biological role of cyanotoxins in the ecosystem is still under debate [4] and current hypotheses include predation defense [5], nitrogen storage [6-8], protein-modulating role [9] and chemical signaling [10,11].

There are different kind of cyanotoxins with differences in their chemistry, biosynthesis, regulation and mode of action. Cylindrospermopsin (CYN) ($C_{15}H_{21}N_5O_7S$) is a water-soluble alkaloid that has cytotoxic effects on animals and humans. This molecule is present in several cyanobacterial species such as *Umezakia natans* [12], *Aphanizomenon ovalisporum* and *Apha. flos-aquae* [13-15], *Raphidiopsis curvata* [16], *Anabaena lapponica* and *Ana. bergii* [1,17], *Lyngbya wollei* [18] and *Cylindrospermopsis raciborskii* [19]. One key factor to explain the distribution of toxins among species and geographical regions is to understand their biological role in the organisms. Although the role of CYN is not yet established, a recent study suggests that CYN in *Apha. ovalisporum* is involved in phosphorus acquisition by inducing the secretion of alkaline phosphatase by other phytoplankters [11].

Among the toxic bloom-forming cyanobacteria, *Cylindrospermopsis raciborskii* (Order Nostocales) has been

focus of considerable attention due to its apparent invasive movement from tropical towards temperate lakes in Europe and the Americas [20-24]. In this species, CYN biosynthesis, regulation and export are encoded by the 43 kb-long *cyr* cluster, which comprises 15 open reading frames (ORFs) [25]. The first three genes in CYN biosynthetic pathway encode for an amidinotransferase (*cyrA*), a nonribosomal peptide synthetase/polyketide synthasa hybrid (NRPS, *cyrB*) and a polyketide synthase (PKS, *cyrC*) [19,26], which correspond to the orthologues *aoaA*, *aoaB* and *aoaC* from *Apha. ovalisporum* [25]. The first enzyme needed to start CYN synthesis is amidinotransferase, via an amidinotransfer onto glycine. According to Kellmann, *et al.* [27], the amidinotransferase occurrence in a cyanotoxin gene cluster would be unique to CYN-producing cyanobacteria. The guanidinoacetate formed in the first step is then recruited by the NRPS (*cyrB* gene product) and passed on to the PKS (*cyrC* gene product).

It has been described that *C. raciborskii* strains produce alternatively one of two kinds of toxins, cylindrospermopsin (CYN, hepatotoxin) or saxitoxin and its analogues (SAX, neurotoxins) [28,29], however there are no reports of strains producing both toxins simultaneously. Furthermore, the relationship between phylogeny and toxicity in *C. raciborskii* has not yet been elucidated. CYN-related toxicity in Australian strains has been shown to be unrelated to phylogenetic affiliation, and genes involved in CYN biosynthesis were only present in hepatotoxic strains, suggesting the absence of mutated or inactivated biosynthetic genes in non-toxic strains [30]. In addition, hepatotoxic or CYN-producing strains have been isolated only from Europe, Australia and Asia [31,32], while in the Americas or in Africa have not yet been found [29,31,33]. In addition, strains isolated from the Americas have been shown to produce only SAX [29,33,34]. However, a recent study detected CYN in natural samples from blooms when *Aphanizomenon* and *Cylindrospermopsis* were detected (Bittencourt-Oliveira *et al*. 2011), suggesting that some Brazilian *Cylindrospermopsis* strains could produce CYN.

Generally, the sequence similarity between toxin-encoding genes is higher than that found for the 16S rRNA. Stucken, *et al.* [35] have recently described the CYN gene cluster from the *C. raciborskii* strain CS-505 isolated from Australia, which showed perfect synteny and high sequence conservation with the CYN cluster of *C. raciborskii* AWT205 described by Mihali, *et al.* [25]. This finding together with the atypical GC content and the evidence of transposase activity would suggest that CYN-producing *C. raciborskii* strains recently acquired the *cyr* cluster by horizontal gene transfer, being Australia the dispersion centre [27,30,36].

In a previous study, we have described two *C. raciborskii* isolates from Uruguay (MVCC14 and MVCC19), which were phylogenetically distinct (based on ITS and *nifH* gene sequences) and showed significantly different morphology and growth behavior under different light intensities and phosphate supply [34]. In that study, when analyzing *nifH* sequences, we could identify subclusters inside the American populations, suggesting an early spread and diversification of the species within the continent. Therefore, we proposed that phenotypic and genetic data support the hypothesis about the existence of different *C. raciborskii* ecotypes whose success is subject to the local environmental conditions. Both ecotypes showed differences in SAX content (under identical conditions MVCC19 had significantly higher SAX concentration than MVCC14) and were unable to produce CYN under sufficient nutrient conditions [34]. In this study, we describe for the first time the presence of putative CYN biosynthesis encoding genes, *cyrA*, *cyrB* and *cyrC*, in saxitoxin-producing *C. raciborskii* strains that were described as being different ecotypes. We also performed phylogenetic analyses of their nucleotide sequences and checked for the production of CYN by quantitative Enzyme Linked Immunosorbent Assays (ELISA).

2. Materials and Methods

2.1. Strains and Culture Conditions

Cultures of *C. raciborskii* strains MVCC14, MVCC19 (Uruguay) and CYP011K (Australia) were grown in BG11 culture medium, modified to have 25% of the $CuSO_4$ of the original recipe after Stanier, *et al.* [37]. Two growth conditions were assayed: with nitrogen (sodium nitrate) (+N) and nitrogen-free (−N), with no addition of sodium nitrate to BG11 medium. Cultures were grown at 26˚C and 80 μmol photons $m^{-1} \cdot s^{-1}$ provided by daylight fluorescent tubes with 16:8-h light:dark photoperiod. Static cultures were maintained in 500 mL borosilicate culture bottles that were continuously bubbled with saturated humid air, pre-bubbled in ultrapure sterilized water and pre-filtered by 0.45 μm. According to previous works, MVCC14 and MVCC19 produce SAX but not CYN and the strain CYP011K is a CYN-producer that was used as positive control [34].

2.2. ELISA Test

Cultures of the three strains (MVCC14, MVCC19 and CYP011K) growing in +N and –N culture medium were harvested when in exponential growth phase for toxin detection and kept frozen until analysis. Quantitative analysis of cylindrospermopsin was performed by ELISA (Abraxis, LLC, Warminster, PA, USA) with a detection limit of 0.05 $\mu g \cdot L^{-1}$. Five milliliters of cultures were frozen and thawed three times to break the filaments and

obtain the toxin. ELISA analyses were performed in accordance with the instructions of the manufacturer. The final CYN concentration was transformed to pg $cell^{-1}$ after cell counting as described in Piccini, *et al.* [34].

2.3. DNA Extraction and PCR

Genomic DNA from *C. raciborskii* strains was extracted according to the protocol described in Piccini, *et al.* [34]. The concentration and purity of DNA were determined spectrophotometrically at 260 and 280 nm (Nanodrop). DNA from MVCC14, MVCC19 and CYP011K was subjected to PCR using primers designed in this study to amplify partial *cyrA* gene sequences of *C. raciborskii*, as well the primers for *cyrA*, *cyrB* and *cyrC* described in Kellmann, *et al.* [27] (**Table 1**). The primers were designed based on the sequence from *C. raciborskii* AWT205 (Australia, Accession number EU140798) and checked against the whole GenBank database using Primer-BLAST (NCBI). All amplifications were performed in a volume of 25 µL containing 1 × PCR buffer (Invitrogen), 1 Unit of Taq (Invitrogen), 0.4 mM of each of the four dNTPs (Invitrogen), 3 mM $MgCl_2$, 10 pM (each) primer, 1.75 µL of bovine seroalbumin (BSA, 30 $mg \cdot mL^{-1}$) and 2 µL of DNA. PCR water was used as a negative control. PCR conditions consisted of an initial 5 min step at 94˚C, followed by 30 cycles of 1 min at 94 ˚C, 30 sec. at 55˚C and 1 min at 72˚C. PCR products were sent to Macrogen Inc. (Korea) for purification and sequencing using the PCR primers (**Table 1**).

2.4. Phylogenetic Analyses

DNA sequences were assembled and edited using DNA Baser Sequence Assembly software (Heracle BioSoft). The multiple sequence alignment tools from ClustalW [38] was used to align the sequences. Alignments were checked manually and maximum-likelihood (ML) phylogenetic analyses were conducted using MEGA version 5 [39]. The number of nucleotides included in the phylogenetic analyses was 459 for *cyrA*, 285 for *cyrB* and 300 for *cyrC*.

3. Results and Discussion

The assessment of CYN-production by the studied strains using an ELISA assay gave results below the detection limit of the technique (0.05 $\mu g \cdot L^{-1}$) for MVCC14 and MVCC19 grown in –N and +N culture medium. On the other hand, CYN concentration of the strain used as positive control, CYP011K, was 5.66 $pg \cdot cell^{-1}$ and 0.44 $pg \cdot cell^{-1}$, under –N and +N growing conditions, respectively. The results found in the CYN-producer strain CYP011K are in agreement with previous findings that showed that CYN concentrations are highest when *C. raciborskii* grow in the absence of nitrogen [40]. However, other environmental constraints, like low temperature, are also proposed to promote the CYN production [41]. Thus, further studies have to be performed to determine if under different environmental conditions the assayed strains are able to express CYN.

When the presence of *cyrA* was assessed by PCR amplifications using the primers described in Kellmann, *et al.* [27] amplicons where not detected neither in MVCC14 nor in MVCC19. However, when DNA amplifications were performed using the newly designed primers, CYLTOX-f and CYLTOX-r, amplicons of the expected size (823 bp) were detected from both strains. Nucleotide sequences corresponding to the obtained amplicons (accession numbers JN014840 and JN014843 for MVCC14 and MVCC19, respectively) shared 99% - 100% similarity with *cyrA* genes from other *C. raciborskii* strains (Blast, NCBI). Amplicons obtained from

Table 1. Primers used in this study. Expected size of amplicons and positions on the *cyrA*, *cyrB* and *cyrC* genes according to GenBank sequences are shown.

Primer name	Sequence (5' – 3')	Target sequence (positions in the gene)	Amplicon size (bp)	Reference
CYLATF	ATTGTAAATAGCTGGAATGAGTGG	*cyrA* (13 - 36)	1105	[27]
CYLATR	TTAGGGAAGTAATCTTCACAG	*cyrA* (1118 - 1098)		[27]
CYLTOX-f	GCGCTTCTAGAATCACAGGG	*cyrA* (157 - 176)	823	This study
CYLTOX-r	TGCGAAAGGGAATTGGATAG	*cyrA* (979 - 960)		This study
CPSF	AGTATATGTTGCGGGACTCG	*cyrB* (338 - 357)	478	[27]
CPSR	CCCGCCAAGACAGAGGGTAG	*cyrB* (816 - 797)		[27]
A205PKF	AATGACAGAGACTTGTGCGGGG	*cyrC* (966 - 987)	558	[27]
A205PKR	TTATCGGTATTGGTGGTAGCAACT	*cyrC* (452 - 429)		[27]

cyrB primers were 478 bp long and the gene sequences (accession numbers JX141619 and JX141620 for MVCC14 and MVCC19, respectively) showed similarities ≥99% with *cyrB* sequences from Australian and Brazilian *C. raciborskii* strains, and from *Raphidiopsis* and *Aphanizomenon* isolates. In the case of *cyrC* the size of the amplicon was 558 bp and the obtained sequences were highly similar (99% identity) to those obtained from CYN-producing Australian and Brazilian strains (accession numbers JX141621 and JX141622 for MVCC14 and MVCC19, respectively).

Phylogeny of *cyrA* gene sequences obtained from the analyzed strains and from GenBank database showed a cluster that was clearly separated from the cluster of sequences belonging to *Aphanizomenon* sp. and confirmed the identity of the genes detected in MVCC14 and MVCC19 (**Figure 1(a)**). These findings also suggest that the reports about the lack of the gene cluster based on the absence of *cyrA* in several *C. raciborskii* strains could be the result of a methodological limitation [27,42], and consequently lacking gene cluster strains should be perhaps re-analyzed.

Phylogenetic analyses based on the gene sequences obtained in this study showed that Uruguayan strains grouped together to *C. raciborskii* strains from Australia and Europe (**Figures 1(b)** and **(c)**). In order to increase the number of sequences in the phylogenetic analyses of *cyr* genes we included sequences belonging to a strain of *Aphanizomenon*. This genus is related to *Cylindrospermopsis* (Nostocaceae) and strains of *Aphanizomenon* have been reported as CYN producers [13,15]. The *cyrC* sequences obtained from the Uruguayan *C. raciborskii* strains grouped in a cluster that included sequences from Australia, Brazil and Poland (**Figure 1(c)**).

When evaluating the molecular phylogeny of *cyrA*, *cyrB* and *cyrC* in strains of *C. raciborskii* from different geographical regions, Kellmann, *et al.* [27] found these genes exclusively in CYN-producing Australian strains, with the exception of a non toxic Hungarian strain having *cyrB* but lacking *cyrA*, *cyrC* and unable to produce the toxin. Similarly, Rasmussen, *et al.* [43] detected the *cyrB* gene in a *C. raciborskii* strain from Australia that lacked CYN production. In a screening involving non-toxic *C. raciborskii* strains isolated from Lake Guiers (Senegal), Berger, *et al.* [44] did not detect the presence of *cyrB* or *cyrC*. Similarly, Fathalli, *et al.* [42] found that Tunisian strains of *C. raciborskii* did not show neither amidinotransferase, NRPS and PKS genes nor the SAX gene *sxtI* encoding for a carbamoyltransferase. Putting together, these data suggest that evolutionary mechanisms leading to toxin production by *C. raciborskii* are quite complex and should be analyzed in a wider biogeographical context.

Genomic studies by Stucken, *et al.* [35] showed that

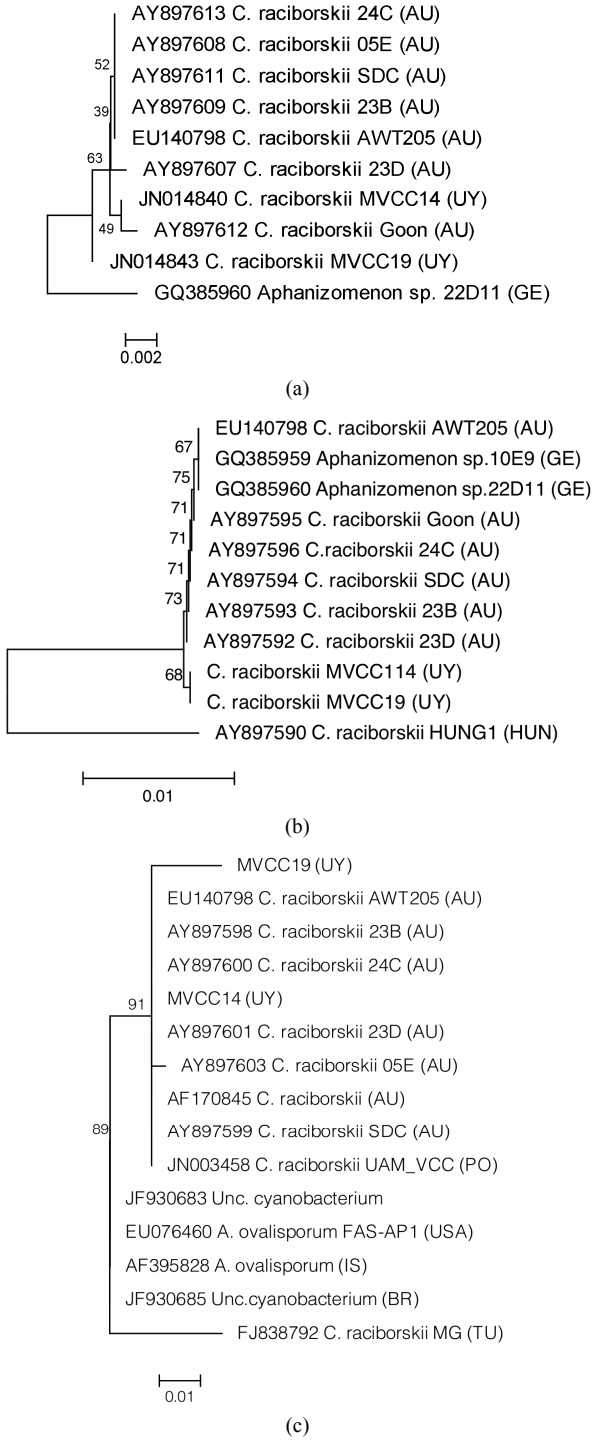

Figure 1. Maximum likelihood phylogenetic trees obtained for *cyrA* (a); *cyrB* (b) and *cyrC* (c) gene sequences from *C. raciborskii* MVCC14 and MVCC19 strains and sequences from GenBank database. Bootstrap values as percentages are given at the nodes (1000 bootstrap resampling). GenBank accession numbers of sequences are shown. Origin of the strains is shown between brackets, AU, Australia; BR, Brazil, GE, Germany; HUN, Hungary; PO, Poland; TU, Tunisia; USA, United States of America; UY, Uruguay; IS, Israel.

cyrA is located between transposase genes (*cyrL*, *cyrM* and CRC_01709) in the CYN-producing *C. raciborskii* strain CS-505. Also, the flanking regions of *cyr* cluster contained *hyp* genes at both ends, with molecular chaperones involved in hydrogenase maturation, suggesting that the toxin gene cluster was likely inserted as a unit in this genome region, interrupting the HYP gene cluster and generating a genetic rearrangement [25,35]. The finding of *cyrA*, *cyrB* and *cyrC* genes in SAX-producing *C. raciborskii* strains unable to synthesize CYN suggests the existence of a non-functional *cyr* gene cluster. In a recent study Ballot, *et al.* [45] detected *cyrA*, *cyrB* and *cyrC* in non-CYN producing *Apha ovalisporum* isolates, suggesting that production of CYN may have been a widespread ability in species of different geographical regions and open the question about which evolutionary mechanisms are involved in the lack of this ability.

According to our previous findings, phylogeny based on ITS, *nifH* and 16S rRNA showed that MVCC14 and MVCC19 ecotypes isolated from Uruguayan lakes are closely related to other American isolates, particularly from Brazil [34]. Phylogenetic analyses of *cyr* sequences obtained from the Uruguayan strains showed that they are highly similar to those from Australian CYN-producing, *Cylindrospermopsis* strains. Moreover, sequences from the three analyzed genes showed to be also closely related to *cyr* sequences from Brazilian populations. Therefore, there are two possible scenarios for explaining these findings. The first one is in agreement with the hypothesis of early acquisition of CYN-encoding genes in the evolution of this species [27,30,36] before its spreading, with a loss of genes by selection mechanisms. The second scenario would be the acquisition of the *cyr* cluster by horizontal gene transfer after the species has spread to the American continent. Further research using *C. raciborskii* strains from the entire American continent and addressing not only genetic but also ecophysiological traits are needed.

To date, available information indicates that *C. raciborskii* strains have different toxicity patterns related to their biogeography, with most Australian [41,46] and some Asian strains [47] producing CYN, South American strains (Brazil and Uruguay) producing SAX [29,34] and non toxic African or European strains [31,32]. Yılmaz, *et al.* [14] tested Florida isolates of this species and found that they do not have the ability to produce CYN. Putting together, these findings would indicate a high diversification of this species within this continent; which agrees with the evidence of subclusters within the *nifH* American cluster of *C. raciborskii* described by Piccini, *et al.* [34].

4. Conclusion

Here we report for the first time the presence of genes belonging to the CYN gene cluster, *cyrA*, *cyrB* and *cyrC* in two *C. raciborskii* ecotypes that produce SAX. Current dispersal hypothesis for *C. raciborskii* suggests that the ability to produce CYN originated in Australia and subsequently radiated to Europe and Asia but not to the Americas [27]. In addition, transcontinental radiation of the species might have occurred at an early stage of evolution, as evidenced by three clear clusters in ITS-based phylogenies (America, Europe, Africa-Australia) [33,48]. It has been reported that CYN genes are evolutionary more conserved than genes encoding for other toxins [36]. The presence of *cyr* genes highly similar to those belonging to current CYN-producers in *C. raciborskii* strains that cannot produce the toxin could indicate either that this cluster may have been present in American populations in the past and are being lost or that these genes have been recently acquired by horizontal transfer.

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