Applied and Environmental Microbiology, May 2002, p. 2567–2571 Vol. 68, No. 5
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.5.2567–2571.2002
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Genetic Characterization of *Cylindrospermopsis raciborskii* (Cyanobacteria) Isolates from Diverse Geographic Origins Based on *nifH* and *cpcBA*-IGS Nucleotide Sequence Analysis

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Received 6 November 2001/Accepted 20 February 2002

Isolates of the toxic, N_2-fixing species *Cylindrospermopsis raciborskii* from various geographic locations were analyzed with respect to their genetic diversity based on the *nifH* and *cpcBA*-IGS genes. Gene sequences clustered according to their geographic origin, with the *nifH* sequences separating into European, Australian, and American groups and the *cpcBA*-IGS sequences separating into American and European or Australian groups. PCR primers for both genes were designed to exclusively amplify DNA from *Cylindrospermopsis* species, and an additional primer set for *cpcBA*-IGS was designed to specifically amplify the American *C. raciborskii* strains.

Cylindrospermopsis raciborskii is a cosmopolitan, nitrogen (N_2)-fixing cyanobacterial species found in temperate to tropical freshwater habitats. The widespread proliferation of *C. raciborskii* in some drinking and recreational water supplies has caused international public health concerns (11). This concern is due to the potential for some strains to produce the alkaloid hepatotoxin cylindrospermopsin. Cyanobacterial toxins have been implicated in a range of animal and human health issues (1, 11, 13, 14, 21, 22, 32, 36). Regional characterization of *C. raciborskii* isolates is necessary for the detection of these strains at prebloom densities in areas susceptible to toxic cyanobacterial growth. Furthermore, analyzing strains from geographically diverse origins may help elucidate the mechanisms of their expansion and dispersal.

Molecular approaches are particularly useful in the detection and identification of specific strains, especially those that are morphologically identical at the species level. Genetic identification has been used to discriminate nuisance species in cyanobacterial genera, including *Microcystis*, *Anabaena*, *Nodularia*, and *Cylindrospermopsis* (5, 8, 10, 23–26, 28, 40). This information can also be used to characterize the degree of genetic similarity among populations. For example, *C. raciborskii* strains originating from different parts of Australia have been compared based on genetic analysis of the 16S rRNA gene (32, 33) and the *rpoC1* (RNA polymerase) gene (39). This is the first study to compare *C. raciborskii* isolates originating from a wider geographic area.

Genetic differences between *C. raciborskii* cultures were identified with two environmentally relevant genes. One of the genes utilized was *nifH*, a highly conserved gene that encodes dinitrogenase reductase, a protein subunit in the nitrogenase complex involved in N_2 fixation. Common to all N_2 fixers, the 324-bp *nifH* fragment is useful in characterizing diazotrophic communities and for differentiating cyanobacterial genera (6–7, 40). The other genetic locus used in this survey was *cpcBA*-IGS, which includes the highly variable intergenic spacer (IGS) region between two phycobilisome (bilin) subunits (*cpcB* and *cpcA*) within the phycocyanin operon (24). Phycocyanin is an accessory pigment that gives cyanobacteria their characteristic blue-green color and, together with chlorophyll *a*, is contained in the photosynthetic apparatus (17). A 685-bp fragment within the phycocyanin operon was chosen because of its potential for greater variability that may be useful in differentiating cyanobacterial strains (8, 24). Both *cpcBA*-IGS and *nifH* appear to be more useful in discriminating between strains than the commonly employed 16S rRNA gene, which exhibits low intrageneric variability in many cyanobacteria (20, 30).

PCR amplification and sequencing. *C. raciborskii* cultures from Australia (northern Queensland and Sydney), Germany, Portugal, Hungary (Lake Balaton), Brazil, and the United States (Florida) (12, 19, 31) were analyzed. The origin, morphology, and GenBank accession number for *nifH* and *cpcBA*-IGS sequences of each isolate used are given in Table 1. DNA extraction was performed by the XS method (37). A negative control in which no culture was added was run with each extraction set. From each of the *C. raciborskii* DNA isolates, the 324-bp *nifH* and the 685-bp *cpcBA*-IGS gene fragments were PCR amplified (in a 20-μl reaction volume containing 1× manufacturer's buffer [Fisher Biotech, Perth, Australia], 2.5 mM $MgCl_2$, 200 μM concentrations of each deoxyribonucleoside triphosphate, 10 pmol of each primer, 1 U of *Taq* DNA polymerase [Fisher Biotech], and 1 μl [ca. 10 ng] of isolated DNA). The primers used were the cyanobacterial *nifH* primers (27) and cyanobacterial *cpcBA*-IGS primers (24). The amplification parameters for *nifH* were 94°C for 5 min, with 30 cycles of 94°C for 10 s, 55°C for 20 s, 72°C for 1 min, and then an extension at 72°C for 7 min. PCR parameters were the same for *cpcBA*-IGS, except that a 52°C annealing temperature was used. The presence of the PCR product was confirmed on a 1% agarose gel (Progen, Brisbane, Australia) run at 75 V with

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TABLE 1. Origins, morphologies, and GenBank accession numbers of the *C. raciborskii* cultures used in this study

Strain	Origin		Date isolated	Morphology	*nifH* accession no.	*cpcBA*-IGS accession no.
	Country	Water body				
Aqc	Australia	Aquaculture pond, Townsville	1997	Coiled	AF426782	AF426788
Aqs	Australia	Aquaculture pond, Townsville	1997	Straight	AF426783	AF426789
Sdc	Australia	Solomon Dam	1996	Coiled	AF426780	AF426803
Sds	Australia	Solomon Dam	1996	Straight	AF426781	AF426804
Mk	Australia	Lake McKinley	1997	Straight	NT[b]	AF426802
Goon	Australia	Goonyella Dam	1998	Straight	NT	AF426799
LJ	Australia	Lake Julius	1995	Straight	AF426778	AF426800
A205	Australia	Ornamental pond, Sydney	NA	Straight	AF426768	AF426787
Germany 1	Germany	NA[a]	NA	Straight	AF426776	AF426797
Germany 2	Germany	NA	NA	Straight	AF426777	AF426798
Bal 5	Hungary	Lake Balaton	1984	Straight	AF426769	AF426790
Bal 6	Hungary	Lake Balaton	1994	Straight	AF426770	AF426791
Caia	Portugal	Caia reservoir	NA	Straight	AF426773	AF426794
4799	Portugal	NA	NA	Straight	AF426767	AF426786
Marau 1	Portugal	Marau reservoir	NA	Straight	AF426779	AF426801
Brazil 1	Brazil	Paranoa Lake	NA	Straight	AF426771	AF426792
Brazil 2	Brazil	Bilings reservoir	NA	Straight	AF426772	AF426793
Florida D	United States	Lake Dora, Fla.	1999	Coiled	AF426774	AF426795
Florida F	United States	Lake Dora, Fla.	1999	Straight	AF426784	AY078437
Florida G	United States	Lake Dora, Fla.	1999	Straight	AF426775	AF426796
Florida I	United States	Lake Dora, Fla.	1999	Coiled	AF426785	AY078438

[a] NA, information not available for this isolate.
[b] NT, not enough DNA was available to sequence *nifH* for this isolate.

3 μl of PCR product and 100 ng of a φX174/*Hae*III molecular size marker. The PCR products were ethanol precipitated and sequenced in both the forward and reverse directions with Big Dye terminators according to the manufacturer's instructions (Applied Biosystems Inc., Foster City, Calif.) and with an automated sequencer (PRISM cycle sequencing system and the ABI 373 sequencer [Applied Biosystems Inc.]). Sequences were aligned with the SeqLab program and were checked manually. Phylogenetic trees were generated with the Dayhoff PAM matrix and neighbor-joining algorithm with PHYLIP software (University of Wisconsin Genetic Computer Group). Consensus sequences were identified from these alignments by using the CONSENSE protocol in PHYLIP (16).

Primer design. Primers specific to *C. raciborskii* were designed based on sequences amplified from the cultures used in this study. Oligonucleotides were synthesized by Genset Oligos Pty. Ltd (Lismore, Australia). For *nifH*, the following primers were used to amplify *Cylindrospermopsis* species to the exclusion of all other heterocystous cyanobacteria and resulted in a 225-bp PCR product: cylnif F (5′-TAARGCTCAAACTAC CGTAT) and cylnif R (5′-ATTTAGACTTCGTTTCCTAC). For *cpcBA*-IGS, two forward primers were designed and used with the general cyanobacterial reverse primer from the original amplifications. One forward primer, cylcpc F (5′-GGCT TACGCGAAACCTATATA) (a 638-bp PCR product), was genus specific, and the other, FBcpc F (5′-AGCAGCAGCT GTTGCATAGTCCA) (a 464-bp PCR product), was specific to Florida and Brazil isolates. The specificity of these primers was tested against the isolates listed in Table 1 as well as against other heterocystous and nonheterocystous cyanobacteria.

Phylogenetic analysis of the *nifH* and *cpcBA*-IGS sequences. A 324-bp fragment from the *nifH* gene and a 685-bp fragment of the phycocyanin operon (*cpcBA*-IGS) were amplified from *C. raciborskii* cultures isolated from Australia, Europe, and the Americas (Table 1). PCR amplification products were detected for both genes from all isolates analyzed. Sequencing these products revealed phyletically significant differences in the nucleotide sequences for *C. raciborskii* from different regions.

Variation within the *nifH* gene reflected a distinct geographic grouping of isolates. All the *C. raciborskii nifH* nucleotide sequences from Europe were identical and were 100% similar to the consensus sequence. The Australian sequences were also 100% similar to each other and deviated from the European sequences by $<0.7\%$. The Brazilian sequences were identical to each other and contained four sites where they were distinct from the consensus nucleotide sequence (1.34% sequence dissimilarity), two of which were shared with the Florida isolates. The Florida isolates were 99% similar to each other and displayed a 2% overall divergence from the consensus sequence. Phylogenetic analysis of these *nifH* nucleotide sequences confirmed a distinct clustering of *C. raciborskii* based on geographic origin. The six sequences from Australian isolates formed one cluster, the sequences from European isolates (Germany, Hungary, and Portugal) formed a second cluster, and the sequences from American isolates (Brazil, Florida, and North Carolina) formed a third cluster (Fig. 1).

For *cpcBA*-IGS, there was more genetic variation between the Florida isolates of *C. raciborskii* but less variation in isolates from other locations. Sequences from the European and Australian isolates were $>99.8\%$ similar to each other at the nucleotide level. Brazilian sequences were 100% similar to each other, but only 97.8% similar to the sequences from European and Australian isolates. Florida sequences had

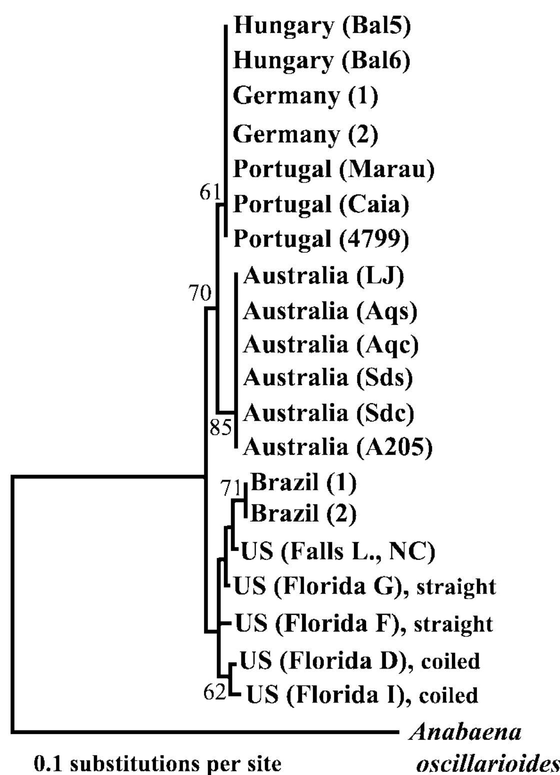

FIG. 1. Phylogenetic tree based upon *nifH* sequences of *C. raciborskii* isolates originating from different geographic locations. Bootstrap values (>50) are given by the corresponding nodes and were generated with distance methods. *Anabaena oscillarioides* (GenBank accession no. M63686) was used as the outgroup.

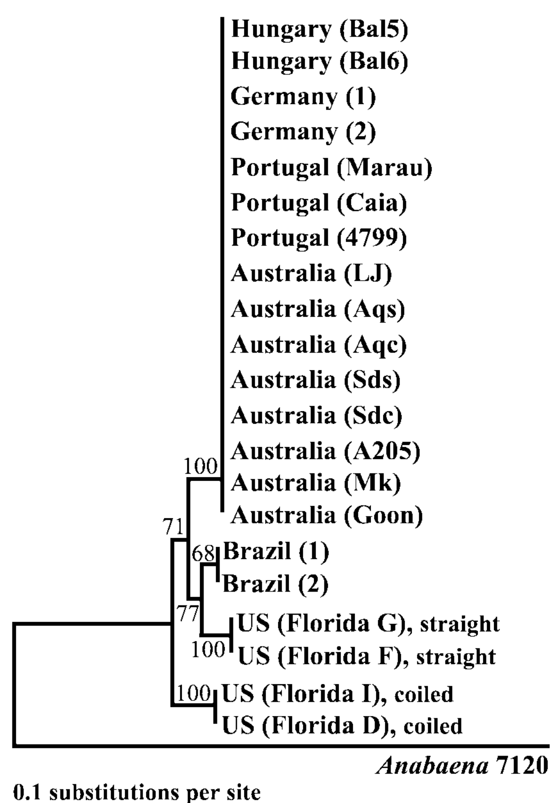

FIG. 2. Phylogenetic tree based upon *cpcBA*-IGS sequences of *C. raciborskii* isolates originating from different geographic locations. Bootstrap values (>50) are given by the corresponding nodes and were generated with distance methods. *Anabaena* 7120 (GenBank accession no. X05239) was used as the outgroup.

much higher genetic variability (96.2% similarity to each other) and were only 94.5% similar to those from Australia and Europe at the nucleotide level. There were nine distinct sites shared by Florida and Brazilian *cpcBA*-IGS nucleotide sequences to the exclusion of those originating from other regions. The phylogenetic analysis based upon *cpcBA*-IGS for these isolates did not delineate European and Australian sequences due to the high percentage of similarity, but they did separate sequences from American isolates into a distinct cluster (Fig. 2).

Thus, the variation among global *C. raciborskii* populations was reflected differently in the *nifH* and *cpcBA*-IGS nucleotide sequences. Although *nifH* was the smaller of the two gene fragments sequenced, regional distinctions could be made based on these sequences. *C. raciborskii* isolates originating from Europe, Australia, or the Americas were separated phylogenetically into distinct clusters based upon the *nifH* sequence data. While there were distinctions between regions revealed by the *nifH* sequence data (variation of up to 2.5%), the amount of variation within each of these three geographic regions was very low (<1%). Phylogenetic analyses based upon *cpcBA*-IGS nucleotide sequences only differentiated American *C. raciborskii* isolates. The higher degree of similarity between European and Australian isolates within this part of the phycocyanin operon did not allow distinctions to be made among these groups. In previous studies, variation in *cpcBA*-IGS has been sufficient to differentiate strains of *Nodularia spumigena*, *Anabaena circinalis*, and *Microcystis aeruginosa* originating from the same geographic region (i.e., Australia) as well as those from other regions (such as North America and the Baltic Sea) (8). However, the delineating power of *cpcBA*-IGS does not appear uniform for all cyanobacterial species or strains (8), as is evident from the *C. raciborskii* sequences of this study. Bolch et al. (10) found a sequence dissimilarity of less than 1% in *cpcBA*-IGS between *Nodularia* strains and suggested that this indicates a single morphospecies. Using this criteria, the <1% variation between the Australian and European *nifH* and *cpcBA*-IGS sequences indicates these two *C. raciborskii* populations may be defined as a single species cluster.

The American *C. raciborskii* isolates displayed greater genetic variability, and it appears that within some Florida lakes there are multiple distinct genotypes. The relatively low similarity between *C. raciborskii* isolates within a single Florida lake is noteworthy, especially when compared to the high similarities between isolates originating from different European countries or Australian states. Morphologically similar but genetically distinct cyanobacteria may coexist in one body of water and form separate blooms, or there may be a mixture of cyanobacteria within one bloom (e.g., *Nodularia* [10], *Synechococcus* [15], *Microcystis* [9, 35, 38], and *Cylindrospermopsis* [4, 34, 39]). A genetically mixed population, such as those examined from the Florida lakes, may be the result of accelerated

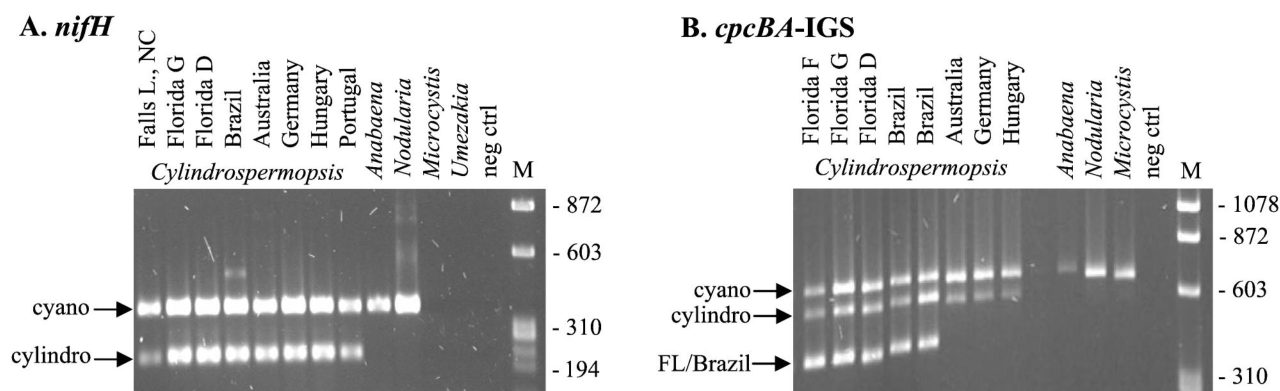

FIG. 3. PCR products amplified with the *nifH* and *cpcBA*-IGS primer sets. Each set of primers was used in a separate PCR, and then the two (for *nifH*) or three (for *cpcBA*-IGS) PCR products from each isolate were run together in the same lane of the gel. A ϕX174/*Hae*III molecular size marker (M) was run on each gel. (A) For *nifH*, the 324-bp product was amplified by the general cyanobacterial primer set (cyano) which had been used in the original sequencing of the *C. raciborskii* isolates. Each of these samples was also amplified with the *Cylindrospermopsis*-specific primer set (cylindro), which resulted in a 225-bp product exclusively from *C. raciborskii* samples. The *C. raciborskii* isolates used were the following: Falls Lake NC, Florida G, Florida D, Brazil 1, A205, Germany 1, Bal 6, and Marau 1. (B) For *cpcBA*-IGS, the 685-bp product was amplified by the general cyanobacterial primer set (cyano) which had been used in the original sequencing of the *C. raciborskii* isolates. Each of these samples was also amplified with the *Cylindrospermopsis*-specific primer set (cylindro), which resulted in a 638-bp product exclusively from *C. raciborskii* samples, and an American strain-specific primer set (FL/Brazil) which amplified a 464-bp band exclusively from the Florida and Brazil isolates. The *C. raciborskii* isolates used were Florida F, Florida G, Florida D, Brazil 1, Brazil 2, A205, Germany 1, and Bal 6.

molecular evolution in highly favorable environmental conditions (warm temperatures, abundant nutrients, and sufficient light year-round) or the more recent introduction of new strains. Possible mechanisms for the introduction and spread of this species include transport on the feet or in the guts of migratory birds that would be present in lakes and reservoirs and would travel long distances (2–3), human-related transport (i.e., recreational boats, commercial ship ballast water, or aquaria), or oceanic currents. *C. raciborskii* would most likely be transported as an akinete that would then germinate when suitable growth conditions were encountered (18, 29). The high degree of genetic similarity between *C. raciborskii* collected from Brazil and Florida could potentially be explained if one of these mechanisms allowed the two populations to be continually mixed or if they originated from the same source population relatively recently.

C. raciborskii isolates from Florida had two morphological variations, coiled and straight. Previous genetic comparisons of these two morphologies in Australian isolates have shown them to be nearly genetically identical (99.8%) based on 16S rRNA sequences (33), and this study demonstrated that they were genetically identical based on *nifH* and *cpcBA*-IGS sequences. However, for the Florida isolates there was a genetic distinction between these two morphologies. Sequences from the two coiled isolates (Florida D and I) clustered separately from isolates with straight morphologies (Florida G and F) in both *nifH* and *cpcBA*-IGS phylogenies (Fig. 1 and 2). While morphology may not be strictly controlled by genetics (33), it is interesting that it appears to be reflected in the nucleotide sequences for two separate genes. The presence of this distinction only in the more genetically diverse Florida isolates and not in those from Australia suggests that there may be other factors besides morphology being regulated. Analysis of additional *C. raciborskii* strains with coiled morphologies is necessary to determine the extent of this relationship between genetic and morphological variation.

Specificity of designed primers. The specificity and accuracy of the primers designed to amplify *nifH* from *Cylindrospermopsis* spp. and *cpcBA*-IGS from *Cylindrospermopsis* spp. and American strains were tested by using other cyanobacterial isolates. The 324-bp PCR product for *nifH* was seen in all cyanobacterial N_2 fixers by the general cyanobacterial primer set (positive control), while the 225-bp band amplified by the *Cylindrospermopsis*-specific primer set was seen only in *C. raciborskii* isolates (and excluded the closely related species *Anabaena circinalis* and *Nodularia spumigena*) (Fig. 3A). These primer sets did not amplify *nifH* from the non-N_2 fixers (*Microcystis aeriginosa* and *Umezakia natans*). For *cpcBA*-IGS, the general cyanobacterial primers amplified a 685-bp band in all the cyanobacterial isolates. The *Cylindrospermopsis*-specific forward primer in combination with the general cyanobacterial reverse primer amplified a 638-bp PCR product from just the *C. raciborskii* cultures and not from the other cyanobacterial cultures. The American strain-specific primer (FBcyl) in combination with the general cyanobacterial reverse primer successfully amplified a 464-bp *cpcBA*-IGS band exclusively from *C. raciborskii* isolates from Florida and Brazil (Fig. 3B). Thus, the designed primer sets appeared to yield the desired specificity. The *nifH Cylindrospermopsis*-specific primer set was tested on DNA extracted from environmental water samples from the Falls Lake reservoir in North Carolina to evaluate its effectiveness in a mixed phytoplankton population. The sequence of the amplified PCR product was unique but was grouped within the *C. raciborskii* cluster. The Falls Lake sequence was most similar to the American (Florida and Brazil) *C. raciborskii nifH* sequences.

Prior to this study, primer sequences had been reported for the PCR amplification of cyanobacterial *nifH* and *cpcBA*-IGS, but none was designed specifically for *Cylindrospermopsis* spp. The development of both genus-specific and region-specific primers is useful for two reasons. First, it confirms that the regional origin of isolates is evident at the genetic level and *C.*

raciborskii from specific geographic regions can be selectively amplified. The high specificity of the designed primers verifies that they have close homology with *C. raciborskii* and confirms that there is sufficient variation within these two genes for the distinction of the *Cylindrospermopsis* genus. Secondly, since these primers have been demonstrated to amplify *C. raciborskii* to the exclusion of other closely related heterocystous cyanobacterial species, they can be used for rapid, unequivocal detection of *C. raciborskii* in water supplies, particularly when there is a mix of cyanobacterial species present. The use of highly sensitive and specific molecular methods in conjunction with traditional microscopic screening will improve the speed and accuracy of detecting *C. raciborskii* and other toxic species in waters prone to cyanobacterial blooms.

Nucleotide sequence accession numbers. The GenBank database accession numbers for *C. raciborskii nifH* sequences from isolates in this study are AF426767 to AF426785 and for the *cpcBA*-IGS sequences are AF726786 to AF426804 and AY078437 and AY078438.

This study was financially supported by the St. Johns River Water Management District, National Science Foundation, U.S. Department of Agriculture, and the Australian Research Council. J.D. was supported by a National Science Foundation Graduate Research Fellowship.

We thank T. Steppe and M. Piehler for critically reading the manuscript, and we give special thanks to colleagues who contributed cultures for this study.

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