Molecular Characterization of the Toxic Cyanobacterium *Cylindrospermopsis raciborskii* and Design of a Species-Specific PCR

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*Cylindrospermopsis raciborskii* is a toxic-bloom-forming cyanobacterium that is commonly found in tropical to subtropical climatic regions worldwide, but it is also recognized as a common component of cyanobacterial communities in temperate climates. Genetic profiles of *C. raciborskii* were examined in 19 cultured isolates originating from geographically diverse regions of Australia and represented by two distinct morphotypes. A 609-bp region of *rpoC1*, a DNA-dependent RNA polymerase gene, was amplified by PCR from these isolates with cyanobacterium-specific primers. Sequence analysis revealed that all isolates belonged to the same species, including morphotypes with straight or coiled trichomes. Additional *rpoC1* gene sequences obtained for a range of cyanobacteria highlighted clustering of *C. raciborskii* with other heterocyst-producing cyanobacteria (orders *Nostocales* and *Stigonematales*). In contrast, randomly amplified polymorphic DNA and short tandemly repeated repetitive sequence profiles revealed a greater level of genetic heterogeneity among *C. raciborskii* isolates than did *rpoC1* gene analysis, and unique band profiles were also found among each of the cyanobacterial genera examined. A PCR test targeting a region of the *rpoC1* gene unique to *C. raciborskii* was developed for the specific identification of *C. raciborskii* from both purified genomic DNA and environmental samples. The PCR was evaluated with a number of cyanobacterial isolates, but a PCR-positive result was only achieved with *C. raciborskii*. This method provides an accurate alternative to traditional morphological identification of *C. raciborskii*.

Cyanobacterial blooms have become an increasing worldwide problem in aquatic habitats such as lakes, rivers, estuaries, and oceans and in man-made water storage systems. These occurrences can be partially attributed to gradual eutrophication of waterways. Certain species of cyanobacteria produce toxins, and as a result, blooms create major threats to animal and human health, tourism, recreation, and aquaculture.

A range of cyanobacterial toxins have been described in detailed reviews (8, 17). Hepatotoxins (liver damaging), neurotoxins (nerve damaging), cytoxins (cell damaging), and toxins responsible for allergic reactions have all been isolated from cyanobacteria. We are particularly interested in the toxic-bloom-forming cyanobacterium *Cylindrospermopsis raciborskii*. This species produces the alkaloid hepatotoxin cylindrospermopsin, which is also produced by the cyanobacteria *Aphanothece menon ovalisporum* (3) and *Umezakia natans* (13).

*C. raciborskii* is infamous for its association with a human poisoning incident on Palm Island, Australia, in 1979 (14). The illness, which produced hepatitis-like symptoms, lasted between 4 and 26 days and required the hospitalization of most of the 148 reported victims (7). The outbreak occurred immediately after treatment of a dense cyanobacterial bloom in the domestic water supply reservoir with copper sulfate. Copper sulfate is known to cause lysis of cyanobacteria and the release of any toxic cellular components into the water. Although the organisms in the original bloom were not identified before treatment with copper sulfate, follow-up studies indicated *C. raciborskii* as the most likely causative agent of this outbreak (6, 14). More recently, toxic *C. raciborskii* blooms have also been implicated in the death of cattle in regions of northern Australia (39).

*C. raciborskii* is a cosmopolitan species found in tropical, subtropical, and temperate climatic regions (2). *C. raciborskii* is identified by the presence of gas vacuoles and by the shape and dimensions of terminal heterocysts, vegetative cells, and trichomes (20). However, the microscopic identification of *C. raciborskii* by morphological characteristics can sometimes be influenced by selective culturing techniques. In addition, descriptions of *C. raciborskii* encompass two distinct morphotypes, both straight (Fig. 1A) and coiled (Fig. 1B) trichomes. The morphological taxonomy of *C. raciborskii* is not supported by any genetic information, and little is known about the level of genetic similarity between the morphotypes and their phylogenetic relationship to other closely related taxa. A molecular test to identify this toxic cyanobacterial species would therefore be advantageous.

The use of DNA sequences for the taxonomic and phylogenetic analysis of cyanobacterial isolates has been reviewed (43). A number of genes have been used as evolutionary markers in the delineation of cyanobacterial taxonomy, with the 16S rRNA gene analyzed most extensively because of its ubiquitous distribution throughout prokaryotic phylogenetic groups (11, 27, 36). Although this technique is well established, the DNA-dependent RNA polymerase (*rpoC1*) gene is suggested to be more discriminatory than 16S rRNA gene analysis at the species level (30). The cyanobacterial *rpoC1* gene encodes the γ subunit of RNA polymerase and exists as a single copy in the genome (5).

Nongenotypic approaches to cyanobacterial strain typing have been used (4, 9, 10, 23, 38), with the major limitation of these techniques being the phenotypic variations under different culture conditions. Improved molecular approaches to study cyanobacterial diversity at the strain level have been described.
(22, 25, 28, 42), but they require the use of axenic cultures, which are difficult to obtain. Cyanobacterium-specific strain-genotyping methods that do not require axenic cultures have also been described. DNA polymorphisms within the intergenic spacer region of the phycocyanin gene locus have been used to infer the genetic relatedness and evolution of toxic and bloom-forming cyanobacteria (26). Short tandemly repeated repetitive (STRR) sequences found to occur at high frequency in the genomes of filamentous, heterocystous cyanobacteria (24) have also been used to establish strain-specific DNA fingerprints. STRR sequences have been used either as oligonucleotide probes (34) or as primers in the generation of PCR-amplified DNA profiles (32).

The present study examines the level of diversity among Australian isolates of *C. raciborskii*. Strains isolated from geographically diverse populations are compared with respect to their *rpoC1* sequences and STRR sequence-generated PCR fingerprints. The phylogenetic relationship of *C. raciborskii* to other taxonomic groups of cyanobacteria is also presented, based on *rpoC1* gene sequences. In addition, this paper de-

<table>
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<sup>a</sup> Refers to *C. raciborskii* trichome structure.
<sup>b</sup> Australian Water Quality Centre culture collection.
<sup>c</sup> National Institute of Environmental Studies culture collection.
<sup>d</sup> NSW, New South Wales.
scribes the design of a PCR test specific for the identification of \textit{C. raciborskii}. The PCR test targets sequences unique to the \textit{C. raciborskii rpoC1} gene, and its robustness is evaluated with laboratory isolates and environmental samples known to contain \textit{C. raciborskii}.

\textbf{MATERIALS AND METHODS}

\textbf{Strains and growth conditions.} The cyanobacterial strains examined in this study are listed in Table 1, including information on their countries of origin and trichome morphologies. The strains were grown under constant light intensity (20 \text{mMm}^{-2} \text{s}^{-1}) for up to 14 days at 25°C in ASM-1 medium (12), with the exception that a nitrogen source was omitted and Na$_2$MoO$_4$ was added to a final concentration of 0.01 mg ml$^{-1}$. Environmental samples from Fred Haigh Dam, Queensland, Australia (courtesy of Glenn McGregor, Queensland Department of Health), and Currency Creek, South Australia, Australia, were frozen until they were required.

\textbf{DNA extraction.} DNA techniques were carried out according to standard procedures (35). Genomic DNA was extracted from cyanobacterial cells essentially as described by Porter (31). Briefly, 50-ml cell cultures were pelleted by centrifugation and resuspended in 0.5 ml of lysis solution (25\% sucrose, 50 mM Tris-HCl, 100 mM magnesium chloride) and stored at –20°C. For environmental samples, DNA was extracted with the InstaGene matrix (Bio-Rad). Briefly, 10-ml samples were pelleted by centrifugation and resuspended in 200 ml of solution containing 90\% InstaGene matrix and 10\% Triton X-100. The cells were incubated at 55°C for 30 min, vortexed for 1 min, then heated to 95°C for 10 min. Following centrifugation, DNA was extracted once with an equal volume of phenol-chloroform and once with chloroform. The DNA was precipitated, resuspended in 50 ml of water, and used directly in PCRs.

\textbf{PCR and DNA sequence analysis.} All PCRs were performed on a Perkin-Elmer GeneAmp 2400 PCR system. Each 50-ml reaction mixture contained 1 to 10 ng of genomic DNA, 20 pmol of each PCR primer, 200 \text{mM} \text{dNTPs}, 250 \text{mM} \text{MgCl}_2, 1 \times \text{PCR buffer II}, and 2.5 U of AmpliTaq Gold (Perkin-Elmer). Oligonucleotides were purchased from GeneWorks Pty. Ltd. For amplification of the \textit{rpoC1} gene from cyanobacterial strains, the following primers were used: rpoC1-1 (5'-GAGCTCNGGNCATCAAGTCNGG) and rpoC1-T (5'-GGTACCNAAYGGNSARRTNGTTGG) (30).

\textbf{FIG. 2.} Amino acid alignment of \textit{C. raciborskii}, \textit{N. spumigena} PCC7194, \textit{Anabaenopsis circularis}, \textit{A. bergii} ANA283A, and \textit{A. circinalis} ANA118C \textit{rpoC1} sequences and additional \textit{rpoC1} sequences obtained from the GenBank database: \textit{Fischerella} sp. strain PCC7414 (accession no. FSPRPOC1), \textit{Synechocystis} (Syn.) sp. strain PCC6308 (accession no. U53244), \textit{Synechoysis} sp. strain PCC7002 (accession no. U53245), \textit{Synechococcus} sp. strain PCC7942 (accession no. SPPRPOC1), \textit{Dermocarpa} sp. (accession no. U53241), \textit{E. coli} (accession no. ECPRPOC1), and \textit{P. putida} (accession no. M3819). Amino acids identical to those of \textit{C. raciborskii} are indicated by dots; the dashes represent gaps introduced into the alignment. The relative locations of primers cyl2 and cyl4 are also indicated (underlined).
Thermal-cycling conditions for the PCR were 95°C for 10 min, 1 cycle; 92°C for 90 s, 58°C for 1 min, and 72°C for 2 min, 35 cycles; and holding of the sample at 4°C.

The ropC1 PCR products were sequenced either directly or following ligation into the PCR cloning vector PCR 2.1 (Invitrogen). DNA sequencing was performed on both strands with the T7 DyeDeoxy Terminator cycle-sequencing kit and an automated model 373A DNA sequencer (Applied Biosystems) according to the manufacturer's instructions. Sequences were analyzed with a GeneJockey II sequence processor (Biosoft), and homology searches were performed in the National Center for Biotechnology Information database with the BLAST network service. Sequence alignments were performed with ClustalX (36).

STRR sequence profiles. A method that identified genetic variation among C. raciborskii strains was developed by using primers derived from previously identified cyanobacterial repeat sequences (24). The following primers were used: STRR1F (5′-GGCATTCCTAGTTATATTGCATTAC), STRR2F (5′-GGGAYTOGGGGAYT), STRR3F (5′-TTGGCACTGTGCA), STRR2R (5′-TTGGCACTGTGCA), STRR3R (5′-TACACTACAGT), and STRR3R (5′-ACTGTTGACTGTTGTTGTTG).

Thermal-cycling conditions were 94°C for 10 min, 1 cycle; 94°C for 30 s, 4°C for 1 min, and 65°C for 4 min, 35 cycles; 65°C for 5 min, 1 cycle; 4°C, hold. All PCR conditions were performed in at least two independent experiments. STRR profiles were converted to binary data by scoring the presence or absence of bands for each isolate as one or zero. These data were used to calculate total character differences, which were subsequently used to construct a neighbor-joining tree with PAUP* (37).

C. raciborskii-specific PCR. A PCR test was developed for the specific identification of C. raciborskii. The primers cyl2 (5′-GGGATCCCTAGTTATATTGCACTTAC), cyl4 (5′-GGGATCCCTAGTTATATTGCATTAC), and cyl-int (5′-TATTGGCACAATCTGTGTAATCGTACACACTCG) were used. An internal control fragment (ICF) was produced with the primer cyl-int to spike into PCRs. The cyl-int primer was designed to match a contiguous 22-base sequence 63 bp downstream of primer cyl2. A 13-base sequence at the 3′ end of cyl2 exactly matched a 13-nucleotide overhang at the 5′ end of cyl-int. The ICF was constructed by performing PCRs with cyl-int and cyl4, and the PCR product was used in a final PCR with cyl2 and cyl4 to give a 247-bp ICF. Each 50-μl PCR reaction contained 100 ng of genomic DNA, 20 pmol of cyl2, 20 pmol of cyl4, 200 μM deoxynucleoside triphosphates, 250 μM magnesium chloride, 1% PCR buffer II, 2.5 U of Ampli Taq Gold, and 20 μg of ICF. The thermal-cycling conditions were 95°C for 10 min, 1 cycle; 94°C for 30 s, 45°C for 30 s, and 72°C for 30 s, 35 cycles; 72°C for 15 min, 1 cycle; 4°C, hold. All PCRs were performed in at least two independent experiments. STRR profiles were converted to binary data by scoring the presence or absence of bands for each isolate as one or zero. These data were used to calculate total character differences, which were subsequently used to construct a neighbor-joining tree with PAUP* (37).

Phylogenetic analysis. Phylogenetic analysis of the DNA sequence data was performed with the MEGA analysis platform (21). Briefly, pairwise distances were calculated by the Jukes-Cantor method, and a tree was constructed with the neighbor-joining algorithm. The pairwise-deletion option was used for missing data and gaps in the alignment. Bootstrap analyses were performed with 500 replicates.

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this work have been deposited in the GenBank database under the following accession numbers. C. raciborskii consensus ropC1, AF159371; Nodularia spumigena PCC73104 ropC1, AF159372; Anabaena circinalis ANA118C ropC1, AF159373; Anabaenopsis circularis ropC1, AF159374; and Anabaena bergii ANA203A ropC1, AF159375.

RESULTS

ropC1 gene sequence analysis. A 609-bp fragment of the ropC1 gene was amplified and sequenced from each of 19 C. raciborskii isolates. The nucleotide sequences differed at only two sites, with 99 to 100% nucleotide sequence identity observed among strains of C. raciborskii. At position 351, 3 strains of 19 showed a synonymous substitution of T for C. At position 427, 7 strains of 19 showed a change from C to A that caused a change from glutamine to lysine. Neither sequence change could be associated with the trichome morphology or the geographic origin of the isolate. Importantly, the ropC1 gene in C. raciborskii was found to be highly conserved, indicating that all isolates examined are the same species, including both coiled and straight morphotypes. The ropC1 gene fragment was also amplified from a selection of other cyanobacteria, namely, A. bergii, A. circinalis, Anabaenopsis circularis, and N. spumigena, and the corresponding amino acid sequences were compared to a selection of cyanobacterial ropC1 sequences obtained from the GenBank database (Fig. 2). At the amino acid level, the C. raciborskii ropC1 sequence exhibited 84 to 93% identity to other cyanobacterial species, indicating significant variation in the ropC1 sequences among species to examine the phylogenetic position of C. raciborskii.

Phylogenetic analysis of C. raciborskii. Jukes-Cantor distances, generated by pairwise comparisons of the isolates, were used to create a phylogenetic tree by neighbor-joining analysis (Fig. 3). In this analysis, Escherichia coli and Pseudomonas putida were included as outgroup taxa to root the tree. The phylogenetic relationships inferred from ropC1 nucleotide sequences. The neighbor-joining tree was constructed by using corrected Jukes-Cantor distances. Bootstrap percentages (calculated from 500 resamplings) are indicated for the nodes. I, cluster I; II, cluster II; and III, cluster III

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<td>Escherichia coli</td>
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<td>Pseudomonas putida</td>
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</table>

FIG. 3. Phylogenetic position of C. raciborskii in relation to other cyanobacteria (sequences resulting from this work and obtained from the GenBank database) and to E. coli and P. putida based on analysis of aligned ropC1 nucleotide sequences. The neighbor-joining tree was constructed by using corrected Jukes-Cantor distances. Bootstrap percentages (calculated from 500 resamplings) are indicated for the nodes. I, cluster I; II, cluster II; and III, cluster III.
and STRR3R (Fig. 4C) yielded very similar patterns for all C. raciborskii isolates, while the greatest variation was seen with the primer combination of STRR1F and STRR3F (Fig. 4A). Differences were also observed among C. raciborskii strains isolated from the same population, i.e., strains CYP003A and CYP003K (Fig. 4, lanes 1 and 2) and strains CYP023A and CYP023E (Fig. 4A and B, lanes 9 and 10). Interestingly, two of the primer combinations yielded fragments unique to the three C. raciborskii isolates with coiled trichomes (Fig. 4A and B, lanes 1, 3, and 4). Information from the STRR sequence banding patterns was represented graphically by converting the bands to binary data, which was then used to construct a tree (Fig. 5). The tree shows similarities among different isolates according to their banding patterns. Interestingly, all of the isolates examined that had coiled trichomes grouped together.

C. raciborskii-specific PCR. Although little difference was found among the rpoC1 sequences of C. raciborskii isolates, there was sufficient difference between the rpoC1 sequence of C. raciborskii and those of other species to design a specific PCR test for C. raciborskii. Primers cyl2 and cyl4 (Fig. 2) were used to amplify a 305-bp diagnostic PCR product from the rpoC1 genes of C. raciborskii isolates. Twenty femtograms of ICF per reaction was found to be the minimum amount required to yield a discernible product following gel electrophoresis of PCR products. The PCR test was used to screen all of the strains listed in Table 1, and the results are shown in Fig. 6. All C. raciborskii isolates produced positive reactions, with amplification of both the diagnostic 305-bp product and the ICF. Chromosomal DNA extracted from two Brazilian isolates, which had previously been tentatively identified as C. raciborskii (Table 1), also tested positive by PCR. The diagnostic product was absent in all other cyanobacterial strains tested, from which only the ICF was amplified. The PCR test was then applied to the direct analysis of two environmental samples, obtained from South Australia and Queensland, that were known to contain C. raciborskii. Significantly, both the diagnostic product and the ICF were amplified from both samples. The test described here is the first report of a rapid method for the identification of C. raciborskii directly from environmental samples.

DISCUSSION

In this study the level of genetic diversity in a selection of Australian C. raciborskii isolates was examined. Although rpoC1 sequences were unable to distinguish among several strains of C. raciborskii, STRR sequence-generated PCR fingerprints were able to discriminate coiled from straight trichomes of C. raciborskii. A comparison of C. raciborskii rpoC1 profiles with those of other cyanobacteria resulted in a more comprehensive analysis of its cyanobacterial phylogeny than was previously available. As a consequence of this study, a rapid method has also been developed for the specific identification of C. raciborskii directly from environmental samples.

The 16S rRNA gene represents the most highly studied gene for identification and phylogenetic analysis. rpoC1 gene anal-
Ysis has been shown to be more discriminatory than 16S rRNA analysis (30). PCR primers designed from conserved regions of the cyanobacterial *rpoC1* gene (30) were used to analyze the *C. raciborskii* isolates. In a previous study, these primers were used in PCRs for strain-level identification of a number of taxonomic groups (41). In addition, they have been used to examine the phylogenetic relationship of prochlorophytes to each other and to the green chloroplasts (29) and to study the diversity of the cyanobacterial genus *Synechococcus* (41). We therefore hypothesized that sequence analysis of the *rpoC1* gene might enable differentiation among strains of *C. raciborskii* isolated from both mixed cyanobacterial communities and monospecific blooms over a 10-year period from tropical and temperate regions in Australia and might even identify a genetic difference between coiled and straight morphotypes. However, although there was sufficient sequence variation at the amino acid level to distinguish and group other cyanobacteria in relation to *C. raciborskii* (Fig. 2), there was insufficient discrimination even at the nucleotide level to distinguish among *C. raciborskii* isolates.

Phylogenetic analysis of the partial *rpoC1* gene sequence among these cyanobacterial species selected in this study indicated one distinct cluster (Fig. 3) and agreed with an earlier phylogenetic classification of cyanobacteria based on both the *rpoC1* gene and the 16S rRNA gene (26, 29, 43). Cluster I (Fig. 3) consisted mostly of representative genera of the order *Nostocales* (*Anabaenopsis circularis*, *N. spumigena*, *A. circinalis*, *A. bergii*, and *C. raciborskii*). Genera of the order *Chroococcales* (*Synecochystis, Dermocarpa*, and *Synechococcus*) were placed external to this cluster. Unlike the simple aggregation of vegetative cells in the *Chroococcales*, representative taxa of the *Nostocales* are characterized by differentiation of cells with a specialized function (e.g., heterocysts). Within the *Nostocales* cluster there was no apparent grouping of common phenotypic features, such as the position and mode of heterocyst differentiation or trichome morphology. The grouping of *Fischerella* (order *Stigonematales*) within the *Nostocales* cluster does not reflect the significant morphological differences that separate them in the traditional classification hierarchy (i.e., *Fischerella* is characterized by the production of multiseriate branched filaments), although taxa of the *Stigonematales* are morphologically more similar to the *Nostocales* than to the *Chroococcales* (1, 18, 19). Further studies would be required to genetically characterize other species of the order *Stigonematales* and validate their phylogenetic relationship to the cluster of *Nostocales* taxa. The paralyphetic distribution of both *Synechocystis* and *Anabaena* species also contradicted the traditional classification system.

In a previous study, primers based on the STRR1 repeat sequence were used in PCRs to fingerprint symbiotic cyanobacterial isolates from the angiosperm *Gunnera* (32). These results demonstrated both high genetic diversity and distinct clustering of symbiotic *Nostoc* isolates. Our attempts to fingerprint *C. raciborskii* isolates with the same primers did not generate any PCR products, a result which may reflect the number, position, and orientation of these repeat sequences within the *C. raciborskii* genome. In order to overcome this problem, we used different combinations of the three known STRR cyanobacterial sequences as primers in PCRs. The STRR primer combinations described here produced clear and reproducible PCR banding patterns among our *C. raciborskii* isolates. Only minor or no PCR products were obtained from control bacterial strains, including *E. coli*, *Pseudomonas, Bacillus subtilis*, and *Klebsiella pneumoniae* (data not shown). Our results reveal genetic heterogeneity among Australian *C. raciborskii* isolates and demonstrate that this strain variation also exists within a single cyanobacterial population or bloom.

The efficient management of water bodies currently relies on obtaining an accurate identification of problematic cyanobacterial species. With traditional microscopic methods, identification by morphological criteria can result in errors of subjective judgment by operators, and in the past isolates have been assigned to the wrong genus. This inconsistency in identification highlights the requirement for better identification methods. The *rpoC1* gene sequence data presented here showed sufficient variation between *C. raciborskii* and closely related cyanobacteria to design primers specific to the *C. raciborskii* *rpoC1* gene. A PCR test was developed to amplify a 305-bp *C. raciborskii*-specific *rpoC1* fragment from both laboratory and environmental samples. It was necessary to pretreat environmental samples with phenol and chloroform to reduce the level of inhibitors to PCR present in the samples. As an additional control, the ICF served to verify that negative reactions were indeed negative and not due to PCR inhibition by some other factor. This test is the first report of a molecular method to identify *C. raciborskii* directly from environmental samples without the need for axenic culture conditions. Our PCR test will allow early detection of *C. raciborskii*, thus enabling efficient management before a bloom occurs and tracking of *C. raciborskii* throughout a water body.

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REFERENCES


