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Varied Diazotrophies, Morphologies, and Toxicities of Genetically Similar Isolates of *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyceae) from Northern Australia

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The potentially toxic freshwater cyanobacterium *Cylindrospermopsis raciborskii* has become increasingly prevalent in tropical and temperate water bodies worldwide. This paper investigates the effects of different nitrogen sources (NO_3^-, NH_4^+, and omission of a fixed form of nitrogen) on the growth rates, morphologies, and cylindrospermopsin (CYL) concentrations (expressed as a percentage of the freeze-dried weight) of seven *C. raciborskii* isolates obtained from a range of water bodies in northern Australia and grown in batch culture. In general, growth rates were lowest in the absence of a fixed-nitrogen source and highest with NH_4^+ as the nitrogen source. Conversely, the highest concentrations of CYL were recorded in cultures grown in the absence of a fixed-nitrogen source and the lowest were found in cultures supplied with NH_4^+. Cultures supplied with NO_3^- were intermediate with respect to both CYL concentration and growth rate. Different nitrogen sources resulted in significant differences in the morphology of *C. raciborskii* trichomes. Most notable were the loss of heterocysts and the tapering of end cells in cultures supplied with NH_4^+ and the statistically significant increase in vegetative cell length (nitrogen depleted < NO_3^- < NH_4^+). The morphological changes induced by different nitrogen sources were consistent for all isolates, despite measurable differences in vegetative-cell and heterocyst dimensions among isolates. Such induced morphological variation has implications for *Cylindrospermopsis* taxonomy, given that distinctions between species are based on minor and overlapping differences in cell lengths and widths. The close phylogenetic association among all seven isolates was confirmed by the high level (>99.8%) of similarity of their 16S rRNA gene sequences. Another genetic technique, analysis of the HIP1 octameric-palindrome repeated sequence, showed greater heterogeneity among the isolates and appears to be a useful method for distinguishing among isolates of *C. raciborskii*.

The freshwater cyanobacterium *Cylindrospermopsis raciborskii* (order Nostocales), first named by Seenayya and Subba Raju (33), was initially assigned to the genus *Anabaenopsis* as *A. raciborskii* Woloszynska (41) but was subsequently recommended for exclusion from that genus because of its quite different pattern of heterocyst development, which more closely resembles that of the genus *Cylindrospermum*. On this basis, the genus *Cylindrospermopsis* was proposed (33). This was later supported by Horecká and Komárek (17), who distinguished *Cylindrospermopsis* from *Cylindrospermum* by the presence in the former of gas vacuoles, attenuated and pointed ends of trichomes, and spores (akinetes) positioned near one or both ends of the trichomes, with one to three vegetative cells between the terminal heterocysts and akinetes. Using numerical taxonomic methods based on a wide range of morphological features, Horecká and Komárek (17) also confirmed the close relationship between the two genera and a considerably more distant relationship between the genera *Cylindrospermopsis* and *Anabaenopsis*. In his description of natural populations of these two genera from western Slovakia, Hindák (16) confirmed that while in *Cylindrospermopsis* heterocysts develop primarily from terminal cells, in *Anabaenopsis* they are formed in pairs in an intercalary position.

Eight *Cylindrospermopsis* species have now been described: *Cylindrospermopsis africana, C. cuspis, C. philippinensis, C. raciborskii* (19), *C. allantoidispora, C. catemaco, C. tavernae* (20, 21), and *C. curvispora* (37). These species, most of which have been described from natural populations, are distinguished by minor and overlapping differences in vegetative-cell and heterocyst dimensions and by akinete shape, although akinetes have not been found in all species (e.g., *C. curvispora*).

C. raciborskii, the most frequently reported species in this genus, is of interest from a water quality perspective due to its ability to produce a potent hepatotoxic alkaloid, cylindrospermopsin (CYL) (13, 14, 28). This toxin, which has been implicated in outbreaks of human sickness (4, 5, 15) and in cattle mortality (30), can accumulate in the tissues of aquatic organisms (29). The ability of *C. raciborskii* to produce paralytic shellfish-poisoning toxins, similar to those found in dinoflagellates and the cyanobacterium *Anabaena circinalis*, has also been demonstrated (22). Given the potential for serious health concerns, there is a clear need to investigate the limits of morphological variation within *C. raciborskii* so that natural populations of this cyanobacterium can be identified correctly for the purposes of ecological monitoring and toxicological studies.

In this paper we describe investigations into the effects of

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TABLE 1. Sources and trichome morphologies of *C. raciborskii* isolates used in this study

Isolate	Source	Impoundment type	Date isolated	Trichome morphology	Reference
*CR*1	Solomon Dam (18°45′S, 146°35′E)	Potable supply	February 1996	Straight	31
*CR*2	Solomon Dam (18°45′S, 146°35′E)	Potable supply	February 1996	Coiled	31
*CR*3	Townsville (19°16′S, 146°49′E)	Aquaculture pond	August 1997	Straight	29
*CR*4	Goonyella Dam (21°48′S, 147°58′E)	Potable supply	April 1998	Straight	NA[a]
*CR*5	McKinlay (21°16′S, 141°18′E)	Farm dam	August 1997	Straight	30
*CR*6	Townsville (19°16′S, 146°49′E)	Aquaculture pond	August 1997	Coiled	29
*CR*7	Lake Julius (20°08′S, 139°44′E)	Potable supply	November 1995	Straight	NA

[a] NA, not available.

different nitrogen sources on the growth, morphology (including those characteristics which distinguish the different species of the genus *Cylindrospermopsis*), and gravimetric CYL concentrations of seven isolates of *C. raciborskii* taken from a range of water bodies in northern Australia and grown in pure culture. The isolates have been characterized genetically by two techniques. Firstly, 16S rRNA gene (rDNA) sequence analysis was performed. This has been shown to be useful in determining differences among cyanobacterial genera (39) and species (25). Second, genomic polymorphism analysis, employing cyanobacterium specific highly iterative palindrome (HIP1) repeats (11), was carried out. This technique has been applied to other cyanobacteria and has been shown to be useful as a typing technique at the strain level for many genera of cyanobacteria (35). This is the first report of the application of this technique to the genus *Cylindrospermopsis*.

MATERIALS AND METHODS

Isolation and culturing of *C. raciborskii*. Seven isolates of *C. raciborskii* (five with straight trichomes and two with coiled trichomes), all with vegetative and heterocyst cell dimensions within the reported range for that species (1, 19), were brought into pure culture as previously described (31). These isolates originated from a range of water bodies in northern Australia. Cultures were grown in ASM-1 medium (6) (pH 7.6) modified by the exclusion of the primary nitrogen source ($NaNO_3$). The sources of isolates, types of source water body, trichome morphologies, and relevant publications are given in Table 1.

Experimental culturing conditions. To investigate the influence of the nitrogen source on the growth rates, morphologies, and gravimetric CYL concentrations of the seven isolates, the primary nitrogen source of the ASM-1 media (2 mM $NaNO_3$) was either included (NO_3^-), omitted, or replaced by NH_4Cl (NH_4^+) to give equivalent final concentrations of nitrogen. Urea was previously shown to be unsuitable for the growth of this cyanobacterium (31). Media for the three nitrogen treatments were also modified by the addition of a buffer (0.02 M HEPES, pH 7.6).

Triplicate 150-ml cultures in sterile 250-ml Erlenmeyer flasks, initiated by the aseptic transfer of 1 ml of stock culture (containing ca. 100,000 cells ml^{-1}), were placed in a controlled-environment cabinet at 25°C with a light intensity of 50 $\mu mol\ m^{-2}\ s^{-1}$ (12 h of light/12 h of darkness) provided by cool white fluorescent tubes.

Growth, CYL concentration determinations, and morphological analyses. Maximum growth rates of cultures, in divisions per day, were determined from growth curves based on periodic (24- to 48-h) estimation of cell concentrations as indicated by previously calibrated optical density (750 nm) measurements (31). Cyanobacterial biomass at the end of the exponential growth phase (determined individually for each of the isolates and nitrogen treatments) was measured as freeze-dried weight following the filtration (0.45-μm-pore-size Whatman GF/C membranes) of pooled triplicate cultures. CYL concentrations in the harvested cultures were analyzed by mass spectrometry coupled with high-performance liquid chromatography (9) and expressed per unit of freeze-dried weight.

For each of the isolates and nitrogen treatments, representative subsamples of pooled culture (ca. 20 ml) were preserved in Lugol's iodine solution for microscopic (Olympus CH-2) measurement of morphological features including vegetative-cell length (VCL), vegetative-cell width (VCW), heterocyst length (HL), and heterocyst width (HW). For each of the isolates and nitrogen treatments, measurements were made on at least 30 heterocysts and vegetative cells. End cells were excluded from the analysis due to the greater variability in end-cell shape (34).

Statistical analysis of the data (appropriately transformed to satisfy the requirements for a parametric test) was by two-way analysis of variance using SPSS version 6.1 (SPSS, Inc., Chicago, Ill.). The experimental design allowed evaluation of differences between isolates, the effect of the nitrogen source, and the interaction between these two variables.

DNA extraction. Total genomic DNA was extracted from lyophilized samples of the seven isolates grown under stock culture conditions (as described above) by using a modification of a technique for purification of DNA from Gram-negative bacteria (24). Briefly, lyophilized samples were suspended in 500 μl of a solution containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), and 50 mM NaCl. Lysozyme was added to a final concentration of 1 mg ml^{-1}, and the solution was incubated at 55°C for 30 min. After addition of 10 μl of a proteinase K solution (10 mg ml^{-1}) and 20 μl of 10% sodium dodecyl sulfate, the mixture was incubated at 55°C for 10 min or until the solution cleared (indicating complete cell lysis). The solution was then chilled on ice and extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol). The supernatant was then added to an equal volume of 4 M ammonium acetate, and total genomic DNA was precipitated by addition of 2 volumes of isopropanol and centrifugation (12,000 × *g*) for 10 min at room temperature. The integrity and concentration of the extracted genomic DNA were determined spectrophotometrically at 260 and 280 nm.

16S rDNA amplification. PCR amplification of the 16S rRNA gene was performed using primers 27F1(UFP) and 1494Rc(URP) together with PCR reagents as previously described (25). Thermal cycling was performed at 94°C for 4 min followed by 30 cycles of 94°C for 20, 50°C for 30, and 72°C for 2 min. The amplification reaction products were purified by using the Wizard PCR purification system (Promega, Madison, Wis.) to remove reaction components including unincorporated primers, enzyme, and nucleotides. Approximately 100 ng of PCR product and 10 pmol of previously described 16S rRNA gene sequencing primers (25) were used to determine the primary structure of the *Cylindrospermopsis* 16S rDNA. Automated DNA sequencing was performed with the PRISM cycle sequencing system and an ABI 373 sequencer (Applied Biosystems Inc., Foster City, Calif.). Oligonucleotide primers were synthesized on an Oligo 1000 DNA synthesis system (Beckman, Fullerton, Calif.) and purified by reverse-phase chromatography.

16S rDNA phylogenetic analysis. DNA sequences were aligned by using the 6CG Pileup program (Genetics Computer Group, Madison, Wis.) and the multiple sequence alignment tool from Clustal X (36). Manual confirmation of the sequence alignment was performed and checked against both primary- and secondary-structure considerations of the 16S rRNA molecule. The aligned sequences were applied to genetic distance and maximum-parsimony methods for phylogenetic inference. Ambiguous characters, where a deletion, insertion, or unidentified state was recorded for any isolate, were not subjected to further analysis. For all multiple sequence alignments and phylogenetic inference programs, the input order of each of the taxa was randomized. Genetic distances (D) were calculated (18) with the formula $D = -3/4\ \ln(1 - 4/3d)$, where d is the sequence dissimilarity. Phylogenetic inference protocols DNADIST, NEIGHBOR, DNAPARS, CONSENSE, and SEQBOOT were supplied in the PHYLIP package (version 3.57c) (10). All sequence manipulation and phylogeny programs were made available by the Australian National Genomic Information Service (Sydney, Australia).

Cyanobacterial repeated-sequence PCR. HIP1 PCR amplifications were performed with primers HipCA and HipTG (35). Twenty-microliter reaction volumes contained 2 μl of a 2 mM deoxynucleoside triphosphate solution, 2 μl of 25

TABLE 2. Effects of different nitrogen sources on growth rates and CYL concentrations (measured at the end of the period of exponential growth) of seven *C. raciborskii* isolates in pure cultures

Isolate	Value obtained with N source:					
	No nitrogen addition		NO_3^-		NH_4^+	
	GR[a]	CYL[b]	GR[a]	CYL[b]	GR[a]	CYL[b]
CR1	0.63 ± 0.05 (4)	0.46	0.65 ± 0.05 (4)	0.35	0.83 ± 0.16 (3)	0.23
CR2	0.75 ± 0.16 (4)	0.30	0.93 ± 0.01 (3)	0.27	1.05 ± 0.01 (3)	0.21
CR3	0.65 ± 0.09 (4)	0.38	0.69 ± 0.12 (3)	0.42	0.51 ± 0.03 (3)	0.34
CR4	0.77 ± 0.13 (4)	2.9×10^{-2}	0.64 ± 0.3 (3)	2.8×10^{-2}	1.30 ± 0.18 (3)	2.1×10^{-2}
CR5	0.87 ± 0.31 (3)	1.2×10^{-4}	1.41 ± 0.27 (3)	7.0×10^{-5}	1.44 ± 0.31 (3)	ND[c]
CR6	0.89 ± 0.15 (3)	ND	1.20 ± 0.14 (3)	ND	1.49 ± 0.29 (3)	ND
CR7	0.86 ± 0.06 (4)	ND	0.71 ± 0.10 (4)	ND	1.10 ± 0.31 (3)	ND

[a] Growth rate in divisions day^{-1} ± 1 of standard deviation. Values in parentheses are numbers of replicates.
[b] Percentage of freeze-dried weight.
[c] ND, not detected.

M $MgCl_2$, 2 μl (100 ng) of DNA preparation, 1 μl (10 pmol) of each primer solution, 11.8 μl of H_2O, and 0.2 μl (1 U) of *Taq* polymerase. Reactions were cycled using a temperature profile consisting of 95°C for 5 min; 30 cycles of 95°C for 10 s, 40°C for 20 s, and 72°C for 60 s; and 1 cycle of 72°C for 5 min. Reactions were also performed as described above but with only the HipTG primer employed to initiate strand extension. PCR products were separated by 1.5% agarose gel electrophoresis in Tris-borate-EDTA buffer according to standard protocols (32). The quantities of PCR products loaded onto the gel differed slightly (between 3 and 6 μl) for the seven isolates so as to result in approximately equivalent intensities of banding patterns. Gel electrophoresis was performed in triplicate to confirm the resultant profiles. Gels were corrected for brightness and contrast and photographed. The photographic image was then used to construct a binary matrix based on the visual presence or absence of DNA bands on the electrophoresis gel. Phylogenetic analysis of this binary data matrix was achieved by using the DNAPARS, CONSENSE, and SEQBOOT programs of the phylogenetic inference package (PHYLIP, version 3.57c) (10).

RESULTS

Effect of nitrogen source on growth rate and CYL concentration of *C. raciborskii*. Five of the seven isolates, *CR1, CR2, CR3, CR4*, and *CR5*, produced detectable concentrations of CYL. Isolates *CR6* and *CR7* did not produce detectable concentrations of this toxin under any of the growth conditions investigated (Table 2). There was no correlation between the ability of the isolates to produce CYL and whether the isolates had coiled or straight trichomes, since both coiled and straight morphotypes were found among those isolates producing high concentrations (0.21 to 0.46% of freeze-dried weight) and those that did not contain detectable concentrations (less than ca. 0.00002%) of CYL. The highest concentrations of this toxin were found in cultures grown in the absence of a fixed nitrogen source, for which growth rates were lower (with the exception of *CR3*) (Table 2). The lowest concentrations of CYL occurred in cultures supplied with NH_4^+, for which growth rates were highest. Cultures supplied with NO_3^- were, in general, intermediate with respect to both CYL concentration and growth rate (Table 2) with the exception of isolate *CR3*, for which this nitrogen source resulted in the highest CYL concentration.

Morphological variation among isolates of *C. raciborskii*. The characteristic gross morphology of the inoculum (i.e., straight or coiled trichomes [Table 1]) was maintained through successive generations in culture. There was no evidence of straightening of coiled trichomes or of coiling of straight trichomes. The numerical taxonomic analysis of morphological characteristics detected a number of statistically significant differences in VCL, VCW, VCL:VCW ratio, HL, HW, and HL:HW ratio among the seven isolates ($P < 0.00$) (Table 3), indicating that there were measurable morphological differences among isolates taken from different water bodies. Nitrogen source had no effect on VCL ($P = 0.3$) or HL:HW ratio ($P = 0.2$) but had a significant effect on all other morphological variables (Table 3). VCW increased as follows: no added N $< NO_3^- < NH_4^+$ (Fig. 1). In general there was a strong consistency in the responses of all isolates to different nitrogen sources. The most striking morphological change induced by different nitrogen sources was the complete loss of heterocysts in cultures that occurred with NH_4^+. The terminal cells of trichomes grown in this treatment also became more tapered in appearance.

None of the differences in vegetative-cell and heterocyst dimensions could be related either to the ability of the isolates to produce CYL (Table 2) or to gross morphology (i.e., straight or coiled trichomes) (Fig. 1).

Genetic comparison. Analysis of 16S rRNA gene nucleotide sequences confirmed a strong (>99.8%) genetic similarity among all seven isolates of *C. raciborskii* (Fig. 2). It was not possible to define Australian *Cylindrospermopsis* isolates by specific sequence signatures. Therefore, the design of specific 16S rDNA-directed PCRs for the delineation of strains used

TABLE 3. Results of a two-way analysis of variance comparing some morphological characteristics of seven *C. raciborskii* isolates grown with different N sources

Variable	Total sample size	Statistical results when evaluating:					
		Differences between isolates		Effect of N source		Isolate differences × N source effects[a]	
		F	*P*	F	*P*	F	*P*
VCL[b]	779	33.79	0.00	1.21	≥0.30	2.54	0.00
VCW[b]	779	170.87	0.00	1,098.4	0.00	18.88	0.00
VCL:VCW[b]	779	70.82	0.00	182.57	0.00	5.30	0.00
HL	521	15.30	0.00	54.49	0.00	2.0	≥0.06
HW	521	35.83	0.00	80.74	0.00	4.19	0.00
HL:HW[b]	521	31.44	0.00	1.68	≥0.20	0.22	≥0.97

[a] Interaction of isolate differences and nitrogen source effects.
[b] Data required $\log_{10}$ transformation to improve normality.

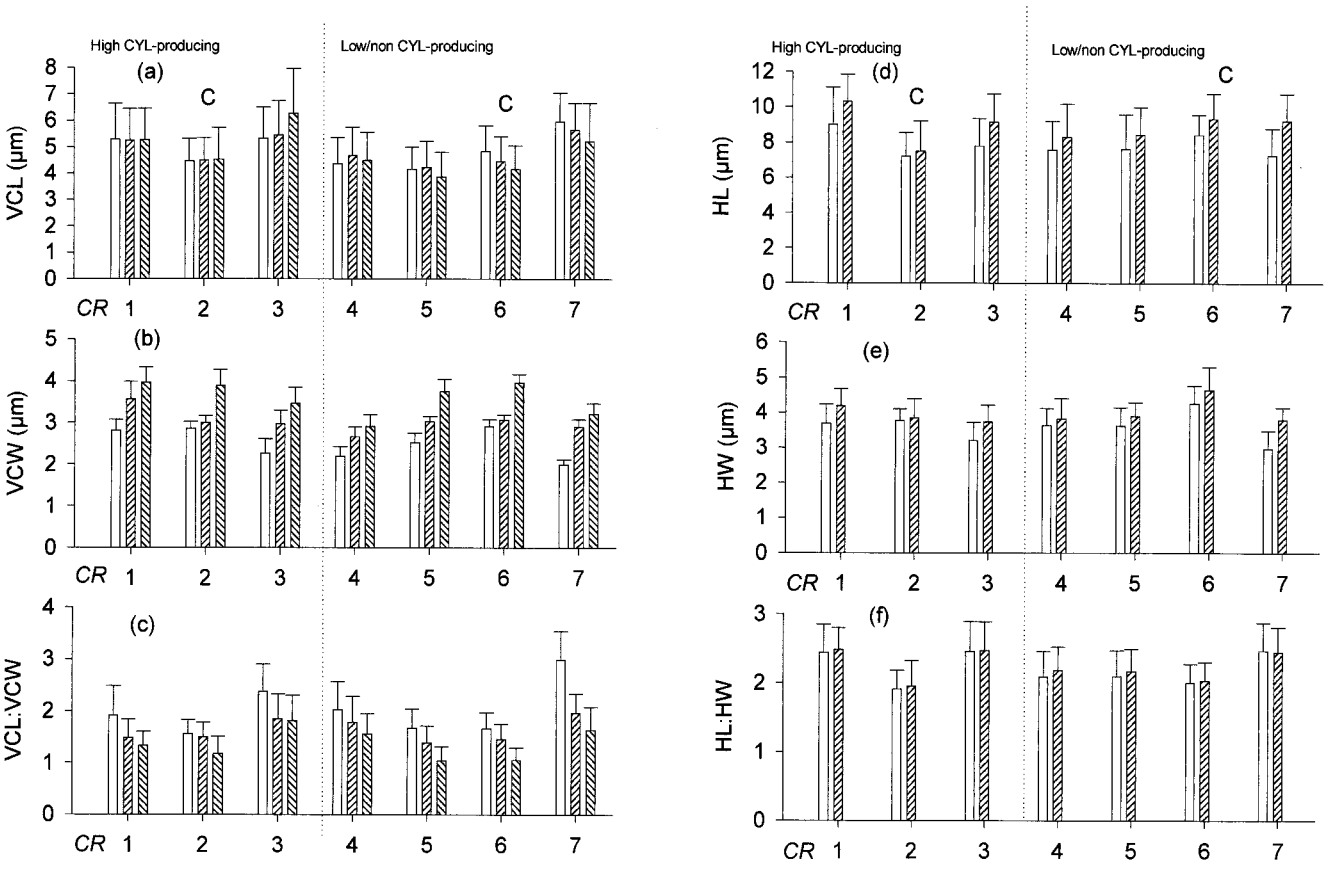

FIG. 1. VCL (a), VCW (b), VCL:VCW ratio (c), HL (d), HW (e), and HL:HW ratio (f) for seven isolates of *C. raciborskii* (*CR1* to *CR7* as described in Table 1) grown in the presence of NO_3^- (▨) or NH_4^+ (▧) or without the addition of a combined nitrogen source (□). The coiled forms, *CR2* and *CR6*, are labeled (C) in the upper panels. In panels d to f, only two bars are shown because cultures grown in the presence of NH_4^+ did not produce heterocysts.

here with regard to toxicity was not undertaken. Other cyanobacteria which are known to produce CYL, including *Aphanizomenon ovalisporum* ILC-146 (93.8% similar), *Umezakia natans* TAC101 (87.5% similar), and *Anabaena bergii* AWQC283A (92% similar), showed divergent 16S rDNA sequences in comparison to those of *C. raciborskii*. These other species were more distant from the *C. raciborskii* strains than *Anabaena cylindrica* and therefore were not used in the phylogenetic reconstruction.

The technique using short tandem-repeat sequences, HIP-PCR, was more sensitive in detecting genetic heterogeneity among isolates. Clear differences in banding patterns were observed among some of the isolates when both HipAC and HipTG primers were used in the one PCR (Fig. 3A). These genomic profiles were employed for the construction of a binary matrix which was subsequently used as the basis for the construction of a phylogenetic tree with *Anabaena cylindrica* serving as the outgroup. *CR1* and *CR3* (both of which possess straight trichomes and produce CYL) were distinct from the other isolates (Fig. 3B). This distinction was validated statistically by (i) the lack of significant bootstrap values separating *CR1* and *CR3* (10%) and (ii) consistent clustering of the remaining strains distinct from the *CR3* lineage in 64.6% of resampled trees (Fig. 3B). The remainder of the isolates clumped together in two closely related groups, the first consisting of three isolates (*CR2, CR7*, and *CR4*) and the second containing two (*CR5* and *CR6*). Both of these closely related groups contained representatives of straight and coiled isolates as well as toxic and nontoxic members. Only three fragments with no polymorphism were revealed for each of the seven strains when only the HipGT primer was used in a PCR (data not shown).

DISCUSSION

Five of the seven isolates produced detectable concentrations of CYL. This proportion (ca. 70%) is in fairly close agreement with the results of mouse toxicity tests of isolates of *C. raciborskii* taken from water bodies in southern Australia (2) and suggests that the CYL toxin is widespread in many Australian water bodies. Only *CR1, CR2*, and *CR3* were found to produce CYL at concentrations of >0.1% of the dry weight. The nitrogen source was found to have a significant effect on CYL concentration. While the growth rate was significantly reduced in the absence of a fixed-nitrogen source, growth under these conditions produced the greatest gravimetric toxin concentration. Similar findings have been reported for some other cyanobacterial species, including *Aphanizomenon flos-aquae* and *Anabaena flos-aquae*, both of which have been shown to produce higher concentrations of the propane alkaloid anatoxin-a under nitrogen-depleted conditions (26).

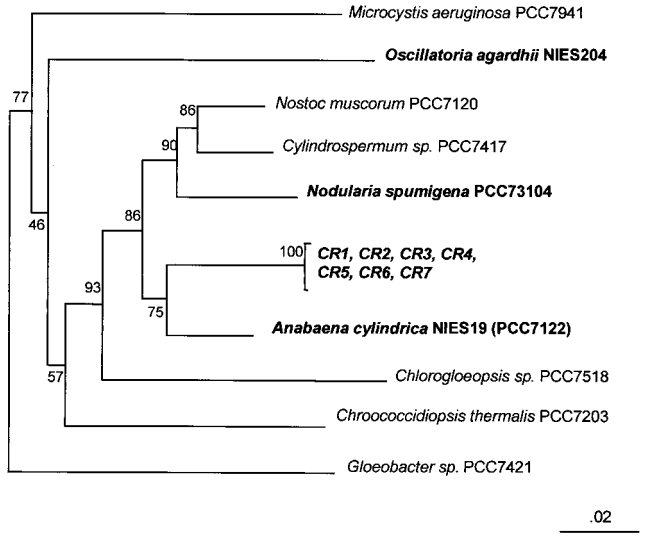

FIG. 2. Phylogenetic affiliations between seven isolates of *C. raciborskii* (*CR1* to *CR7*) (Table 1) and other cyanobacteria, derived from complete 16S rRNA gene sequences. The phenogram was reconstructed from a pairwise distance matrix (18) by the neighbor-joining method (27). The scale represents two substitutions per 100 nucleotide positions. Bootstrap values (1,000 resampling cycles) indicate the statistical significance of each node. GenBank accession numbers for the 16S rDNA sequences of strains *M. aeruginosa* PCC7941, *N. muscorum* PCC7120, and *Chlorogloeopsis* spp. strain PCC7518 are U40340, X59559, and X68780, respectively. Other sequence data were obtained from the Ribosomal Database Project under the accession codes cyls. 7417 (*Cylindrospermum* spp. strain PCC7417), chrc. 7203 (*C. thermalis* PCC7203), and glb.violac (*Gloeobacter* spp. strain PCC7421). Strains in boldface type were characterized during the present study.

Different nitrogen sources were found to induce statistically significant changes to the morphologies of all seven isolates. The loss of heterocysts, as occurred in cultures supplied with NH_4^+, considerably increases the difficulty in identifying members of the genus *Cylindrospermopsis*, considering that the terminal nature of heterocysts is the primary diagnostic feature of the genus. Furthermore, the provision of NH_4^+ as the primary nitrogen source led to a 33 to 61% increase in VCW and effected a 23 to 45% reduction in the VCL:VCW ratio. Considering that species in the genus *Cylindrospermopsis* are distinguished by minor differences in vegetative-cell and heterocyst dimensions, there are clear implications for the intrageneric taxonomy of *Cylindrospermopsis* strains. Similar observations have been reported for other genera of cyanobacteria in which the primary taxonomic characteristics vary under different culture conditions (7, 8, 38). The morphological variants induced by the different nitrogen supplies as reported here are in strong correspondence with the seasonal variants described by Singh (34). This observation is further supported by the findings of Komárková et al. (21) showing that populations of *C. raciborskii* lacking heterocysts were predominant in a tropical reservoir during periods of higher NH_4^+ concentration. Clearly, cell length and width measurements are insufficient to distinguish between strains or species of the genus *Cylindrospermopsis*, an observation in agreement with the suggestion that many morphological features of microorganisms in general may not be under tight genetic control (12).

All isolates, despite exhibiting statistically significant differences in many morphological characteristics, were found to be extremely similar in terms of their 16S rRNA gene nucleotide sequences. While *Anabaena cylindrica* grouped closely to *C. raciborskii, Aphanizomenon ovalisporum* and *U. natans*, two species also known to produce CYL, were considerably more genetically distant. With our increasing recognition of the ubiquity of some cyanobacterial toxins, such as microcystin and saxitoxin, throughout a range of distantly related cyanobacterial groups, it is not surprising that the CYL toxin is also produced by other genera of cyanobacteria. Given the data presented here, it would seem that the genus *Cylindrospermopsis* is a genetically well-defined population exhibiting considerable morphological

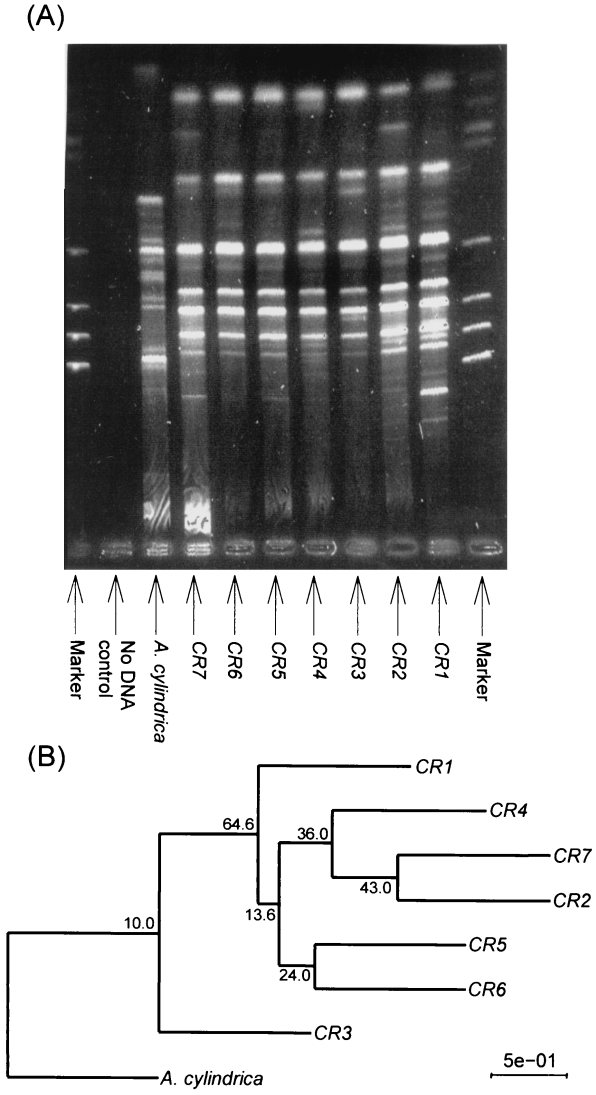

FIG. 3. (A) Electrophoretic comparison of the PCR products formed in reactions primed with HipCA and HipTG primers for the seven isolates of *C. raciborskii* (*CR1* to *CR7*), *Anabaena cylindrica*, and a no-DNA control. (B) Phenogram constructed from analysis of electrophoresis gels resulting from the HIP-PCR in panel A. A binary matrix was tabulated based on the presence or absence of bands and consisted of 25 characters across the eight operational taxonomic units. Tree reconstruction procedures are described in the text. All bootstrap values (1,000 resampling events) are shown.

and toxicological plasticity, unlike some other nostocalean cyanobacteria (3, 23).

The HIP-PCR analysis was found here to be useful for distinguishing among isolates of *C. raciborskii* at the strain level. Interestingly, this technique detected a significant difference between isolate *CR3* and all other isolates. This isolate, which was obtained from an aquaculture pond (Table 1), responded quite differently than the other isolates to different nitrogen sources, most notably with respect to growth rate responses.

Among the isolates which could not be distinguished by this technique were representatives with both straight and coiled trichomes and those which were toxic or nontoxic. The HIP-PCR analysis supports the proposal that morphologies, including heterocyst differentiation, trichome coiling, and CYL production, are inducible or repressible characters and are not necessarily linked to the phylogeny of *Cylindrospermopsis*. This confirmed the earlier finding (31) that neither of these characteristics is a valid taxonomic criterion, even though preliminary DNA profiling based on heptamer repeats indicates some linkage between trichome coiling and genotype (40). Identification and characterization of the genetic basis for CYL biosynthesis will assist in the detection of toxigenic strains and provide evidence for the evolution of CYL production in *C. raciborskii*. This may be achievable given the state of proteomics and the effect of altered diazotrophy on CYL production.

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