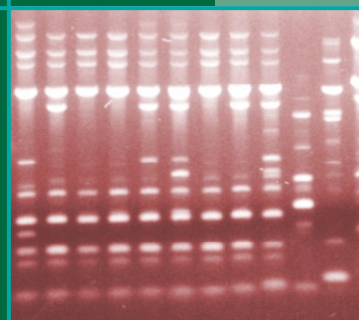




Regulation of lindrospermopsin Production by the Cyanobacterium *Cylindro- spermopsis raciborskii*



Research Report

32

Regulation of Cylindrospermopsin Production by the Cyanobacterium *Cylindrospermopsis raciborskii*

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Australian Water Quality Centre

Research Report No 32
March 2007

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Regulation of Cylindrospermopsin Production

Research Report 32
ISBN 1876616571

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PUBLICATIONS

Significant refereed international publications related to this work:

Wilson, K., Schembri, M. A. and Saint C. P. (2000) Genetic analysis of the toxic cyanobacterium *Cylindrospermopsis raciborskii*. *Applied and Environmental Microbiology* **66**: 332-338.

Fergusson, K., and Saint, C. P. (2000) Molecular phylogeny of *Anabaena circinalis* and its identification in environmental samples using PCR *Applied and Environmental Microbiology* **66**: 4145-4148.

Schembri, M. A., Neilan, B. A. and Saint C. P. (2001) Identification of genes implicated in toxin production in the cyanobacterium *Cylindrospermopsis raciborskii*. *Environmental Toxicology* **16**: 413-421.

Saint, C. P. and Fergusson, K. M. (2001) Molecular Identification of Toxic Cyanobacteria. In: *Application of photosynthetic microorganisms in environmental biotechnology*. Y. K. Lee & H. Kojima (Eds.) Ch. 15 pp 247-264. Springer-Verlag, Heidelberg.

Fergusson, K. M. and Saint, C. P. (2002) A Multiplex PCR Assay for *Cylindrospermopsis raciborskii* and Cylindrospermopsin Producing Cyanobacteria. (submitted to *Environmental Toxicology*)

Neilan, B. A., Saker, M. L., Fastner, J., Torokne, A., Dyble, J., Paerl, H. W. and Burns, P. B. (2002) Phylogeography of the invasive cyanobacterium *Cylindrospermopsis raciborskii* (submitted to *Molecular Ecology*)

Dyble, J., Paerl H. W., and Neilan, B. A. (2002) Phylogenetic relationships among global strains of the toxic cyanobacterium *Cylindrospermopsis raciborskii* based on *nifH* and *cpcBA*-IGS (*Applied and Environmental Microbiology* in press)

Foreword

This project was initiated with the following 3 objectives in mind:

- To characterise the types and distribution of *C. raciborskii* strains
- Examine the environmental factors which regulate toxin production
- To gain an understanding of the pathways of toxin production and their regulation

As this document reveals we have been successful in characterising a range of *C. raciborskii* isolates and have gained a basic knowledge of the genetic determinants involved in cylindrospermopsin production. The direction of the work did take a different emphasis when the first research officer, Ms Renate Velzeboer, was replaced by Dr Mark Schembri. Renate had experience in the area of algal culture and ecology and it was the intention that she would perform studies on the environmental factors affecting toxin production. Mark's expertise lay in the area of molecular biology, which resulted in a greater emphasis on this area of work. Subsequently on Mark's departure Dr Sarah Baker continued with the molecular studies. Having a succession of 3 research officers working on the project has not been ideal but continued involvement of the project leader and the input of Ms Kim Fergusson, firstly as an honours student and latterly as a PhD student, have helped to provide continuity. The input of the UNSW group under the direction of Dr Brett Neilan has also made a significant contribution to this end. Consequently significant progress has been made resulting in several publications in international peer reviewed journals and a provisional patent.

It is fully intended that the work reported here be continued and ways of achieving this aim are currently being investigated. In terms of characterisation of the genes involved in cylindrospermopsin production a PhD project to continue this area of research has been proposed. As the original project progressed it became clear that there is great potential in using gene technology as a basis for a rapid diagnostic test for *C. raciborskii*. Such tests could avoid the need for costly and protracted analytical chemistry to characterise toxin production and have potential for the development of field based assays. It is the intention that these aspects of the work will be further developed in a new jointly funded AWWARF/CRC project entitled, "Early Detection of Cyanobacterial Toxins Using Genetic Methods". The project will involve the same 2 groups of researchers at AWQC and UNSW and include collaboration with university and water authority researchers in the USA.

Executive Summary

The cyanobacterium (blue-green algae) *Cylindrospermopsis raciborskii* is best known for its problem bloom formation in tropical and sub-tropical regions of the world. In Australia the best known incidence of human poisoning occurred on Palm Island, Queensland in the 1970's, resulting in the hospitalisation of 148 people. In recent times toxic blooms of this organism have been reported throughout Australia and internationally in countries such as the USA, South Africa, Brazil, Hungary and Thailand. Problems with this organism are compounded when blooms are treated with control agents or the water treatment process itself causes cells to lyse releasing cylindrospermopsin into the water supply. In this project genetic methods have been applied to characterise toxic and non-toxic isolates of *C. raciborskii* from Australia and around the world. This characterisation has directly assisted identification of the organism which has been difficult using traditional methods. This genetic characterisation has also permitted the development of tests to rapidly identify the organism in the laboratory with clear definition. In addition genetic determinants likely to be involved in production of the toxin cylindrospermopsin have been identified and their initial characterisation commenced. Genetic analysis of this organism has not been straightforward but the results presented here provide a basis for the rapid acceleration of this work and its applications in the next few years.

A total of 19 Australian isolates identified by microscopy as *C. raciborskii* were characterised by examining the DNA sequence of a fragment of the DNA dependent RNA polymerase gene *rpoC1*. The sequence was highly conserved (>99%) between isolates. This conservation of sequence was maintained across both coiled and straight isolates of the organism. The characteristic (consensus) sequence for *C. raciborskii* was compared to equivalent sequences for a range of other cyanobacterial genera and species. As expected *C. raciborskii* grouped with other filamentous nitrogen fixing cyanobacteria and was most closely related to *Anabaena*. In an equivalent study examining 16S rRNA gene sequence a similar pattern was seen with >99% sequence identity in strains examined. That study also included a range of internationally acquired strains and although geographic grouping was evident the level of dissimilarity between the groups was very low.

Neither *rpoC1* nor 16S rRNA gene analysis distinguished coiled from straight morphotypes or toxic from non-toxic isolates of *C. raciborskii*, so an alternative genetic profiling technique was applied. Cyanobacteria have short stretches of repeated DNA sequence distributed randomly throughout their genomes. The repeats are known as short tandemly repeated repetitive sequences, or STRR sequences. These were targeted in polymerase chain reaction (PCR) tests and resulted in genetic profiles for the isolates. Computer analysis revealed specific profiles that were able to distinguish coiled from straight isolates but not toxic from non-toxic, i.e. this method failed to specifically identify cylindrospermopsin producers.

By analogy to what is known regarding the genetic determinants involved in microcystin production in *Microcystis aeruginosa*, DNA primers were designed in an attempt to amplify and identify similar DNA sequence from *C. raciborskii*. This was successful with candidate polyketide synthase (*pks*) and peptide synthetase (*ps*) determinants identified. Further genetic analysis revealed that these determinants were always present in cylindrospermopsin producing isolates and absent in non-toxic isolates. In addition they were also identified in a strain of *Anabaena bergii* known to produce cylindrospermopsin.

Using the genetic information gathered during this project diagnostic tests were developed to specifically identify *C. raciborskii* and assess its toxin producing potential. The tests were all based on gene amplification protocols using PCR. A quantitative PCR was developed to identify *C. raciborskii* and estimate cell numbers in 2 – 3 hrs. This test incorporated an internal control that permitted quantification, and ensured that a negative result for *C. raciborskii* was not due to the reaction being inhibited by environmental contaminants carried over from the raw sample. The test was successfully applied to several bloom samples. Similarly a test was developed termed a multiplex PCR that indicated whether the sample contained *C. raciborskii* and also whether the two determinants linked to cylindrospermopsin production, *pks* and *ps*, were present. This test also incorporated an internal control.

Taken as a whole the data derived from this project have established a strong foundation to develop further rapid DNA based tests for *C. raciborskii* and a range of other toxic cyanobacteria including *Anabaena circinalis* and *Microcystis aeruginosa*. Genetic analysis of the *pks* and *ps* determinants was hampered by the difficulty of performing manipulations in the filamentous cyanobacterium *C. raciborskii* which in some ways delayed the project. However, the work has been left at a stage where further detailed characterisation of the genes should now be possible, permitting a detailed understanding of how, if at all, cylindrospermopsin production is regulated at the genetic level. Finally, the information so far acquired will be extremely useful in developing improved rapid DNA based tests for these nuisance organisms with the ultimate aim of producing simple kits that can be used in the field. This will be of particular value to water authorities charged with the maintenance of remote water supplies.

1 Introduction

1.1 Cyanobacteria (Blue-green Algae)

Cyanobacteria are photosynthetic bacteria that are widespread in marine and freshwater aquatic environments. They are able to cause significant and potentially hazardous toxic blooms on the surface of water bodies, particularly under conditions of eutrophication. Toxic bloom-forming cyanobacteria have been found amongst the genera *Microcystis*, *Anabaena*, *Oscillatoria*, *Aphanizomenon*, *Cylindrospermum*, *Cylindrospermopsis*, *Nostoc*, *Nodularin*, *Lyngbya* and *Phormidium* (Carmichael and Falconer, 1993; Hunter, 1998). The types of toxins isolated and characterised from these cyanobacteria include those with hepatotoxic, neurotoxic and dermatotoxic effects.

1.2 The Significance of *C. raciborskii*

The cyanobacterium *Cylindrospermopsis raciborskii* is found predominantly in tropical to sub-tropical regions, although more recently it has been isolated from cooler, temperate climates (Komárek, 1985; Baker and Humpage, 1994; Kleinkauf and von Döhren, 1996). Blooms of this species have been found throughout Australia, Brazil, Europe and the USA. *C. raciborskii* can produce a potent hepatotoxin termed cylindrospermopsin. The toxic compound is a novel alkaloid, thought to be of polyketide origin, with a molecular weight of 415 incorporating a tricyclic guanidine moiety combined with hydroxymethyluracil (Fig. 1; Ohtani et al., 1992). Norris et al. (1999) demonstrated that deoxycylindrospermopsin produced by *C. raciborskii* was non-hepatotoxic. They suggested the presence of the hydroxyl on the uracil bridge, or the keto-enol status of the uracil moiety, is essential for toxicity. Recent work by Banker et al. (2001) demonstrated simple substitution of the vinylic proton at position 5 of the uracil ring by chlorine is sufficient to abolish known toxicity.

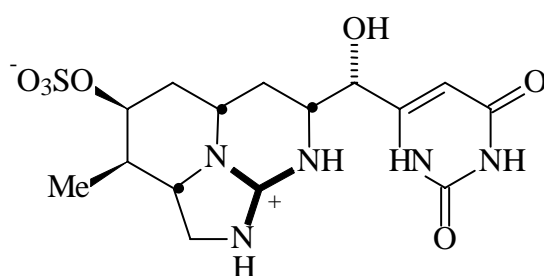


Fig. 1. Structure of cylindrospermopsin.

In 1979, *C. raciborskii* was implicated in a significant human poisoning incident on Palm Island, Australia. There were 148 reported cases of illness lasting between 4 and 26 days, symptoms being hepatitis like (Byth, 1980). Cyanobacteria from the original bloom were not identified prior to copper sulphate treatment of the contaminated reservoir, but subsequent investigations indicated *C. raciborskii* was the likely cause of the outbreak (Bourke et al., 1983; Hawkins et al., 1985). An isolate of *C. raciborskii* was cultured from the water supply reservoir and shown to be severely hepatotoxic in mice, with extensive necrosis observed in the liver and various degrees of damage also observed to the kidneys, adrenal glands, lungs and intestine (Hawkins et al., 1985). Subsequent studies using purified cylindrospermopsin have shown that this toxin is hepatotoxic *in vivo*, directly

cytotoxic, and can result in significant depletion of cell glutathione levels (Runnegar et al., 1994; Runnegar et al., 1995). In addition to human illness, toxic *C. raciborskii* blooms have been implicated in the death of cattle in regions of northern Australia (Thomas et al., 1998). Apart from *C. raciborskii*, recent studies have demonstrated the cyanobacteria *Aphanizomenon ovalisporum* (Banker et al., 1997; Shaw et al., 1999) and *Umezakia natans* (Harada et al., 1994; Teraro et al., 1994) also produce cylindrospermopsin.

1.3 Genetic Studies on Cyanobacterial Toxins

Cyanobacteria produce a chemically diverse array of peptides and polyketides with varying properties that are collectively referred to as secondary metabolites. These compounds are synthesised when normal, balanced growth ceases and are associated with toxic, hormonal, antineoplastic and antimicrobial effects (Carmichael, 1992; Moore et al., 1993; Erhard et al., 1997). Microcystins, nodularins, cylindrospermopsin, anatoxins and saxitoxins are all examples of harmful secondary metabolites produced by toxic bloom forming cyanobacteria. Polyketide synthase (PKS) and peptide synthetase (PS) determinants are involved in secondary metabolite biosynthesis in bacteria and fungi (Hutchison, 1995). PKSs are multifunctional enzyme assemblies which synthesise polyketide metabolites by catalysing repeated decarboxylative condensations between enzyme bound acylthioesters (Hopwood and Sherman, 1990; Donadio et al., 1991; O'Hagan, 1991; Hutchinson, 1995). Methods to exploit the use of PKS genes to synthesise novel medically important products have also been investigated (Hutchinson, 1999; Gokhale et al., 1999). The PSs are composed of distinct modules and employ a thiotemplate mechanism for non-ribosomal peptide synthesis (Lipmann, 1980). Each module encodes a specific peptide synthetase unit that catalyses one elongation step of non-ribosomal peptide synthesis. The role of multimodular PS genes in the synthesis of peptide antibiotics has been demonstrated in microorganisms (Belshaw et al., 1999).

While cyanobacteria produce a number of potentially important polyketide products, few genetic studies have been performed (Black and Wolk, 1994; Campbell et al., 1997) and little is known of the molecular processes involved. Genes involved in the synthesis of microcystin have recently been characterised in *Microcystis aeruginosa* (Dittmann et al., 1997; Nishizawa et al., 1999; Nishizawa et al., 2000; Tillet et al., 2000). A suite of genes spanning 55 kbp of the chromosome are involved. *mcyA-C* constitute the PS component whilst *mcyD-J* form the PKS. Mutant analysis revealed the PKS to be responsible for production of the Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) side chain and the PS production and condensation of the remaining six amino acids to form the complete heptapeptide.

1.4 Key Initiatives of this Study

1.4.1 Identification of *C. raciborskii*

Of interest is that the species referred to as "*C. raciborskii*" clearly has different morphotypes which can be conveniently termed coiled and straight (Fig. 2). We set out to apply a range of molecular typing techniques to clarify whether these forms are indeed one and the same species. In this study we have used DNA sequence analysis of the 16S rRNA gene, the DNA dependent RNA polymerase gene (*rpoC1*) and short tandemly repeated repetitive (STRR) sequence analysis to characterise a range of coiled and straight isolates. It also was not known whether both straight and coiled isolates of the cyanobacterium were capable of producing toxin. Assessment of toxin production amongst a range of isolates was made using HPLC-MS.

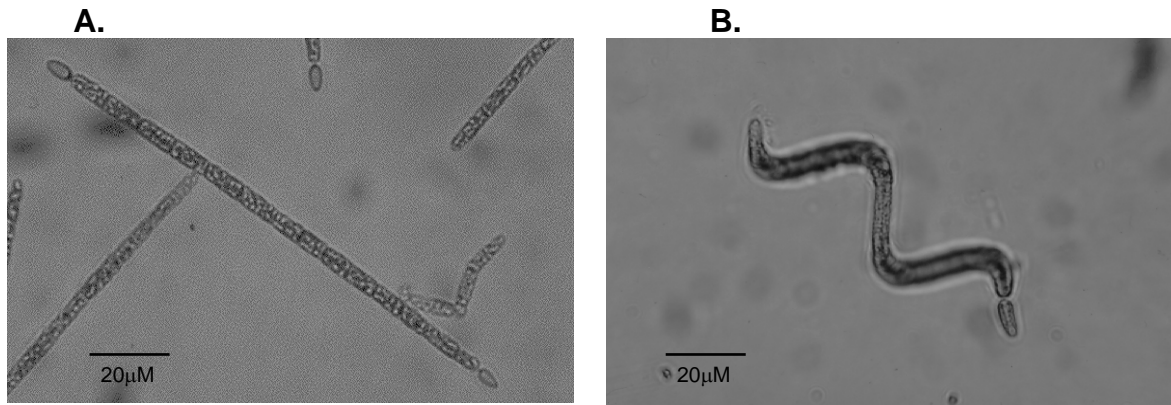


Fig. 2. Morphotypes of *C. raciborskii* at 268x magnification.

1.4.2 Identification of Genes Implicated in Toxin Production

From what was known concerning PS and PKS determinants in other microorganisms degenerate primers were designed to attempt amplification of specific regions of these genes. The work reported here on *Microcystis* was progressing along the same lines at about the same time. Reported here is the identification of putative PS and PKS determinants in *Cylindrospermopsis* that are only evident in toxic strains. In addition these determinants were also present in *Anabaena bergii* and *Aphanizomenon ovalisporum* isolates that also produced cylindrospermopsin. Efforts have been made to produce a mutant deficient in cylindrospermopsin production by genetic recombination. The approaches attempted are

detailed but so far these have not been successful. *C. raciborskii* is a filamentous cyanobacterium and genetic manipulations are notoriously difficult in such species. However, alternative approaches such as attempting to clone the complete suite of genes involved in cylindrospermopsin production have been attempted with some success. The expression of these genes in a foreign host may be an alternative way of proving their involvement in cylindrospermopsin production. This may also pave the way for increased production of cylindrospermopsin and its purification for commercial purposes.

1.4.3 Rapid Testing for Toxic *Cylindrospermopsis*

Data from this project has contributed to the rapid identification of *C. raciborskii* using polymerase chain reaction (PCR) based tests. A range of tests which identify and quantify *C. raciborskii* and reveal the presence/absence of toxin genes have been developed and trialed on isolates and environmental samples. This work is important as it establishes a basis for further work investigating the development of DNA based tests for field applications.

2 Methods and Materials

2.1 Bacterial Strains and Growth Conditions

Lyophilised samples of uni-algal cultures of *C. raciborskii* and other cyanobacteria were obtained from a range of different countries, Australian isolates were derived largely from the AWQC culture collection. Information including strain source location, gross morphology and references to toxicity of these strains are provided in Table 1. Strains were grown under constant light intensity ($20 \mu\text{Mm}^{-1}\text{s}^{-1}$) for up to 14 days at 25°C in ASM-1 medium (Gorham et al., 1964), with the exception that a nitrogen source was omitted and Na_2MoO_4 was added to a final concentration of 0.01 mg mL^{-1} . Environmental samples from Fred Haigh Dam, Queensland (courtesy of Glenn McGregor, Queensland Department of Health) and Currency Creek, South Australia, were frozen until required.

Table 1. List of strains used in this study.

Strain	Origin	Morphology ^a	Toxins ^b	Reference ^c
<i>Cylindrospermopsis raciborskii</i>				
AWT205	NSW, Australia	straight	TM, CYL	Hawkins et al. (1997)
CYP003A	Victoria, Australia	coiled	NOCYL	AWQC
CYP003K	Victoria, Australia	straight	CYL	AWQC
CYP005E	NSW, Australia	coiled	CYL	AWQC
CYP005F	Australia	coiled	Not tested	AWQC
CYP010A	Australia	coiled	Not tested	AWQC
CYP010C	Australia	coiled	Not tested	AWQC
CYP014A	Queensland, Australia	straight	NOCYL	AWQC
CYP015A	Queensland, Australia	straight	NOCYL	AWQC
CYP020A	Palm Island, Australia	straight	CYL	AWQC
CYP020B	Palm Island, Australia	straight	CYL	AWQC
CYP023A	Queensland, Australia	straight	CYL	AWQC
CYP023B	Queensland, Australia	straight	Not tested	AWQC
CYP023D	Queensland, Australia	coiled/straight	Not tested	AWQC
CYP023E	Queensland, Australia	straight	CYL	AWQC
CYP024C	Queensland, Australia	straight	CYL	AWQC
CYP025B	Queensland, Australia	coiled	CYL	AWQC
CYP025E	Queensland, Australia	coiled	Not tested	AWQC
CYP026J	NSW, Australia	straight	CYL	AWQC
SDS	Queensland Australia	Straight	TM, CYL	Saker et al. (1999a)
SDC	Queensland, Australia	Coiled	TM, CYL	Saker et al. (1999a)
AQS	Queensland, Australia	Straight	TM, CYL	Saker & Eaglesham (1999)
GOON	Queensland, Australia	Straight	TM, CYL	Saker and Griffiths (2000)
LJ	Queensland, Australia	Straight	NM, NOCYL	Saker (2000)
MCKIN	Queensland, Australia	Straight	TM, CYL	Saker et al. (1999b)

AQC	Queensland, Australia	Coiled	NM, NOCYL	Saker (2000)
brazil 1	Brazil	unknown	Not tested	S. Azevado
brazil 2	Brazil	unknown	Not tested	S. Azevado
BRAZ1	São Paulo, Brazil	Straight	TM, PSP	Lagos <i>et al.</i> (1999)
BRAZ2	São Paulo, Brazil	Straight	TM, PSP	Lagos <i>et al.</i> (1999)
GERM1	Lake Melangsee, Germany	Straight	NOCYL	J. Fastner
GERM2	Lake Melangsee, Germany	Straight	NOCYL	J. Fastner
BAL5	Lake Balaton, Hungary	Straight	Not tested	O. Skulberg
BAL6	Lake Balaton, Hungary	Straight	Not tested	A. Kovacs
MARAU1	Maranhão Reservoir, Portugal	Straight	TM, NOCYL	Saker <i>et al.</i> (unpublished)
MARAU2	Maranhão Reservoir, Portugal	Straight	TM, NOCYL	Saker <i>et al.</i> (unpublished)
4799	Odivelas Reservoir, Portugal	Straight	TM, NOCYL	Saker <i>et al.</i> (unpublished)
CAIA	Caia Reservoir, Portugal	Straight	TM, NOCYL	Saker <i>et al.</i> (unpublished)
FL- D	St Johns River, Florida, USA	Coiled	Not tested	P. Moisander
FL-G	St Johns River, Florida, USA	Straight	Not tested	P. Moisander
FL-F	St Johns River, Florida, USA	Straight	Not tested	P. Moisander
FL-I	St Johns River, Florida, USA	Straight	Not tested	P. Moisander
<i>Anabaena circinalis</i> ANA118C	Australia		NOCYL	AWQC
<i>Anabaena circinalis</i> ANA173A	Australia			AWQC
<i>Anabaena bergii</i> ANA283A	Australia		CYL	AWQC
<i>Microcystis aeruginosa</i> PCC7806	The Netherlands		NOCYL	Ripka & Herdman (1992)
<i>Microcystis aeruginosa</i>	Australia			AWQC
<i>Nodularia spumigena</i> PCC73104	Canada		NOCYL	Ripka & Herdman (1992)
<i>Anabaenopsis circularis</i>	Japan			NIES

- a. Refers to *C. raciborskii* trichome structure
- b. TM - Toxicity confirmed by mouse bioassay; NM - non-toxic in mouse bioassay; CYL - presence of cylindrospermopsin confirmed by HPLC; NOCYL - absence of cylindrospermopsin confirmed by HPLC; PSP - production of paralytic shellfish poisons confirmed by HPLC
- c. AWQC, Australian Water Quality Centre culture collection; NIES, National Institute of Environmental Studies culture collection

2.2 DNA Extraction

DNA techniques were carried out according to standard procedures (Sambrook et al., 1989). Genomic DNA was extracted from cyanobacterial cells essentially as described by Porter (1988). 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 EDTA). Three freeze-thaw cycles were performed at -80°C and room temperature respectively. The cells were treated with 5 mg lysozyme for 30 minutes at 37°C. Sodium dodecyl sulfate and proteinase K (Sigma) were added to final concentrations of 1% (w/v) and 100 µg mL⁻¹ respectively, and the samples incubated at 45°C overnight. The DNA was extracted three times with phenol-chloroform and twice with chloroform. The DNA was precipitated, washed with 70% ethanol, resuspended in 100 µL of TE buffer and stored at -20°C. For environmental samples, DNA was extracted using the InstaGene matrix (Bio-Rad). Briefly, 10 mL samples were pelleted by centrifugation and resuspended in 200 µL of solution containing 90% InstaGene matrix and 10% TritonX-100. Cells were incubated at 55°C for 30 minutes, vortexed for 1 minute then heated to 95°C for 10 minutes. Following centrifugation, DNA was extracted once with an equal volume of phenol-chloroform then once with chloroform. The DNA was precipitated, resuspended into 50 µL of water and used directly in PCR's.

2.3 PCR and DNA Sequence Analysis

All PCR's were performed on a Perkin-Elmer GeneAmp 2400 PCR system. Each 50 µL reaction contained 1-10 ng genomic DNA, 20 pmole of each PCR primer, 200 µM deoxynucleoside triphosphates (dNTP's), 250 µM magnesium chloride, 1 x PCR BufferII and 2.5 units of Ampli Taq Gold (Perkin-Elmer). Oligonucleotides were purchased from GeneWorks Pty. Ltd. For amplification of the *rpoC1* gene from cyanobacterial strains, the following primers were used: *rpoC1*-1: (5'-GAGCTCYAWNACCATCCAYTCNGG) and *rpoC1*-T: (5'-GGTACCNAAYGGNSARRTNGTTGG) (Palenik and Haselkorn, 1992). Thermal cycling conditions for the PCR were: 95°C 10 min, 1 cycle; 92°C 90 s, 58°C 1 min, 72°C 2 min, 35 cycles; 4°C, hold. For amplification of putative PKS and PS determinants the following primers were used: PKS degenerate primers: DKF, 5'-GTGCCGGTNCC(AG)TGNG(TC)(TC)TC and DKR, 5'-GCGATGGA(TC)CCNCA(AG)CA(AG)(CA)G; PS degenerate primers: MTF2, 5'-GCNGG(CT)GG(CT)GCNTA(CT)GTNCC and MTR, 5'-CCNCG(AGT)AT(TC)TTNAC(TC)TG (Neilan et al., 1999); PKS specific primers: M4, 5'-GAAGCTCTGGAATCCGGTAA and M5, 5'-AATCCTTACGGGATCCGGTGC; PS specific primers: M13, 5'-GGCAAATTGTGATAGCCACGAGC and M14, 5'-GATGGAACATCGCTCACTGGTG. For PCR using degenerate primers the following protocol was used: 94°C 4 min, 1 cycle; 94°C 10 s, 50°C 20 s, 72°C 1 min, 30 cycles; 4°C hold. Where either the PKS or PS specific primers were used the protocol was the same with the exception that the annealing temperature was increased to 55°C.

PCR products were either sequenced directly or following ligation into the PCR cloning vector pCR[®]2.1 (Invitrogen). DNA sequencing was performed on both strands with the Taq DyeDeoxy Terminator Cycle Sequencing Kit and an automated model 373A DNA sequencer (Applied Biosystems) according to the manufacturer's instructions. Sequences were analysed with GeneJockeyII sequence processor (Biosoft) and homology searches performed with the National Centre for Biotechnology Information (NCBI) database using the BLAST network service. Sequence alignments were performed using ClustalX (Thompson et al., 1997).

2.4 Southern Hybridisation Analysis

Southern blot analysis was performed according to standard techniques (Sambrook et al., 1989). Gene probes were amplified from *C. raciborskii* AWT205 using primers specific for the PKS (M4 and M5) and the PS (M13 and M14) fragments and labelled using the digoxigenin random priming kit (Boehringer Mannheim) according to the manufacturer's instructions. Screening for the presence of these genes in other *C. raciborskii* strains was performed under high stringency conditions (60°C hybridisation temperature; 0.5 x SSC, 0.1% SDS wash at 60°C). The screening of other cyanobacterial strains for the presence of these genes was performed at lower stringency (45°C hybridisation temperature; 1 x SSC, 0.1% SDS wash at room temperature). Hybridisation signals were observed by colorimetric detection using BCIP/NBT as described by the manufacturer (Boehringer Mannheim).

2.5 Cylindrospermopsin Analysis

Analysis of *C. raciborskii* and *A. bergii* extracts for the presence of cylindrospermopsin was performed by reverse phase HPLC and mass spectroscopy as previously described (Eaglesham et al., 1999). The estimated detection limit for this assay is 0.2 µg L⁻¹.

2.6 STRR Profiles

A method that identified genetic variation between *C. raciborskii* strains was developed using primers derived from previously identified cyanobacterial repeat sequences (Mazel et al., 1990). The following primers were used: STRR1F (5'-CCCCARTCCCCART), STRR1R (5'-GGGGAYTGGGGAYT), STRR2F (5'-TTGGTCATTGGTCA), STRR2R (5'-TGACCAATGACCAA), STRR3F (5'-CAACAGTCAACAGT), STRR3R (5'-ACTGTTGACTGTTG). Thermal cycling conditions were: 94°C 10 min, 1 cycle; 94°C 30 s, 40°C 1 min, 65°C 4 min, 35 cycles; 65°C 7 min, 1 cycle; 4°C, hold. All PCR reactions were performed in at least two independent experiments. STRR profiles were converted to binary data by scoring 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 Neighbour-Joining tree with PAUP* (Swofford, 1999).

2.7 16S rDNA Amplification

PCR amplification of the 16S rRNA gene was performed using primers 27FI(UFP) and 1494Rc(URP) together with PCR reagents as previously described (Neilan et al. 1997). Thermal cycling was performed at 94°C for 4 min., 1 cycle; 94°C, 20 sec., 50°C, 30 sec., 72°C, 2 min., 30 cycles. The amplification reactions were purified using the Wizard PCR purification system (Promega, Madison, WI) 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 (Neilan et al. 1997) were used to determine the primary structure of the *Cylindrospermopsis* 16S rDNA. Automated DNA sequencing was performed using the PRISM™ cycle sequencing system and the ABI373 sequencer (ABI, Foster City, CA). Oligonucleotide primers were synthesised on a Beckman Oligo 1000 DNA synthesis system (Beckman, Fullerton, CA) and purified by reverse phase chromatography.

2.8 *C. raciborskii* Specific PCR

A PCR test was developed for the specific identification of *C. raciborskii*. The primers cyl2 (5'-GGCATTCTAGTTATATTGCCATACTA), cyl4 (5'-GCCCCGTTTTGTCCCTTTGCTGC) and cyl-int (5'-TATTGCCATACTACCTGGTAATGCTGACACACTCG) were used. An internal control fragment (ICF) was produced using the primer cyl-int to spike into PCR reactions. The 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 PCR reactions 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 genomic DNA, 20 pmole cyl2, 20 pmole cyl4, 200 µM dNTP's, 250 µM magnesium chloride, 1 x PCR BufferII, 2.5 units of Ampli Taq Gold and 20 fg internal control fragment. Thermal cycling conditions were: 95°C 10 min, 1 cycle; 94°C 30 s, 45°C 30 s, 72°C 30 s, 35 cycles; 72°C 15 min, 1 cycle; 4°C, hold.

2.9 Phylogenetic Analysis

2.9.1 Analysis of the *rpoC1* Gene

Phylogenetic analysis of the DNA sequence data was performed with the MEGA analysis platform (Kumar et al., 1993). Briefly, pairwise distances were calculated using the Jukes-Cantor method and a tree constructed with the Neighbour-Joining algorithm. The pairwise deletion option was used for missing data and gaps in the alignment. Bootstrap analyses were performed using 500 replicates.

2.9.2 Analysis of the 16S rRNA gene

DNA sequences were aligned using the Pileup program (GCG, Madison, Wisconsin) and the multiple sequence alignment tool from Clustal X (Thompson et al. 1994). Manual confirmation of the sequence alignment was performed and checked against both primary and secondary structure considerations of the 16S rDNA molecule. The aligned sequences were applied to genetic distance, maximum likelihood and parsimony methods for phylogenetic inference. Ambiguous characters, where a deletion, insertion, or unidentified state was recorded for any strain, were removed from 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 (Jukes and Cantor 1969), where $D = -3/4 \ln(1 - 4/3d)$ and d is the sequence dissimilarity. Phylogenetic inference protocols, DNADIST, NEIGHBOR, DNAPARS, CONSENSE, and SEQBOOT were supplied by the PHYLIP package (Version 3.57c) (Felsenstein 1989). All sequence manipulation and phylogeny programs were made available by the Australian National Genomic Information Service (ANGIS, Sydney, Australia).

2.10 Knock-out Mutant Studies

2.10.1 Plasmid pCYL1220 construction

A 2.8 kb fragment of the *C. raciborskii* AWT205 *pks* gene was amplified in two parts by PCR, using primers that contained the *Sma*I restriction site. These PCR products were cloned separately into pCR2.1 (Invitrogen Corp.) cloning vector. Using the *Eco*R1 site unique to the vector, and the *Sma*I site unique to the insert, one of the inserts was cut out and ligated into the other vector. The resultant insert in the

pCR2.1 vector was the 2.8 kb *pks* fragment with an engineered *Sma*I site. pACYC184 was obtained from the University of Adelaide. PCR primers were designed to amplify the chloramphenicol gene from pACYC184, and it was then successfully ligated into the *Sma*I site of pCYL1220.

2.10.2 Electroporation

50 mL cultures of *C. raciborskii* AWT205 were grown at a constant light intensity in ASM-1 media to approximately 10^5 cells/mL, then collected by centrifugation and resuspended in 25 mL ASM-1 media. It was necessary for the physical disruption of *C. raciborskii* filaments to 1-3 cell lengths, in order to minimise the incidence of mixed colonies. Cells were sonicated on ice at 50% for 15-20 blasts until filaments either 'bent' or broke into filaments of 2-3 cell lengths. The cells were then centrifuged, washed twice in 1 mL ice-cold 1mM HEPES, pH 7.2, and resuspended in a final volume of 250 μ L. For electroporation, 40 μ L of cells and 1-5 μ g/mL of the knock-out plasmid pCYL1220 or control plasmid pRL6 were mixed and chilled on ice. Electroporation was performed on the Bio-Rad Gene Pulser in a chilled, sterile cuvette with a 2 mm electrode gap at a range of field strengths:

0.6 kV and 5.2 ms time constant (200 Ω , with the 25 μ F capacitor)

0.8 kV and 2.5 ms time constant (100 Ω , with the 25 μ F capacitor)

0.9 kV and 5.2 ms time constant (200 Ω , with the 25 μ F capacitor)

1.3 kV and 5.2 ms time constant (200 Ω , with the 25 μ F capacitor)

Cells were rinsed immediately into 10 mL ASM-1 or ASM-1 –N+Mo with either 0.5, 1 or 5 μ g/mL chloramphenicol and incubated at 25°C at a continuous light intensity for 5 days. The cells were then plated on 0.75% ASM-1 agar with an overlay of 0.75% ASM-1 agar containing 10 μ g/mL chloramphenicol and sealed with parafilm. After 6 weeks incubation, there were no transformants.

2.10.3 Plasmid DNA Methylation/electroporation Procedure

5 μ L of plasmid DNA was treated with 1 U Sss-I methylase (New England Biolabs) for 2 hours to overnight at 37 °C. The mixture was precipitated with 1/10 volume 4M LiCl and ethanol, then washed with 70% ethanol, then resuspended in 5-15 μ L of MilliQ water. 5 μ L of methylated plasmid DNA (either pRL6 or Tn5 plasmid + transposase in JM109) was used per reaction. Cells were rinsed immediately into 1 mL ASM-1, covered with paper towel and incubated at 25°C overnight at a low light intensity. Cells were added to 4 mL 0.75% ASM-1 agar, overlayed onto ASM-1 agar plates, then the plates were sealed with parafilm and incubated at a constant light intensity for 72 hours at 25 °C. 3 mL of 0.75% ASM-1 agar containing a final concentration of 0.5 μ g/mL chloramphenicol was then overlayed, the plates resealed and incubated for up to 6 weeks, with no transformants being produced.

2.10.4 Rouhiainen's Electroporation Procedure

60 mL samples of *C. raciborskii* AWT205, *C. raciborskii* CYP020A, *C. raciborskii* CYP020B, *A. bergii* ANA366B and *Aph. ovalisporum* APH033C were grown for 18 days (10^7 cells/mL), collected by centrifugation, washed once with 30 mL of 1 mM HEPES, pH 7.2, and three more times with 1 mL of the same buffer. After the final wash, the volume of the cell suspension was adjusted to 600 μ L. For electroporation,

50 μ L cells and 20 μ g of either the knock-out plasmid pCYL1220 or the control plasmid pRL6 in 50 μ L of HEPES, pH 7.2, were mixed and vortexed for 3 minutes. The mixture was centrifuged (1 minute, 15 000 $\times g$) and extra buffer removed to reduce the volume to 50 μ L. The suspension was chilled on ice and electroporated on the Bio-Rad Gene Pulser in a chilled, sterile cuvette with a 2 mm electrode gap by delivering a pulse of either 1.0 kV and 5.0 ms time constant (200 Ω , with the 25 μ F capacitor), 1.5 kV and 4.8 ms time constant (200 Ω , with the 25 μ F capacitor), or 2.0 kV and 2.5 ms time constant (100 Ω , with the 25 μ F capacitor). Cells were kept in the cuvette on ice for 2 minutes then rinsed by pipetting with 2 x 200 μ L HEPES buffer into 2 mL ASM-1 media. After 3 hours at room temperature, cells were pelleted (6000 $\times g$, 1 minute), resuspended in 1.5 mL of ASM-1 media, incubated at 25 $^{\circ}$ C with continuous illumination for 60 hours, then plated on ASM-1 agarose (0.4% agarose) that had been spread with 95 μ L of 0.17 mg/mL chloramphenicol. After 16 days, the chloramphenicol concentration was gradually raised to 1 μ g/mL. Experimental results are still pending.

2.11 Nucleotide Sequence Accession Numbers

The nucleotide sequences obtained in this work have been deposited with the GenBank database under the accession numbers: *C. raciborskii* consensus *rpoC1*, AF159371; *N. spumigena* PCC73104 *rpoC1*, AF159372; *A. circinalis* ANA118C *rpoC1*, AF159373; *An. circularis* *rpoC1*, AF159374; *A. bergii* ANA283A *rpoC1*, AF159375; *C. raciborskii* AWT205, PKS (AF160254) and PS (AF160255); *C. raciborskii* CYP020B, PKS (AF170845) and PS (AF170842); *A. bergii* ANA283A, PKS (AF170844), PS (AF170843) and partial 16S rRNA gene (AF160256).

3 Results

3.1 Are all Isolates Really *C. raciborskii*?

3.1.1 *rpoC1* Analysis and Inferred Phylogeny

A 609 bp fragment of the *rpoC1* gene was amplified and sequenced from 19 *C. raciborskii* isolates. The nucleotide sequences differed at only 2 sites, with 99 % to 100 % nucleotide sequence identity observed between strains of *C. raciborskii*. At position 351, 3 strains out of 19 showed a synonymous substitution from C to T. At position 427, 7 strains out of 19 showed a change from C to A that caused a change from glutamine to lysine. Neither sequence change could be associated with trichome morphology nor the geographic origin of the isolate. Importantly, the *rpoC1* 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 *rpoC1* gene fragment was also amplified from a selection of other cyanobacteria, namely *Anabaena bergii*, *Anabaena circinalis*, *Anabaenopsis circularis* and *Nodularia spumigena*, and the corresponding amino acid sequences were compared to a selection of cyanobacterial *rpoC1* sequences obtained from the GenBank database (Fig. 3). At the amino acid level, the *C. raciborskii* *rpoC1* sequence exhibited 84 % to 93 % identity to other cyanobacterial species, indicating sufficient variation in the *rpoC1* sequences among species to examine the phylogenetic position of *C. raciborskii*.

Jukes-Cantor distances, generated by pairwise comparisons of the isolates, were used to create a phylogenetic tree using Neighbour-Joining analysis (Fig. 4a). In this analysis, *E. coli* and *P. putida* were included as outgroup taxa to root the tree. The phylogenetic relationships inferred from *rpoC1* sequence comparison generally supported the traditional classification of cyanobacteria based on morphological criteria. One distinct cluster was apparent (cluster I, Fig. 4a), dominated by the filamentous forms containing heterocysts representing the Orders *Nostocales* and *Stigonematales*. Genera representing simple coccoid forms that lack differentiation into specialised cell types were external to cluster I. *Fischerella* PCC7414 is grouped within the *Nostocales* cluster despite significant morphological disparities, such as multiseriate trichomes. In addition, further anomalies are evident by the paraphyly of the two *Anabaena* species and the higher affinity between *Anabaena circinalis* and *C. raciborskii*. Morphological support for the latter would appear to be lacking (Horecká and Komárek, 1979).

C. raciborskii EVTKPETINYRTLKPEMDGLFCERIFGPAKDWECHCGKYKRVHRGIVCERCQVEVTESR
N. spumigena
An. circularis
A. bergii
A. circinalis
Fischerella
Dermocarpa
SynechococcusK.....
Syn. PCC6308 ...T.....Q.....K.....S.....W.....
Syn. PCC7002
E. coli ..K.....F...R....A....V..Y..L.....LK...VI..K.....QTK
P. putida ..K.....F...R....AK....V..Y..L.....LK...VI..K.....ALGK

cyl2

C. raciborskii VRRHRMGFIKLAAPVAHVWYLGKIPSYIAILLDMPLRDVEQIVYFNSYVVLDPGNADTLV
N. spumigenaY.....S.....S.....E..T
An. circularisY.....S.....S.....E..T
A. bergiiY.....S.....S.....E..T
A. circinalis ...R.....C..R.....T
FischerellaY.....S.....S.....E..S
DermocarpaY.....T.....LS.....V....A.....GN.S
SynechococcusN...HSE.Q
Syn. PCC6308Y.....T.....LS.....V.....H.....TN.S
Syn. PCC7002Y.....A.T.....LS.....A.....TN.S
E. coli ...E...H.E..S.T..I..F..SL..R.GL.....I..RVL..E....IEG.-MTN.E
P. putida .AAE...H.E..CGL..I..F..SL..R.GL.M..T...I..RVL..E....I...-MT..E

C. raciborskii YKQLLTEDQWLEIEDRIYSEDSQLVGVEVGIGAEALLRLLSGINLEEEAEKLRGEIEAAK
N. spumigenaS.....Q.....L.Q.....AD....Q...
An. circularisS.....Q.....T.Q.....AD....Q...S...E..ST..
A. bergiiS.....Q.....L.Q.....AD.S..Q...N..E.TAN..
A. circinalisS.....A.....E.....AD....Q...S...E..IG..
FischerellaS.....Q.....T.Q.....AD....Q...T..E..TT..
Dermocarpa A.....I....QL...E.E.....VQ...EEV.....EQ.AD..
SynechococcusN...M...Q..A.E.D.E.I.....QQ..QDL..N..S...Q..AES.
Syn. PCC6308 .R...S...I...EQ..A.....E.I.....VE...QE.Q.....E..VKS.
Syn. PCC7002EQL.....ED.....IE...QELE..AV...E..EG.ANS.
E. coli RQ.I...E.Y.DALEEFG-DE----FDAKM...IQA..KSMD..Q.C.Q..E.LNETN
P. putida KG...NDE.YF.ALEEFG-D-----FDAAM...VRE..HA.D..H.IGP..E..PQTN

cyl4

C. raciborskii GQ-KRAKLIKRLRVIDNFIATGSQ
N. spumigena
An. circularis ..-.....K
A. bergii ..-.....
A. circinalis ..-.....K
Fischerella ..-.....K
Dermocarpa ..-.....V....R
Synechococcus ..-.....G.E.R
Syn. PCC6308 ..-.....L.....K
Syn. PCC7002 ..-.....AR
E. coli SET..K..T..IKLLEA.VQS.NK
P. putida SET.IK..S...KLMEA.QGI.NL

Fig. 3. Amino acid alignment of *C. raciborskii*, *N. spumigena* PCC73104, *An. circularis*, *A. bergii* ANA283A, *A. circinalis* ANA118C *rpoC1* sequences and additional *rpoC1* sequences obtained from the GenBank database; *Fischerella* sp. PCC7414 (accession no. FSPRPOC1), *Synechocystis* sp. PCC6308 (accession no. U52344), *Synechocystis* sp. PCC7002 (accession no. U52345), *Synechococcus* sp. PCC7942 (accession no. SRPOC1), *Dermocarpa* sp. (accession no. U52341), *Escherichia coli* (accession no. ECRPOBC) and *Pseudomonas putida* (accession no. M38319). Amino acids identical to those of *C. raciborskii* are indicated by dots; dashed lines represent gaps introduced into the alignment. The relative locations of primers cyl2 and cyl4 are also indicated.

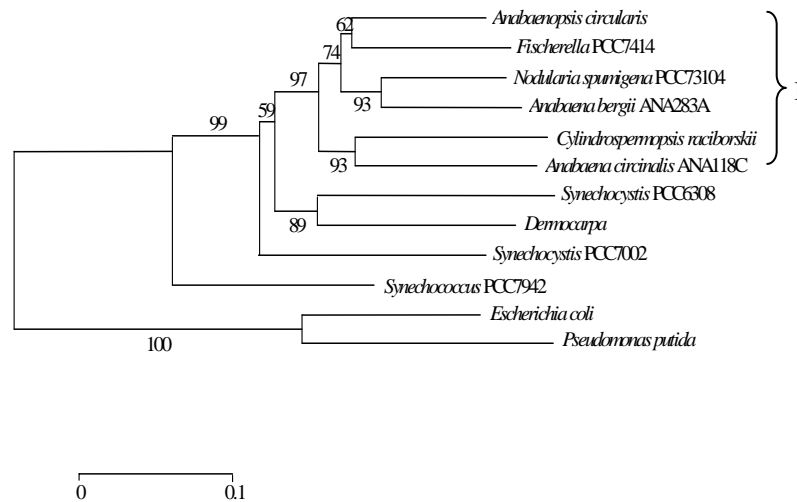


Fig.4a. Phylogenetic position of *C. raciborskii* to other cyanobacteria (sequences resulting from this work and obtained from the GenBank database) and to *E. coli* and *P. putida* based on analysis of aligned *rpoC1* nucleotide sequences. The Neighbour-Joining tree was constructed using Jukes-Cantor corrected distances and assessed by the bootstrap method with 500 replications.

3.1.2 16S rRNA Gene Sequences and the Global Relationships of *Cylindrospermopsis*

Analysis of the 16S rRNA gene nucleotide sequences revealed 99.1 % similarity between the strains of *C. raciborskii* collected from a range of global locations. The lowest similarity was between an Australian strain (CYP024C) and a Brazilian strain (Braz1). Several strains could not be distinguished by their 16S rRNA gene nucleotide sequences (100 % similarity). A phenogram, constructed by alignment of the *C. raciborskii* 16S rRNA gene sequences showed that there was some grouping of phylotypes into geographically distinct groups (Figure 4b). In general, strains from Australia grouped together as did strains from Brazil and the USA. European strains from Germany, Hungary and Portugal were also more closely related to each other with respect to their 16S rRNA gene nucleotide sequences.

The analysis of the 16S rRNA gene sequences confirmed a strong relationship between *Cylindrospermopsis* and other Nostoclean genera. The level of sequence similarity between the *C. raciborskii* cluster and other Nostoclean cyanobacteria was as follows; *Cylindrospermum* sp. PCC7417 (93.7%), *Nostoc* sp. PCC7120 (93.7%), *Anabaena bergii* (93.3%) and *Anabaenopsis* sp. 1A (93.2%). These values were considerably higher than the similarity value obtained for another cyanobacterium known to produce cylindrospermopsin, from the Order Stigonematales, *Umezakia natans* (84.6%).

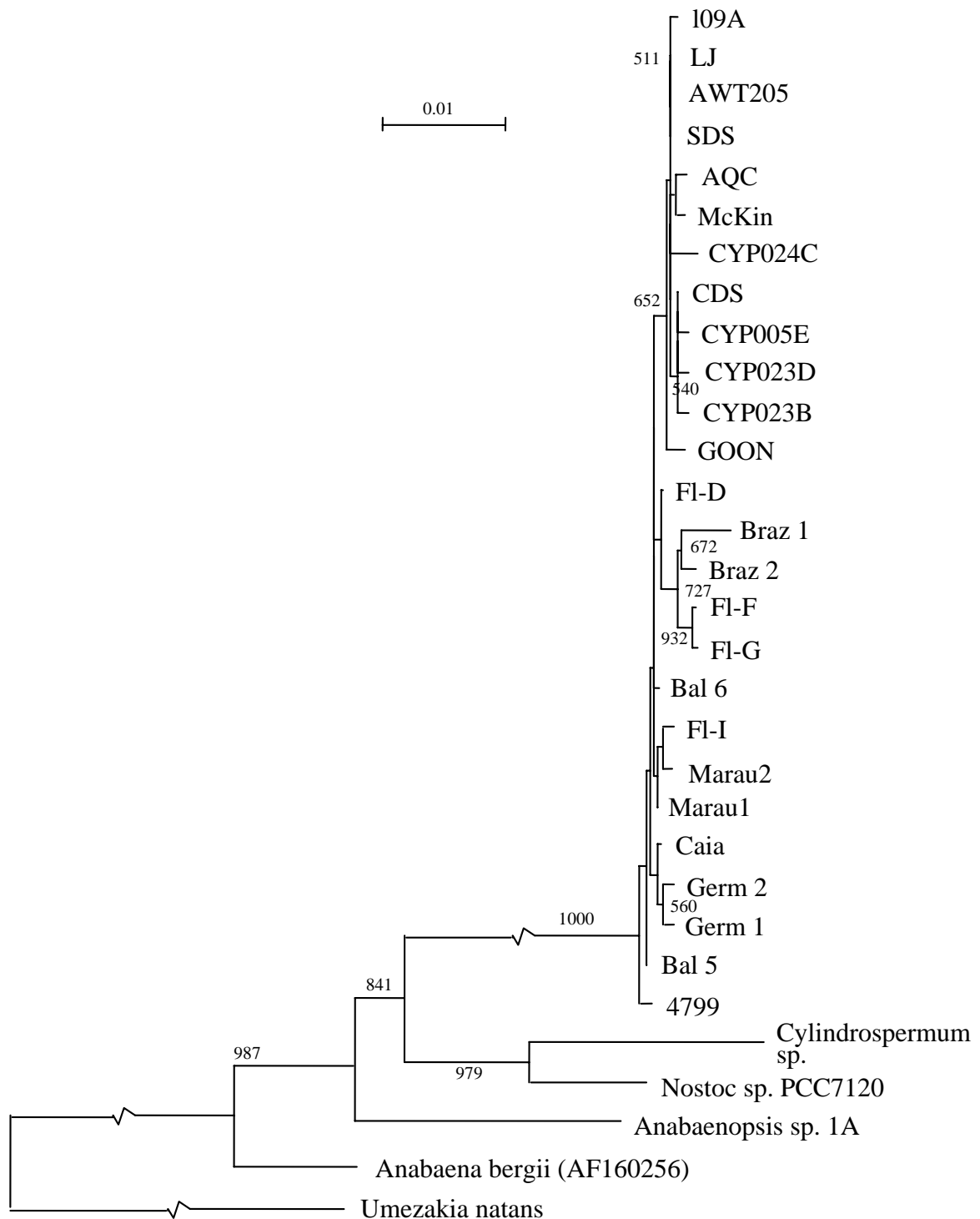


Fig. 4b Phylogenetic affiliations between 26 strains of *C. raciborskii* and other Nostoclean cyanobacteria derived from complete 16S rRNA gene sequences. The Phenogram was reconstructed from a pairwise distance matrix (Jukes and Cantor 1969) using the neighbour-joining method (Saitou and Nei 1987). The scale represents two substitutions per 100 nucleotide positions. Bootstrap values (1000 re-sampling cycles) indicate the statistical significance of each node.

3.1.3 STRR Profiles

Since *rpoC1* sequences did not reveal a significant difference between isolates of *C. raciborskii*, STRR profiles were used to examine the level of genetic diversity.

Thirteen isolates produced growth to sufficient density for use in STRR analysis. PCR amplification with primers derived from cyanobacterial specific STRR sequences were used. Various combinations of these primers established a DNA fingerprint pattern for each isolate (Fig. 5). This analysis revealed unique band profiles among each of the different cyanobacterial genera examined, with the extent of this variation dependent on the primer combination used.

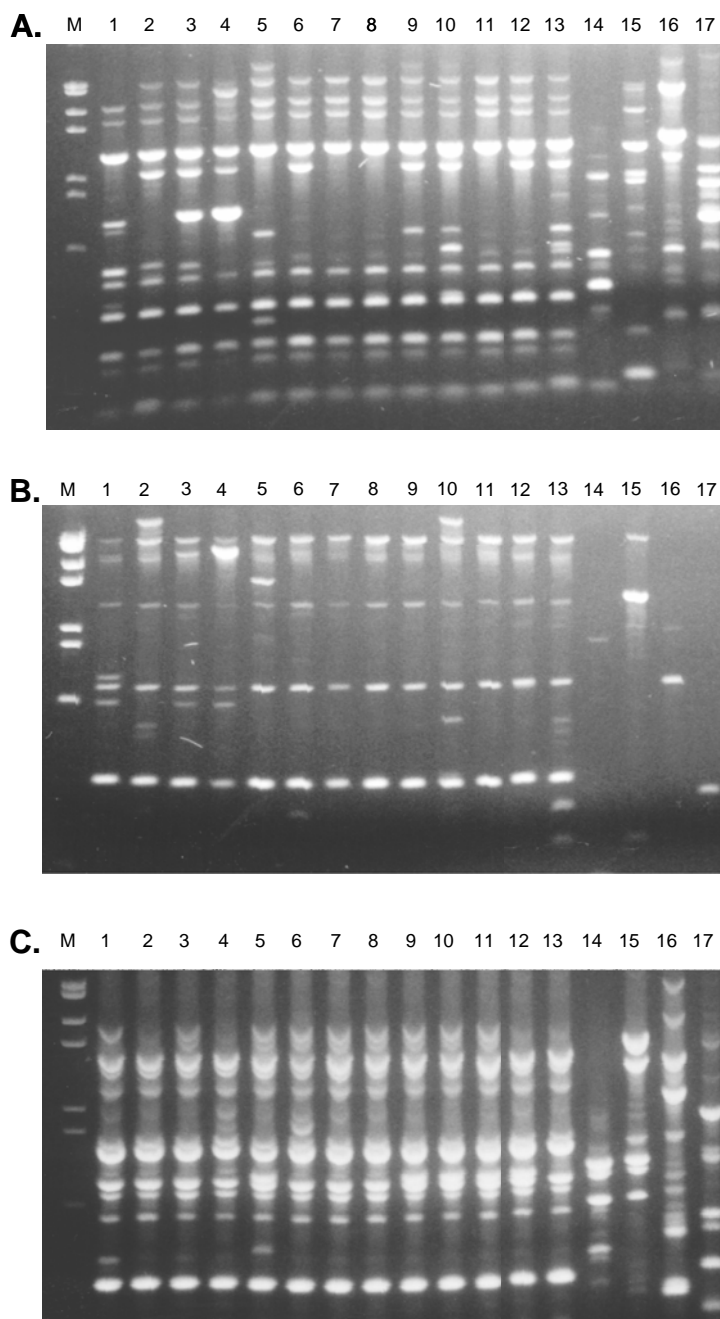


Fig. 5. STRR profile analysis of 13 isolates of *C. raciborskii* and other representative genera obtained by PCR amplification with primers STRR1F and STRR3F (**A**), STRR1F and STRR3R (**B**), and STRR1R and STRR3R (**C**). Lanes 1 CYP003A, lanes 2 CYP003K, lanes 3 CYP005E, lanes 4 CYP010A, lanes 5 CYP014A, lanes 6 CYP015A, lanes 7 CYP020A, lanes 8 CYP020B, lanes 9 CYP023A, lanes 10 CYP023E, lanes 11 CYP024C, lanes 12 CYP026J, lanes 13 AWT205, lanes 14 *A. circinalis* ANA173A, lanes 15 *A. circinalis* ANA118C, lanes 16 *M. aeruginosa* PCC7806, lanes 17 *N. spumigena* PCC73104. Lane M, molecular weight marker sizes (bp): 2027, 1904, 1584, 1375, 947, 831, 564.

The amplified PCR products ranged in size from approximately 0.1 to 2.5 kb. Combinations of STRR1R and STRR3R (Fig. 5C) yielded a very similar pattern for all *C. raciborskii* isolates, while the greatest variation was seen with the primer combination of STRR1F and STRR3F (Fig. 5A). Differences were also observed between *C. raciborskii* strains isolated from the same population, i.e. strains CYP003A and CYP003K (lanes 1 and 2, Fig. 5A, B and C) and strains CYP023A and CYP023E (lanes 9 and 10, Fig. 5A and B). Interestingly, two of the primer combinations yielded fragments unique to the three *C. raciborskii* isolates with coiled trichomes (lanes 1, 3 and 4, Fig. 5A and B). Information from the STRR banding patterns was represented graphically by converting the bands to binary data which was then used to construct a tree (Fig. 6). The tree shows similarities between different isolates according to their banding patterns. Interestingly, all of the isolates examined with coiled trichomes grouped together.

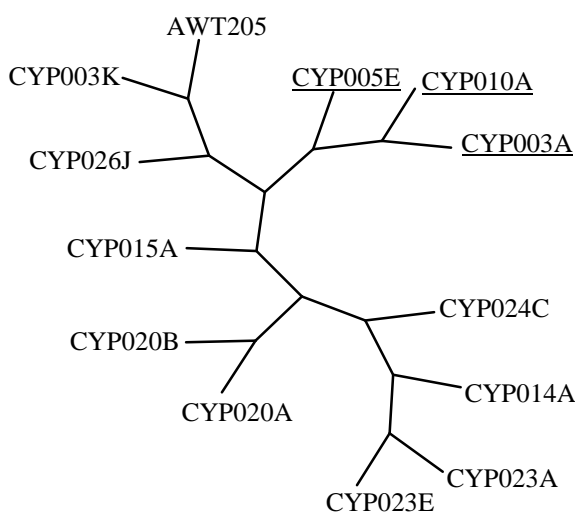


Fig. 6. Graphical representation of the branching pattern of *C. raciborskii* STRR profiles derived from total character differences using the Neighbour-Joining method. Isolates showing coiled morphology are underlined.

3.2 Identification of Genes Responsible for Toxin Production

3.2.1 *Cylindrospermopsis* Production

The first report of toxicity amongst the species *C. raciborskii* occurred through its association with an episode of human poisoning in Australia (Hawkins et al., 1985). Prior to this the species was not known to be toxic. Although the occurrence of both toxic and non-toxic strains of *C. raciborskii* has been inferred (Hawkins et al., 1997), no detailed studies of different isolates have been reported. We examined 13 *C. raciborskii* strains from various toxic blooms around Australia for their ability to produce cylindrospermopsin by HPLC-MS analysis. This analysis demonstrated that all but three of the strains produced the toxic compound cylindrospermopsin (Table 1). Interestingly, our screening of other cyanobacteria for the presence of this toxin revealed it is also produced by *Anabaena bergii* (var *limnetica*) strain ANA283A. This is the first report of cylindrospermopsin production from this genus.

3.3 Identification of PKS and PS Determinants

PKSs and PSs are enzymes involved in secondary metabolite biosynthesis by cyanobacteria. The strategy we used to amplify segments of the PKS and PS genes from *C. raciborskii* strains AWT205 and CYP020B took advantage of the structural organization known to be present within these sequences. Degenerate PKS gene primers (DKF and DKR) were designed from conserved regions of type I class genes available on the GenBank and EMBL databases. PCR using these primers resulted in the specific amplification of a 650 bp DNA product. Using a similar strategy, degenerate primers (MTF2 and MTR, Neilan et al., 1999) directed to the conserved regions of several bacterial and fungal peptide synthetases were used to amplify an 1150 bp PS gene fragment. The PCR fragments were purified and sequenced directly. The nucleotide sequence of the PKS gene fragment from both strains was identical; the PS nucleotide sequence between the two strains differed by one base, however this difference did not alter the amino acid sequence. Analysis of the primary amino acid sequence deduced from each of the amplified products revealed that the putative PKS displays strongest similarity (up to 57% identity) to other PKS proteins from *Stigmatella aurantiaca*, *Polyangium cellulorum* and *Microcystis aeruginosa*, (Fig. 7a); whilst the putative PS is most similar (up to 48% identity) to other PS proteins from *Nostoc* sp., *Pseudomonas aeruginosa*, *Mesorhizobium loti* and *Streptomyces pristinaespiralis* (Fig. 7b). The *C. raciborskii* PKS shares 54% amino acid identity to an equivalent region of *M. aeruginosa* McyE which includes the β -ketoacyl-ACP synthase domain of module 8 (Fig. 7a). Module 8 is reported to supply the amino group to the Adda moiety of microcystin (Tillet et al., 2000). The *C. raciborskii* PS sequence shares 28% amino acid identity with McyB of *M. aeruginosa* over 180 amino acids (Fig. 7b). The homology with McyB falls within module 3, between the ATP binding and ATPase domains (Nishizawa et al., 2000).

(a)

<i>C. raciborskii</i> AWT205 PKS	FIGIGGSNYK	SLMIENRSRI	GKTDLYELSG	TDVSVAAGRI	SYVLGLMGPS	FVIDTACSSS
<i>C. raciborskii</i> CYP020B PKS
<i>A. bergii</i> PKSTD...I.....
<i>S. aurantiaca</i> MtaE	.V.VMLND.A	Q.QMKQADPT	-LL.A.MAL.	N.S.FM....	..I..AQ...	MAVN.....
<i>P. cellulorum</i> EpoE	.V.VCATE.L	HAAVAHQP.E	-ER.A.STT.	NML.I....L	..T...Q..C	LTV.....
<i>P. cellulorum</i> EpoSD	.V.VCATE.L	HAAVAHQP.E	-ER.A.STT.	NML.I....L	..T...Q..C	LTV.....
<i>M. aeruginosa</i> McyE module8	.V..TSIDHA	LKVYGTNYDQ	--I.SFFG..	NAL.A....L	..F.N.H..C	LS..A..A..
<i>C. raciborskii</i> AWT205 PKS	LVSVMQACQS	LRQRECDLAL	AGGVGLLIDP	DEMIGLSQGG	MLAPDGSCKT	FDANANGYVR
<i>C. raciborskii</i> CYP020B PKS
<i>A. bergii</i> PKSC.....
<i>S. aurantiaca</i> MtaEL....	..S...NM.ISVILA.	.GH.VS.RLR	SQS.Q.R...	...S.D....
<i>P. cellulorum</i> EpoE	..AI.L..R.	..A..S....NM.LS.	.T.RA.ARTQ	A.S.N.R.Q.	...S...F..
<i>P. cellulorum</i> EpoSD	..AI.L..R.	..A..S....NM.LS.	.T.RA.ARTQ	A.S.N.R.Q.	...S...F..
<i>M. aeruginosa</i> McyE module8	..A...GIR.	..N...E...	V...N.ILE.	AIT.S...S.	.MS...R...	...S.....
<i>C. raciborskii</i> AWT205 PKS	GEGCGMIVLK	RLSDATADGD	NILAIIRGSM	VNHDGHSSGL	TAPRGPAQVS	VIKQALDRAG
<i>C. raciborskii</i> CYP020B PKS
<i>A. bergii</i> PKS
<i>S. aurantiaca</i> MtaEVV...I....	PV..V...GAP.G...	.V.S....EA	..RR..AS..
<i>P. cellulorum</i> EpoEL....RR...	R.W.L...A	I.Q..R.T..	...NVL..GA	LLRE..RN..
<i>P. cellulorum</i> EpoSDL....RR...	R.W.L...A	I.Q..R.T..	...NVL..GA	LLRE..RN..
<i>M. aeruginosa</i> McyE module8VLI..	T..E.QKN..	H...LL...A	...N.AAA..	.V.S....QE	LLR...AD.R

Fig. 7. (a) Amino acid alignment of the *C. raciborskii* (AWT205 and CYP020B) and *A. bergii* (ANA283A) PKS sequences with homologous sequences derived from gene sequence identified in the database; *S. aurantiaca* mtaE gene (accession no. AF188281), *P. cellulorum* epoE gene (AF217189), *P. cellulorum* epoSD gene (AF210843) and the *M. aeruginosa* mcyE gene, module 8 (U97078).

(b)

<i>C. raciborskii</i> AWT205 PS	WWHQQTRPSV	QGVRTLQFCA	VSFDFSCHEI	FSTLCLGGIL	VLVPEAVRQN	PFALAEFISQ
<i>C. raciborskii</i> CYP020B
<i>A. bergii</i> PS	..L.....
<i>Nostoc</i> sp. NosA	L..RENLKIP	R.AK....AS	IN..V.FQ..	.T.W.S..T.	F.IG.EL.RD	TS..LG.LQ.
<i>P. aeruginosa</i> probable PS	Q.QLRVASG.	P.L....AP	L...MAFQ..G..E.	Q.ISNRE.MD	.S..LHVLER
<i>M. loti</i> PS	D.QIELSTLS	DNAA.A..AP	I...V.FQ..S..SI	..LANEQ.ID	.DL.SDE.LR
<i>S. pristinaespiralis</i> SnbDE	T..ARRF.GG	T....A..T.	IG...VQ..	L.P.VM.KT.	AVPS.E..HS	AEL..GWLET
<i>M. aeruginosa</i> McyB module3	C...DAFEIT	PLDKIT.LAR	IA..AAVW.L	WPC.TA.AS.	...KPEIM.S	.PD.RDWLIA
<i>C. raciborskii</i> AWT205 PS	QKIEKLFLPV	IALQLAEAV	NGNKSTSLAL	CEVITTGEQM	QITPAVANLF	QK-TGAMLHN
<i>C. raciborskii</i> CYP020B
<i>A. bergii</i> PS	T.....
<i>Nostoc</i> sp. NosA	KA..RM...F	V..Q....VA	I.GELVNSH.	R.I..A...LISQWL	S.L.DCT...
<i>P. aeruginosa</i> probable PS	RQVQRVL..F	V..QR....S	.ALGVRPG..	RV.VSS...L	R..ED.RAFC	AAMP..LL.E.
<i>M. loti</i> PS	ARV.R....F	..Q...SNC	VERNLFPPDS.	R.IH.A...L	VVSS.LREF.	I.LPQCR.F.
<i>S. pristinaespiralis</i> SnbDE	.Q.NE..A.N	LVIEA...A	AEAGR.LPD.	TDILQG..AL	AP.ER.RAFT	AAVP.RR...
<i>M. aeruginosa</i> McyB module3	.E.TVS...T	PLVEKILSLE	WDEN---I..	RIIL.G.DKL	HH-----YP	SGLMPFK.I.
<i>C. raciborskii</i> AWT205 PS	HYGATEFQDA	TTHTLKGNPE	GW-PTLVPVG	RPLHNVQVYI	LDEAQQPVPL	GGEGEFCIGG
<i>C. raciborskii</i> CYP020B
<i>A. bergii</i> PSN.....
<i>Nostoc</i> sp. NosA	...PS.SHL.	.SF..TNSV.	T.-.L.P...	..IA.A.I..	..RFL...V	.VP..LY.A.
<i>P. aeruginosa</i> probable PS	Q..P..THQV	.Y.S.S.D.A	HY-.D.P.I.	...DG.E.QV	..A.LR...V	.VT..LYF..
<i>M. loti</i> PS	Q..PS.THVV	.C.E.DS..A	E.-.R.P.I.	...P..VLF.	.G.DGR..R.	.EV..LY...
<i>S. pristinaespiralis</i> SnbDE	V..PA.THAVAAD.A	H-.PSA.I.	...VDHDR..V	..S.LR...P	.VT..LYLA.
<i>M. aeruginosa</i> McyB module3	N..P..NSV	..SG.VRDY.	EGN.PSPSI.	K.VY.TKI..	..QNL..L.I	.VP..LH.SS

Fig 7 (b) Amino acid alignment of the *C. raciborskii* (AWT205 and CYP020B) and *A. bergii* (ANA283A) PS sequences and homologous sequences derived from gene sequence identified in the database; *Nostoc* sp. *nosA* gene (accession no. AF204805), *P. aeruginosa* probable PS gene (AE004755), *M. loti* PS gene (AP003015), *S. pristinaespiralis* *snbDE* gene (X98690) and *M. aeruginosa* *mcysB* gene (AF183408). Residues identical to those of the *C. raciborskii* sequence are indicated by dots; dashed lines indicate gaps introduced into the alignment. Underlined in (a) is the conserved β -ketoacyl-ACP synthase domain.

3.4 PKS and PS Appear Linked to Toxicity in *C. raciborskii*

Secondary metabolite production by cyanobacteria has been shown to exhibit variability at different taxonomic levels. Current evidence suggests a genetic basis for variable toxin production amongst strains of the cyanobacterial genera *Microcystis*, *Anabaena* and *Nodularia* (Meißner et al., 1996; Neilan et al., 1999). We used Southern blot analysis to examine the strain specific distribution of the PKS and PS genes in *C. raciborskii*. Genomic DNA was extracted from the 13 strains whose toxicity had been assessed and probed with either the PKS or PS gene fragment, respectively. The genomic DNA was specifically digested with an enzyme that would cut once within the probe sequence. This was done to determine whether or not the adjacent flanking regions were also similar. Hybridisation analysis revealed both genes were present in 10 of the 13 strains examined (Fig. 8). The hybridisation profile of all of these positive strains was identical. The presence of these two genes appeared to be linked, i.e. each strain either had both or neither of these two genes. Significantly, the possession of these two genes was also directly related to the ability to produce cylindrospermopsin. Taken together, this data suggests these genes are likely involved in secondary metabolite and toxin production by *C. raciborskii*.

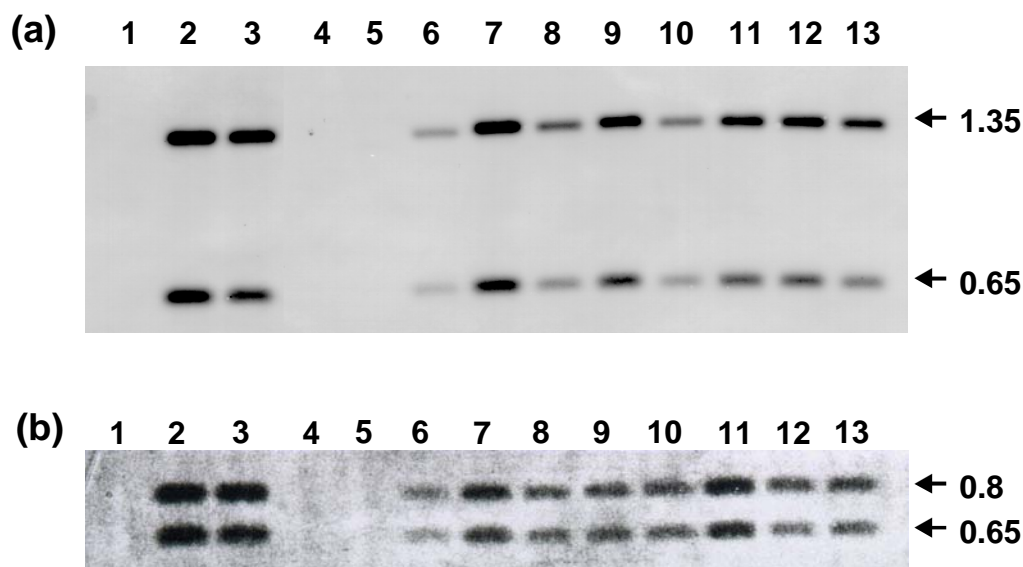


Fig. 8. Screening of *C. raciborskii* isolates for the presence of PKS and PS gene sequences. Genomic DNA from *C. raciborskii* strains was either digested with *EcoRV* and probed with DIG-labelled PKS gene fragment from *C. raciborskii* AWT205 (a) or digested with *HindIII* and probed with DIG-labelled PS gene fragment from *C. raciborskii* AWT205 (b). Lanes 1, CYP003A; lanes 2, CYP003K; lanes 3, CYP005E; lanes 4, CYP014A; lanes 5, CYP015A; lanes 6, CYP020A; lanes 7, CYP020B; lanes 8, CYP023A; lanes 9, CYP023E; lanes 10, CYP024C; lanes 11, CYP025B; lanes 12, CYP026J; lanes 13, AWT205. The sizes of hybridising fragments are indicated in kbp.

3.5 PKS and PS in Other Cyindrospermopsin Producing Cyanobacteria

Although cylindrospermopsin production was first detected in *C. raciborskii*, more recent studies have shown it is also produced by *Aph. ovalisporum* (Shaw et al., 1999), *U. natans* (Harada et al., 1994; Teraro et al., 1994) and now *A. bergii*. Using Southern blot analysis, various cyanobacteria were screened for sequences homologous to the PKS and PS gene fragments from *C. raciborskii* (Fig. 9). At low stringency, we failed to detect PKS or PS homologous sequences in *A. circinalis*, *M. aeruginosa* and *N. spumigena*. However, a hybridisation signal was observed with genomic DNA from *A. bergii* ANA283A, suggesting that these genes are present in this organism. It should be noted that this hybridisation signal was also observed under high stringency conditions (data not shown). Interestingly, the PKS and PS hybridisation profiles from *A. bergii* ANA283A gave a different RFLP pattern to those of all *C. raciborskii* strains examined. DNA sequence analysis of the PKS and PS gene fragments from *A. bergii* revealed that they are highly similar at both the nucleotide and amino acid level to the corresponding sequences of *C. raciborskii* (Fig. 7).

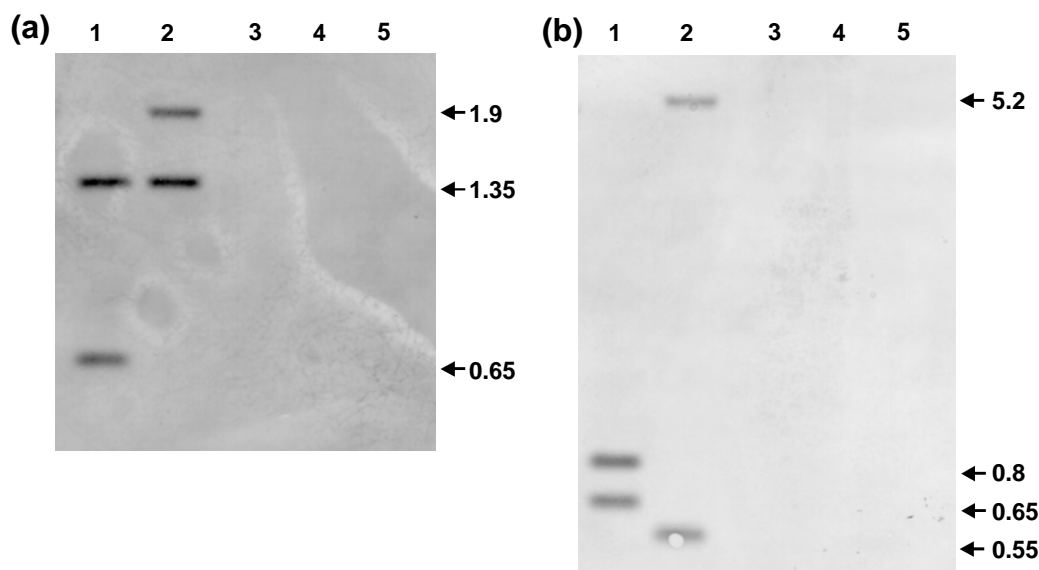


Fig. 9. Screening of other cyanobacterial species for PKS and PS gene sequences. Genomic DNA from cyanobacterial strains was either digested with *EcoRV* and probed with DIG-labelled PKS gene fragment from *C. raciborskii* AWT205 (a) or digested with *HindIII* and probed with DIG-labelled PS gene fragment from *C. raciborskii* AWT205 (b). Lanes 1, AWT205; lanes 2, *A. bergii* ANA283A; lanes 3, *A. circinalis* ANA118C; lanes 4, *M. aeruginosa* PCC7806; lanes 5, *N. spumigena* PCC73104. The sizes of hybridising fragments are indicated in kbp.

3.6 Attempts to Inactivate the PKS Determinant

To date, the genetic manipulation of *Cylindrospermopsis raciborskii* has not been reported. Therefore, it was necessary for experimental conditions for electroporation of *C. raciborskii* to be optimised. The techniques trialled were based on those that have been successful with other filamentous cyanobacteria.

To date attempts to construct a knock-out mutant have not been successful. Possible reasons why this might be the case and other options for confirming the involvement of the *ps* and *pks* determinants in cylindrospermopsin production are considered in the discussion.

3.7 Rapid DNA Based Testing for Toxic *C. raciborskii*

3.7.1 *C. raciborskii* specific PCR

Although little difference was found between the *rpoC1* sequences of *C. raciborskii* isolates, there was sufficient difference between the *rpoC1* sequence of *C. raciborskii* and other species to design a specific PCR test for *C. raciborskii*. Primers *cyl2* and *cyl4* (Fig. 3) were used to amplify a 305 bp diagnostic PCR product from the *rpoC1* gene of *C. raciborskii* isolates. Twenty fg 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 strains listed in Table 1, and the results are shown in Fig. 10. All *C. raciborskii* isolates produced a positive reaction, with amplification of both the diagnostic 305bp 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 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. By adjusting the starting concentration of the ICF the test can be made quantitative, currently we can detect down to the equivalent of 2,000 cells using this procedure. The test described here was the first report of a rapid method for the identification of *C. raciborskii* direct from environmental samples.



Fig. 10. *C. raciborskii* specific PCR reaction. Genomic DNA from laboratory cultures and environmental samples was amplified with primers cyl2 and cyl4 in a PCR reaction spiked with internal control fragment. Lane 1 AWT205, lane 2 CYP003A, lane 3 CYP003K, lane 4 CYP005E, lane 5 CYP005F, lane 6 CYP010A, lane 7 CYP010C, lane 8 CYP014A, lane 9 CYP015A, lane 10 CYP020A, lane 11 CYP020B, lane 12 CYP023A, lane 13 CYP023B, lane 14 CYP023D, lane 15 CYP023E, lane 16 CYP024C, lane 17 CYP025B, lane 18 CYP025E, lane 19 CYP026J, lane 20 brazil 1, lane 21 brazil 2, lane 22 *A. circinalis* ANA118C, lane 23 *A. circinalis* ANA173A, lane 24 *M. aeruginosa* PCC7806, lane 25 *M. aeruginosa*, lane 26 *N. spumigena* PCC73104, lane 27 *N. spumigena*, lane 28 *A. bergii* ANA283A, lane 29 *An. circularis*, lane 30 environmental sample (Fred Haigh Dam, Qld Australia), lane 31 environmental sample (Currency Creek, SA Australia), lane 32 ICF only, lane 33 CYP014A only. Lane M, molecular weight marker sizes (bp): 587, 540, 504, 458, 434, 267, 234, 213, 192, 184.

3.7.2 PCR to Identify Cylindrospermopsin Producing Cyanobacteria

The PCR and Southern hybridisation data indicated a direct link between the presence of the PKS and PS genes and toxin production. We therefore investigated the possibility of designing a PCR based rapid test for the identification of these genes in cylindrospermopsin producing cyanobacterial strains. PKS (M4 and M5) and PS (M13 and M14) specific primers were used to amplify portions of each of these genes. The results are shown in Fig. 11 and are consistent with those obtained in Southern hybridisations. Correct sized PCR products were only obtained from the cylindrospermopsin producing *C. raciborskii* and *A. bergii* isolates. In addition, chromosomal DNA from two cylindrospermopsin negative Brazilian isolates, identified as *C. raciborskii* by PCR (Wilson et al., 2000), gave no PCR product. This data indicates these genes may be useful markers for the rapid identification of cylindrospermopsin-producing cyanobacteria.

3.7.3 Multiplex PCR for Cylindrospermopsin Producing *C. raciborskii*

The *pks* specific PCR product obtained with primers M4 and M5 (540bp) and the *ps* specific PCR product obtained with primers M13 and M14 (534bp) could not be separated and identified by subsequent gel electrophoresis of a PCR reaction mix due their similarity in size. Therefore, a new primer, K18 (5' CCTCGCACATAGCCATTGTC) was used in conjunction with M5 to produce a smaller 450bp *pks* product. The *pks* and *ps* specific primers were combined with the *C. raciborskii* *rpoC1* specific primers cyl2 and cyl4 to produce a multiplex PCR that could simultaneously show the presence of *pks* and *ps* determinants in *C. raciborskii* and other cylindrospermopsin-producing cyanobacteria.

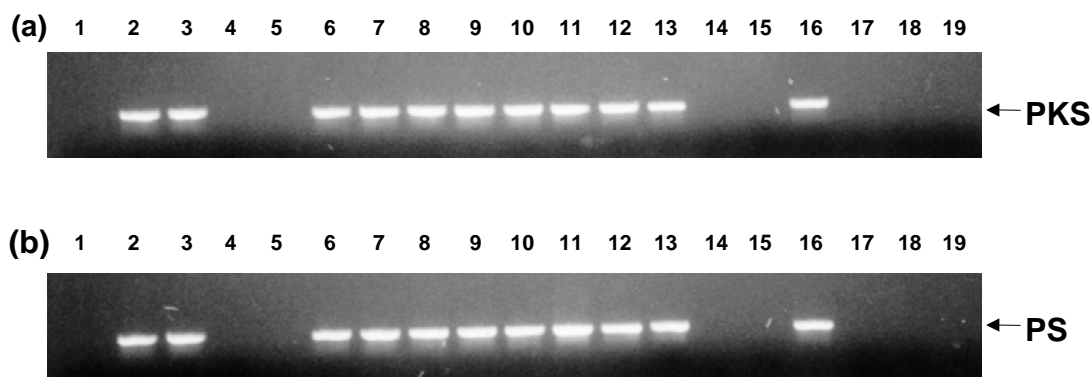


Fig. 11. PCR analysis of cyanobacterial strains for the presence of either the PKS gene fragment (a) or the PS gene fragment (b). Lanes 1, CYP003A; lanes 2, CYP003K; lanes 3, CYP005E; lanes 4, CYP014A; lanes 5, CYP015A; lanes 6, CYP020A; lanes 7, CYP020B; lanes 8, CYP023A; lanes 9, CYP023E; lanes 10, CYP024C; lanes 11, CYP025B; lanes 12, CYP026J; lanes 13, AWT205; lanes 14, Brazil 1; lanes 15, Brazil 2; lanes 16, *A. bergii* ANA283A; lanes 17, *A. circinalis* ANA118C; lanes 18, *M. aeruginosa* PCC7806; lanes 19, *N. spumigena* PCC73104.

The PCR was used to screen 15 cultured isolates of *C. raciborskii* (Wilson et al. 2000; Fig. 12, lanes 2-15 & 17), 3 environmental samples (Fig. 12, lanes 16 & 18-19) and *A. bergii* ANA283A (Fig. 12, lane 20). To ensure primer specificity, isolates of *A. circinalis*, *M. aeruginosa* and *N. spumigena* were also screened. The multiplex PCR successfully identified *C. raciborskii* in all samples previously shown by microscopic analysis to contain this organism. The PKS and PS genes were identified in all 10 of the 15 cultured *C. raciborskii* isolates which have been shown by reverse phase HPLC and mass spectroscopy assay to produce cylindrospermopsin (Wilson et al. 2000). In addition, the Broken Hill environmental sample also possessed the PKS and PS determinants and was also shown by the same techniques to be toxic. *A. bergii* ANA283A has been shown to produce the toxin cylindrospermopsin, and both the *pks* and *ps* determinants were identified when this strain was examined by multiplex PCR. All other cyanobacterial samples tested did not show the presence of *C. raciborskii*, or the presence of *pks* and *ps* determinants. This demonstrates the utility of multiplex PCR for defining the cyanobacterial species and its toxic potential.

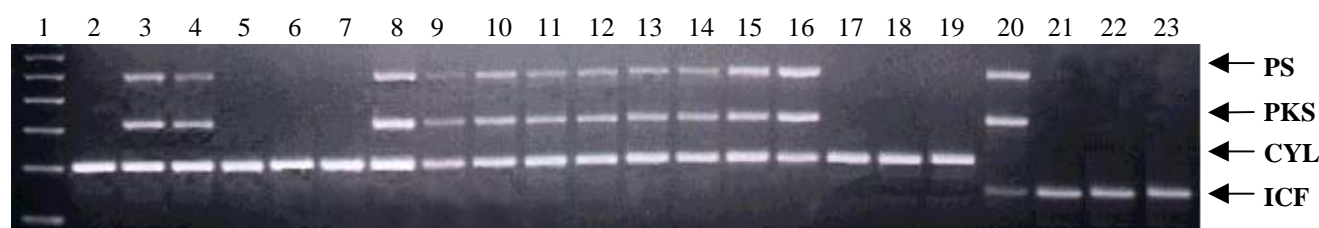


Fig. 12 Detection of *C. raciborskii*, peptide synthetase (*ps*) and polyketide synthase (*pks*) determinants by multiplex PCR. Lane 1, molecular weight marker sizes (bp): 700, 600, 500, 400, 300, 200; *C. raciborskii* Lanes 2-15: CYP003A, CYP003K, CYP005E, CYP010C, CYP014A, CYP015A, CYP020A, CYP020B, CYP023A, CYP023E, CYP024C, CYP025B, CYP026J, AWT205; Lane 16, environmental sample *C. raciborskii* (Broken Hill, South Australia); Lane 17, Brazil 1; Lane 18, environmental sample *C. raciborskii* (Fred Haigh Dam, Queensland); Lane 19, environmental sample *C. raciborskii* (Currency Creek, South Australia); Lane 20, *A. bergii* ANA283A; Lane 21, *M. aeruginosa*; Lane 22, *N. spumigena* PCC73104; Lane 23, *A. circinalis* ANA118C.

4 Discussion

The 16S rRNA gene represents the most highly studied gene for identification and phylogenetic analysis. *rpoC1* gene analysis has been shown to be more discriminatory than 16S rRNA analysis (Palenik and Haselkorn, 1992). PCR primers designed from conserved regions of the cyanobacterial *rpoC1* gene (Palenik and Haselkorn, 1992) were used to analyse the *C. raciborskii* isolates. In a previous study, these primers were used in PCR reactions for strain-level identification of a number of taxonomic groups (Toledo and Palenik, 1997). In addition, they have been used to examine the phylogeny of prochlorophytes to each other and to the green chloroplasts (Palenik and Swift, 1996), and to study diversity of the cyanobacterial genus *Synechococcus* (Toledo and Palenik, 1997). We therefore hypothesized that sequence analysis of the *rpoC1* gene may enable differentiation between 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 may 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. 3), there was insufficient discrimination even at the nucleotide level, to distinguish among *C. raciborskii* isolates.

Phylogenetic analysis of the partial *rpoC1* gene sequence among those cyanobacterial species selected in this study indicated one distinct cluster (Fig. 4a) and concurred with an earlier phylogenetic classification of cyanobacteria based on both the *rpoC1* gene and the 16S rRNA gene (Wilmotte, 1994; Palenik and Swift, 1996; Neilan et al., 1999). Cluster I (Fig. 4a) consisted mostly of representative genera of the Order *Nostocales* (*Anabaenopsis circularis*, *Nodularia spumigena*, *Anabaena circinalis*, *Anabaena bergii* and *Cylindrospermopsis raciborskii*). Genera of the Order *Chroococcales* (*Synechocystis*, *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 specialised 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 *Chroococcales* (Komárek and Anagnostidis, 1986, 1989; Anagnostidis and Komárek, 1990). 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 paraphyletic distribution of both *Synechocystis* and *Anabaena* species also contradicted the traditional classification system.

Cylindrospermopsis raciborskii has been described in the literature as a highly adaptive cyanobacterium capable of considerable physiological and morphological plasticity (Padisák, 1997). Our findings in this study support that view. The strains investigated here showed a range of morphological characteristics (Table1), and an ability to produce a range of toxic compounds including cylindrospermopsin, PSPs and as yet unidentified toxic compounds. It is also clear that this species can no longer be considered a species of only tropical interest, nor can its distribution be considered as "pan tropical" given that, as stated by Padisak (1997), its northern occurrences overlap with many circum-polar phytoplankton species. Blooms of this

species have become a worldwide problem due to their production of toxic compounds (Lagos et al. 1999; McGreggor & Fabbro 2000; Saker et al. 1999b). The 16S rRNA gene nucleotide sequences of the 26 strains investigated here showed 99.1% similarity indicating that *C. raciborskii* is a genetically well-defined group. The genetic divergence determined for these strains of *C. raciborskii* is less than was found for a global study of 19 *A. circinalis* strains, which reported 98 % similarity in 16S rRNA gene nucleotide sequences (Beltran & Neilan, 2000).

Despite the strong similarity between *C. raciborskii* strains, the phenogram constructed by alignment of the 16S rRNA gene nucleotide sequences indicated clustering of strains into geographically distinct groups, with strains from North and South America forming one group, European strains forming another group, and Australian strains constituting the third group. This observed relationship could not be validated statistically. The strains assembled into two statistically validated groups. The first group contained strains from USA and Brazil; the second, European and Australian strains. Within the second group, the Australian and European strains showed statistically significant divergence. Within the European strains, the German and Portuguese strains were more closely related to each other than to the Hungarian strains, and within strains from Australia, the two strains isolated from water bodies in the north at latitudes <20°S were more similar than those isolated from temperate regions of that continent.

While many of the strains investigated here have not been investigated thoroughly for the presence of toxic compounds (Table 1), it is interesting to note that as yet, only Australian strains of *C. raciborskii* have been reported to produce cylindrospermopsin, and only Brazilian strains have been reported to produce PSP's. These two groups showed the greatest divergence with respect to both their 16S rRNA gene sequences (Fig. 4b). Furthermore, both Portuguese and Hungarian strains from the European sub-group (Fig. 4b) have been shown to have a toxic effect in the mouse bioassay despite a confirmed absence of cylindrospermopsin and PSP toxins. It is possible that strains from these two sources could share similar toxic compound/s.

The findings here do not permit any resolution concerning the origin source of *C. raciborskii*, however given the greater genetic similarity between European and Australian strains (as indicated by both 16S rRNA sequences), it is possible that transfer of scientific samples from the Australasian region to Europe could have provided the source of this cyanobacterium which is now invading many previously unaffected European water bodies (Padisák, 1997). Whilst this argument is purely speculative, Padisák (1997) suggested that some physiological characteristics of *C. raciborskii*, including its high temperature demand (Saker & Griffiths, 2000) and its inability to adapt to temperature fluctuations, indicate that this species possibly evolved in tropical lakes, with Australia or Africa as possible radiation points. Alternatively, it was further suggested (Padisák, 1997) that the naturally expanding distribution of *C. raciborskii* might be a useful indicator of global warming. Padisák (1997) also proposed dispersal of *C. raciborskii* akinetes (resting spores) with migratory birds as the most probable dispersal mechanism.

In a previous study, primers based on the STRR1 repeat sequence were used in PCR reactions to fingerprint symbiotic cyanobacterial isolates from the angiosperm *Gunnera* (Rasmussen and Svenning, 1998). 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 PCR reactions. 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 *Escherichia 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 multiplex PCR successfully identified *C. raciborskii* in all samples previously shown by microscopic examination to contain this organism, and identified the *pks* and *ps* genes isolates shown by reverse phase HPLC and mass spectroscopy assay to produce cylindrospermopsin. The ability to identify both *C. raciborskii* and cylindrospermopsin toxicity determinants in a single reaction has provided a unique means to assess environmental samples. In addition, the multiplex PCR assay will detect other cylindrospermopsin-producing cyanobacteria where they may otherwise have been missed.

Secondary metabolites encompass a range of toxic peptides and polyketides produced by cyanobacteria and possess properties that include proteinase inhibition, anticyanobacterial, antialgal and antifungal action, immunosuppression and promotion of cell differentiation (Erhard, 1997). The best characterised compounds are the cyclic peptide microcystins, which act as potent inhibitors of type 1 and type 2A protein phosphatases. Over 60 different types of microcystins have been identified so far (Sivonen, 1996). Cylindrospermopsin is a toxic secondary metabolite produced by *C. raciborskii*. Recent work using radiolabelled precursors has established that cylindrospermopsin has a polyketide origin and is assembled from guanidinoacetic acid and five acetate units (Burgoyne et al., 2000). Given the nature of this compound it seems reasonable to assume the involvement of a PKS in its biosynthesis. While it is not clear what role a PS would have in the synthesis of a polyketide compound, these proteins are involved in the synthesis of secondary metabolites in other cyanobacteria such as *M. aeruginosa* (Meißner et al., 1996). We therefore hypothesised that screening for both of these sequences should enable the identification of genes involved in the synthesis of a range of classes of secondary metabolites by *C. raciborskii*.

The *pks* and *ps* genes were identified from *C. raciborskii* strains AWT205 and CYP020B by PCR using degenerate primers designed from conserved regions within each gene. These 2 *C. raciborskii* strains were chosen as type strains for genetic analysis as they have previously been reported to be highly toxic. *C. raciborskii* AWT205 was isolated from a water bloom (Hawkins et al., 1997), while strain CYP020B was originally isolated from Palm Island after the 1979 water-borne poisoning incident. Several lines of evidence support the hypothesis that the *pks* and/or *ps* genes identified in this study are involved in secondary metabolite biosynthesis in *C. raciborskii*. Firstly, each of these gene fragments displays strong similarity to genes encoding proteins with similar functions from other bacteria. Secondly, these genes are only present in cylindrospermopsin producing *C. raciborskii* strains. Finally, these genes are both present in a strain of *A. bergii* that has been shown for the first time to produce cylindrospermopsin.

Unfortunately *in vitro* genetic manipulation of cyanobacteria is often difficult due to factors such as host restriction and poorly defined transformation systems, and is particularly problematic when dealing with filamentous forms such as *Cylindrospermopsis*. However we are working towards the construction of mutant strains defective in PS and PKS gene activity to determine their exact roles in

cylindrospermopsin production. So far none of the methods attempted have been successful with *C. raciborskii*. However, currently we are trialling the methods with *A. bergii* and *Aph. ovalisporum* known to contain the *pks* and *ps* determinants. It may be that these different species are more easily transformed to the mutant phenotype. One other possibility is the cloning and expression of this suite of genes in another host such as *E. coli*, or even another cyanobacterium, eg *Synechococcus* or *Microcystis*. We are currently constructing a cosmid gene bank of a toxic *C. raciborskii* isolate with this goal in mind

We used Southern hybridisation analysis to screen a number of different cyanobacteria for the presence of sequences similar to the *C. raciborskii* PKS and PS gene fragments. Significantly, these sequences were only detected in the cylindrospermopsin-producing cyanobacterium *A. bergii*. Interestingly, a different RFLP pattern was observed between *C. raciborskii* and *A. bergii*. The high degree of nucleotide sequence conservation between the PKS (535/540 bp or 99%) and PS (511/534 bp or 95%) gene fragments of *C. raciborskii* and *A. bergii* is striking. This result is further emphasised by comparing these values to that of a fragment of the *rpoC1* gene. The *rpoC1* gene encodes for a DNA-dependent RNA polymerase and is highly conserved amongst prokaryotes (Palenik, 1994). This gene shows only 75% (328/433 bp) nucleotide sequence similarity between these two cyanobacteria (Wilson et al., 2000). Taken together, this data is suggestive of a recent gene transfer event between these two genera. The observation that the PKS and PS genes are either both present or absent in the strains we examined also suggests that they are physically linked, akin to the structure of the genetic determinants involved in microcystin production by *M. aeruginosa* (Nishizawa et al., 2000; Tillett et al., 2000). Whilst the role of both PS and PKS in the formation of microcystin has been described the structure of cylindrospermopsin implicates a PKS but not PS in its formation. Further analysis of the PS in *C. raciborskii* will establish its role, if any, in the production of cylindrospermopsin. Unfortunately the two other isolates reported to produce cylindrospermopsin, namely *Aph. ovalisporum* (Shaw et al., 1999) and *U. natans* (Harada et al., 1994), were not available for this study. However analysis of partial 16S rRNA gene sequence for *A. bergii* ANA 283A (data not presented) revealed 476/477 sequence identity to the published sequence for *Aph. ovalisporum* (Shaw et al., 1999), suggesting that these two organisms may be morphological variants of the same bacterium. It would be of interest to test the *Aph. ovalisporum* and *U. natans* isolates for the presence of PS and PKS and compare any derived sequence to the *C. raciborskii* and *A. bergii* determinants.

5 Acknowledgements

The Project Leader (Dr Chris Saint) and Project Proponent (Dr Brett Neilan) would like to thank the following organisations and people for their valued support:

The CRC for Water Quality and Treatment for financial support to this project and for a postgraduate stipend awarded to Kim Fergusson. The University of South Australia for the APA awarded to Kim Fergusson and the Australian Research Council for additional support to Brett Neilan's group. The contribution of the 3 research officers Renate Velzeboer, Dr Mark Schembri and Dr Sarah Baker at AWQC and research assistance provided by Leigh Hardman, Amanda Bass, Brendan Burns and Michelle Moffitt at UNSW. The input of Dr Martin Saker, formerly of James Cook University is also acknowledged. We thank Dr Glendon Shaw, NRCET, for valued discussions conducted at a variety of locations and venues and Geoff Eaglesham, NRCET, for cylindrospermopsin assays. George Turelli, Business Manager CRC for Water Quality and Treatment, is thanked for keeping the project accounts in order and

making us realise why we chose science for a career. Last but not least we are grateful for the support of the CRC Program Leader Dr Dennis Steffensen whose jurisdiction this project fell under.

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