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**Assessing genetic diversity and identification of
Microcystis aeruginosa strains through AFLP and PCR-
RFLP analyses.**

by

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Magister Scientiae

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DECLARATION

I the undersigned hereby declare that the work carried out in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.



Paul Johan Oberholster
23 December 2003

RESEARCH OUTPUT

The following peer-reviewed publications and conference presentations resulted from this study:

1. OBERHOLSTER PJ, BOTHA A-M & GROBBELAAR JU (2004) *Microcystis aeruginosa*: source of toxic microcystins in drinking water. African Journal of Biotechnology 3(1): 159-168.
2. OBERHOLSTER PJ, BOTHA A-M, COETZEE L & GROBBELAAR JU (2004) *Microcystis aeruginosa* strain identification using PCR analysis. Proceedings of the WISA meeting. ISBN 1-920-0172-8-3.
3. OBERHOLSTER PJ, BOTHA A-M & GROBBELAAR JU (2003) *Microcystis aeruginosa* strain identification using molecular tools. Algal Biotechnology meeting, Qiandao, China, October 2003 (poster).
4. OBERHOLSTER PJ, BOTHA A-M & GROBBELAAR JU (2004) Application of molecular tools for the identification of *Microcystis aeruginosa* strains. SAMS meeting, University of Stellenbosch, Stellenbosch, 4-7 April 2004 (poster).

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List of Abbreviations

aa	Amino acid
ABS	Absorbed photon flux
Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
AFLP	Amplified Fragment Length Polymorphism
ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
AP	Alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	Base pair
CCAP	Culture Collection of Algae and Protozoa, UK
CTAB	N-cetyl-N-N-N-trimethyl ammonium bromide
dATP	Deoxyadenine triphosphate
dCTP	Deoxycytidine triphosphate
ddH ₂ O	Double distilled water
dGTP	Deoxyguanosine triphosphate
DIG	Digoxigenin
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dNTP	Deoxynuclein triphosphate
DTE	Dithioerythritol
DTT	Dithiothreitol
dTTP	Deoxythymine triphosphate
dUTP	Deoxyuracil triphosphate
EC	Enzyme code
EDTA	Ethylenediamine tetra-acetic acid, disodium magnesium
ELISA	Enzyme-linked immunosorbent assay
ET	Electron transport past Q _A
e-value	expectancy value
F ₀	Minimal fluorescence of a dark adapted sample
F _m	Maximal fluorescence of a dark adapted sample
GC	Gas chromatography
HPLC	High performance liquid chromatography
I _k	the light intensity at the onset of light saturated photosynthesis in $\mu\text{mol photon m}^{-2} \text{s}^{-1}$
i.p.	intraperitoneally
IPTG	Isopropyl- β -D-galactoside
i.v.	intravenous
kb	Kilobase
kDa	Kilodalton
LB	Luria Bertrani
LD ₅₀	Lethal dose
LDH	Lactate dehydrogenase

MC	Microcystin
Mdha	N-methyl-dehydroalanine
MMPB	3-methoxy-2-methyl-4-phenylbutric acid
mRNA	Messenger ribonucleic acid
NBT	Nitroblue tetrazolium salt
NIES	National Institute for Environmental Studies, Japan
P^B_{max}	maximum biomass specific photosynthetic rate in $\mu\text{mol O}_2 \text{ mg chl a}^{-1} \text{ h}^{-1}$
PCC	Pasteur Culture Collection
PCR	Polymerase Chain Reaction
PCR-RFLPs	Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms
PP	Protein phosphatase
PPi	Inorganic pyrophosphate
RC	Reaction Centre
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
SSC (20X)	0.3 M NaCitrate, 3 M NaCl, pH 7.0
STET	0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 5 % Triton®X-100
TAE (1X)	40 mM Tris-acetate, 1 mM EDTA, pH 8.0
TE	10mM Tris-HCl, 1 mM EDTA, pH 8.0
TOC	Total organic carbon
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
tRNA	Transfer ribonucleic acid
UP	University of Pretoria
UV	Ultraviolet
UV	Strain in the University of the Free State Culture collection
WHO	World Health Organization
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside
X-phosphate	Toluidinium salt

List of Units

Anti-digoxigenin-AP conjugate

One unit is the quantity of enzyme that hydrolyses 1 μM *p*-nitrophenylphosphatase in 1 minute at 37 °C.

LD₅₀

Dose of toxin that kills 50 % of the animals tested.

Klenow

One unit is the enzyme activity that incorporates 10 nmol of total nucleotides into an acid-precipitate fraction in 30 minutes under assay conditions.

Restriction Enzyme

One unit is the enzyme activity that completely cleaves 1 μg λ DNA in 1 h at enzyme specific temperature in a total volume of 25 μL .

Taq DNA Polymerase

One unit is the quantity of enzyme required to catalyze the incorporation of 10 nmol of dNTP's into acid insoluble material in 30 minutes at 74 °C.

Weiss Units

One unit is the quantity of enzyme that catalyses the exchange of 1 nmole of ³²P from pyrophosphate into [γ , β -³²P]ATP in 20 minutes at 37 °C.

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Chapter I

Introduction

Cyanobacteria are one of the earth's most ancient life forms. Evidence of their existence on earth, derived from fossil records, encompasses a period of some 3.5 billion years, i.e. the late Precambrian era (Robarts and Zohary 1987). Cyanobacteria are the dominant phytoplankton group in eutrophic freshwater bodies worldwide. They have caused animal poisoning in many parts of the world and may present risks to human health through drinking and recreational activity (Carmichael and Falconer 1993). Cyanobacteria produce two main groups of toxins namely neurotoxins and peptide hepatotoxins (Carmichael 1992). They were first characterized from the unicellular species *Microcystis aeruginosa*, which is the most common toxic cyanobacterium in eutrophic freshwaters (Carmichael 1992).

The first livestock mortalities in South Africa caused by cyanobacteria blooms were observed by Steyn (1945) who noted that over a period of twenty-five to thirty years, the deaths of many thousands of livestock around pans in the North West and Mpumelanga Provinces, South Africa were reported by farmers in the region, who referred to the condition as 'pan sickness'. The first death suspected to be due to algal poisoning were brought to the attention of staff at Onderstepoort Veterinary Laboratories by farmers from the Amersfoort district in 1927. Since then numerous reports exist, documenting the incidents of stock losses in South Africa such as the poisoning of an entire dairy herd in 1996 near Kareedouw in the Tsitsikamma area.

South Africa is a water-stressed country where water planners and managers are faced with increasingly complex issues. The country is largely semi-arid and prone to erratic and unpredictable extremes of droughts and floods. Rivers are the main source of water in South Africa. Country-wide, the average annual rainfall is a little less than 500 mm, compared to the world average of about 860 mm. On average, only some 9 per cent of all rainfall, reach the rivers. The average annual potential evaporation is higher than the rainfall in all but a few isolated areas where rainfall exceed 1 400 mm per year. Consequently, only about 32 000 million kilolitres of the annual run-off can be economically exploited using current methods. Apart from erratic rainfall and the low ratio of run-off, resistance to the provision of funding for cyanobacterial research is often based on the argument that there are far greater health problems and that funding needs to be directed to the alleviation of diseases (Harding and Plaxton 2001). This argument is in contrast with the fact that the quality of many water sources in South Africa is declining. The decline is primarily a result of eutrophication and pollution by trace metals that are micro-pollutants (DWA 1986).

In this study samples of cyanobacterial blooms were collected from the Hartbeespoort , Rietvlei and Roodeplaat Dams, respectively. These dams are located in the populous and economically important industrial hub of Gauteng and North-West Provinces. The Hartbeespoort Dam was completed in 1925, and was formed by the damming of the Crocodile River below its confluence with the Magalies River 25 km to the west of Pretoria. When the dam is full, the shore-line is 56 km, the surface area is 1 283 ha. and the volume of water is estimated at 13 000 000 m³, with a maximum depth of 9.6 m. The dam lies in a basin of shale and diabase of the Pretoria series. It serves as a source of water for irrigation purposes to the extensive farming area to the north of the Magalies Mountains, as

well as domestic consumption for the town of Brits. The dam lies in an area of summer rainfall, and in the transition between the Highveld and Bushveld vegetation types. As a result it is not subject to seasonal extremes of temperature so typical of the Highveld (Allanson and Gieskes 1961).

Hartbeespoort Dam's eutrophication problems arise largely from two sources, namely treated sewage from Johannesburg's Northern Sewerage Works, and untreated sewage and other pollutants from the Jukskei River, which runs through Alexandra. These sources contribute significant quantities of phosphates and nitrates, causing the dam to be hypereutrophic (Robarts and Zohary 1987). During April 2003 a cyanobacteria bloom of 30 cm thick and covering an area of 4 ha was detected in the Hartbeespoort Dam. This particular bloom did not only pose a health risk to both animals and humans, but could negatively impact on suppliers and users of potable water. The development of undesirable blooms detracts the visual appearance of the dam, obstruct swimmers, fishermen and motorboats; clog irrigation and stock water pipes; and disrupt water treatment plants. When the scum decay, major odour problems result that also affects the taste of the water. The decaying biomass furthermore removes oxygen and could cause fish kills and the deaths of other aquatic life forms. Because of the potential problems the Department of Water Affairs invested half a million Rand to get contractors to remove the cyanobacteria by pumpsuction (Louw 2003).

The Roodeplaat dam was completed in 1950 and was constructed to store water that could be used for irrigation purposes. The dam has a storage capacity of 40 000 000 m³ and is built in the Pienaars River some 20 km north-east of Central Pretoria. The catchment area is 684 km², and the average rainfall in the catchment is 720 mm per annum. The geology

consists of quartzite and shale, with grass and bushveld as vegetation cover. The original natural run-off supplied a good quality raw water to the dam. However, as the catchment area developed, a denser population settled with both accommodated in industrial and domestic areas, giving rise to ever increasing pollution. Over and above natural run-off flowing into the dam, the dam receives treated water from two sewage treatment plants, Zeekoegat and Baviaanspoort (Langenegger and Partners 1997).

The Rietvlei dam is situated approximately 15 km south-east of Pretoria and its catchment, covering an area of 481 km², extends predominantly south-east to include Kempton Park's north-eastern urban area. The Johannesburg International airport forms the catchment's eastern boundary. The river originates in a marshy area east of Kempton Park and *en route* to the Rietvlei dam, passes through a number of wetlands. The catchment area is extensively utilized by agricultural activities where water is withdrawn for irrigation. The water's natural run-off is augmented by springs and effluent from the Hartbeesfontein sewage works. During 1994, the Pretoria Metropolitan Substructure launched a comprehensive study of Rietvlei Dam to consider the available management options to ensure the long-term viability of the Rietvlei system as a source of economical, high quality drinking water to the citizens of Pretoria (Van der Walt et al. 2001).

This study of Rietvlei Dam was completed in 1996 and the conclusion was that the quality of the water in Rietvlei Dam has deteriorated considerably over the past 20 years (Van der Walt et al. 2001). This was attributed to increased effluent discharges into the catchment, reduced effluent quality and the reduction in natural runoff, which dilutes and flushes out pollutants. As a result of the deteriorating water quality, regular blooms of cyanobacteria occur, and will continue to occur with increasing severity, with the consequent bad odours

and tastes. The water from Rietvlei Dam has been utilized as a drinking water source for the City of Pretoria since 1934. Since then, the treatment plant at the Rietvlei Dam had to be repeatedly extended to accommodate changes in the raw water characteristics, particularly to deal with the eutrophication of the dam. The original processes of flocculation, settling, filtration and chlorination had been augmented with dissolved air flotation in 1988, while granular activated carbon [GAC] was added in 1999. The cost for implementing activated carbon filtration as part of the treatment process for the production of potable water from Rietvlei Dam was R 20.4 million with a estimated operational cost increase of 23c/m³ (Van der Walt et al. 2001).

To understand the genetic diversity and population structure of *Microcystis aeruginosa*, it is important to study diversity of isolated strains and their counterparts in nature, and only then can physiological data gained from culture studies begin to be confidently extrapolated to natural conditions (Castenholz and Waterbury 1989). Inadequate culture conditions leading to the loss of various morphological characteristics, with researchers inability to grow certain organisms in the laboratory, and misidentifications of strains in culture collections make it difficult in many cases to apply taxonomic assignments based on cultures to field populations (Wilmotte 1994). Both classification systems for the bacteriological approach, as well as the traditional botanical approach, rely primarily on morphological characteristics of cells and colonies, and do not necessarily lead to the identification of phylogenetically coherent taxa. At all taxonomic levels, the DNA based methodology (i.e. polymorphisms in genomic DNA or specific gene sequences) is currently the most promising approach. Variation in genomic DNA sequences is independent of cultivation or growth conditions. The other advantage is that the information can be retrieved by PCR from small quantities of DNA extracted from laboratory cultures or from

the natural environment (Pan et al. 2002). The purpose of the present study was thus, to compare the genetic diversity of geographically unrelated *Microcystis aeruginosa* strains in culture, to that of *Microcystis* strains obtained in nature (i.e. Hartbeespoort, Roodeplaat and Rietvlei dams) using amplified fragment length polymorphisms (Chapter 3). The second objective of the study was to produce a fast screening method based on the polymerase chain reaction to detect the presence or absence of the mycotoxins in water, based on the premise that the presence of the *mcyB* gene is indicative of toxicity. Differences in the *mcyB* gene sequence was further used to differentiate between the different *Microcystis* strains (Chapter 4).

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Chapter II

Literature review

2.1 Cyanobacteria

Cyanobacteria are the dominant phytoplankton group in eutrophic freshwaters (Davidson 1959; Negri et al. 1995). They are prokaryotes possessing a cell wall composed of peptidoglycon and lipopolysaccharide layers instead of the cellulose of green algae (Skulberg et al. 1993). All Cyanobacteria are photosynthetic and possess chlorophyll *a* (Chl *a*). Morphological diversity ranges from unicells; to small colonies of cells to simple and branched filamentous forms (Weier et al. 1982).

The cytoplasm contains many ribosomes and appears granular. In filamentous forms, fine plasmodesmata connect adjacent cells. The plasmalemma may form invaginations but in addition, there are a series of parallel membranes within the cytoplasm that are separate from the plasmalemma. The process of photosynthesis occurs on these membranes, which contain Chl *a*, and a few other accessory pigments are grouped together in rods and discs that are called phycobilisomes, that are attached to the outside of the membranes (Weier et al. 1982). These pigments capture light between wavelengths 550 to 650 nm, and pass their light energy on the Chl α .

Other cytoplasmic inclusions are gas vesicles, granules of glycogen, lipid droplets, granules of arginine and aspartic acid polymers and polyhedral carboxysomes. Gas vesicles are especially prominent in floating aquatic species and it is likely that they contribute to buoyancy. The nucleoplasm is sharply delimited from the cytoplasm, even though there is no nuclear membrane as in bacterial cells, it is composed of a circular, double-stranded molecule of DNA. Cell volume ranges from 5 to 50 μm^3 , in contrast to

0.01 to 5 μm^3 for bacteria. They have about twice as much DNA as does *E. coli*, with one chromosome (Weier et al. 1982).

About one third of all cyanobacteria species are able to fix atmospheric nitrogen. In most of the cases, nitrogen fixation occurs in specialized cells called heterocysts. These are enlarged cells with an envelope. The internal membranes no longer lie in parallel arrays, and these cells may have lost photosystem II, hence do not generate O_2 . A plasmodesmata connect the heterocysts to adjacent cells within a filament. It is possible that the thick wall maintain an anaerobic condition in the cytoplasm (Weier et al. 1982).

Cyanobacteria are especially abundant in shallow, warm, nutrient rich or polluted water that is low in oxygen, and can grow to form thick scums that could colour the water, creating blooms (Figure 2.1)(Stotts et al. 1993). Most blooms disappear in a few days, but the cells can release toxins lethal to animals that swim in or drink the water (Weier et al. 1982).

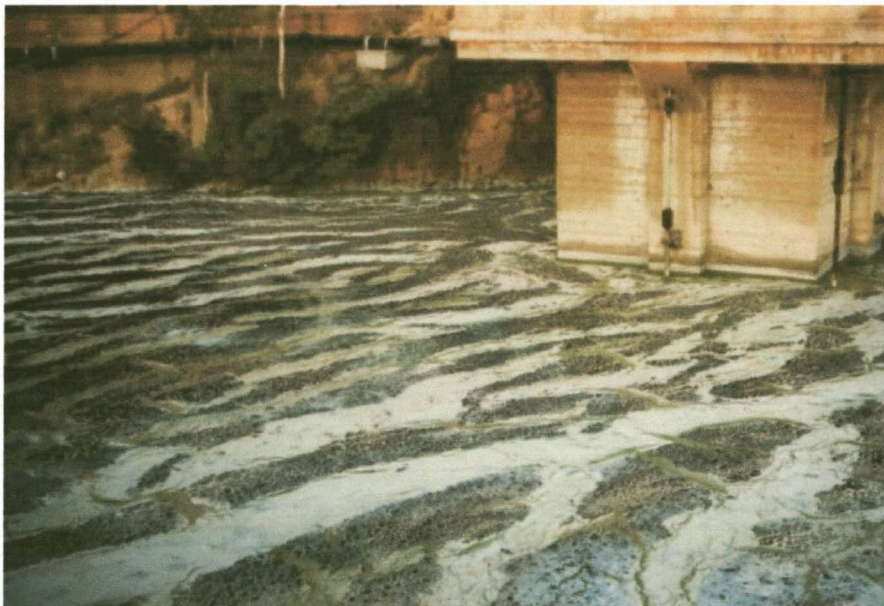


Figure 2.1 Cyanobacterial bloom visible as green scum on the water of the Hartbeespoort Dam (December 2002).

2.2 Association of environmental parameters with cyanobacterial blooms and toxicity of microcystin

Fieldstudies in South Africa (Wicks and Thiel 1990) have shown that certain environmental factors are associated with the quantity of toxins found in cyanobacterial blooms. The effects of environmental factors on toxin production by cyanobacteria have also been shown by laboratory studies (Sivonnen 1990; Utkilen and Gjolme 1992).

2.2.1 Physical factors

Temperature

In general, cyanobacteria prefer warm conditions, and low temperatures are one of the major factors that end cyanobacterial blooms. Robarts and Zohary (1987) found that *Microcystis* was severely limited at temperatures below 15°C and were optimal at temperatures around 25°C. Temperature alone may only partly determine bloom formation and it is accepted that a combination of factors are responsible for a bloom to develop. These are increasing temperatures, decreasing nutrients and increased water column stability. This also explains why succession of algae usually follow patterns in freshwater bodies from diatoms through chlorophytes to cyanobacteria.

Van der Westhuizen and Eloff (1985) determined that temperature has a most pronounced effect on toxicity. The highest growth rate was obtained at 32°C, while the highest toxicity was found at 20°C, but declined at temperatures higher than 28°C. At temperatures of 32°C and 36°C toxicity was 1.6 and 4 times, respectively less than cells cultured at 28°C, suggesting that highest growth rate is not correlated with highest toxicity. They considered

the decreased toxin production to be possibly related to decreased stress levels at temperatures above 20°C.

Temperature changes were found to induce variations in both the concentration and peptide composition of the toxin (Yokoyama and Park 2003). A third toxic peptide [C] was discovered at a higher concentration than either peptides A or B at 16°C. Peptide C was suspected of containing aspartic acid rather than B-methylaspartic acid. Small quantities of phenylalanine and arginine were detected in peptide C, as well as alanine [23%], leucine [26%], aspartic acid [23%] and glutamic acid [27%]. The percentage content of peptide A increased between 16°C and 36°C, while overall toxicity decreased sharply. This being due to a decrease in the concentration of peptides A and B. Peptide C disappeared gradually at higher temperatures, Van der Westhuizen and Eloff (1985) ascribed this to reduced synthesis or increased decomposition, rather than leaching, since cells were still growing after the growth phase.

Light and buoyancy

The effect of light intensity on the fine structure of *M. aeruginosa* was investigated under laboratory conditions. The optimal growth rate for *M. aeruginosa* cells was 3 600-18 000 lux ($k \text{ lux} \times 18 \approx 1 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$) (Abelovich and Shilo 1972). The lag phases lasted approximately 5 days, followed by an 11-day period of exponential growth. At light levels in the excess of 18 000 lux the growth rate declined rapidly. Pigment ratios and visual pigmentation were found to change considerably at different light intensities. At 3 600 lux and lower, cultures were green for the duration of the experiment period of 28 days. At 5 700 lux, cultures were yellow, and at 18 000 lux they were orange. The ratio of Chl a to carotenoids, plotted against light intensity showed that as light intensity increased,

carotenoid pigments increased relative to Chl *a*. A reduction in this ratio occurred with ageing. Carotenoid pigments shield cells from high light intensity, preventing the destruction of Chl *a* and the photo-oxidation of photosynthetic pigments (Abelovich and Shilo 1972). In a recent publication it was reported that the quality of light (i.e. 16 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in the red light spectrum) increase toxin production in a *M. aeruginosa* strain (Kaebernick et al. 2000).

It was also found that the effect of light intensity affected the gasvacuole content and thylakoid configuration. The gasvacuole content increased as light intensity increased to 6 000 lux, thereafter decreasing between 6 000 and 8 000 lux (Waaland et al. 1971), suggesting that the vesicles could act as light shields in addition to their possible buoyancy functions. The absence of gasvacuoles grown at low light intensities of 400 lux supported this observation.

Buoyancy is regulated by a number of mechanisms, such as the form of stored carbohydrates and turgor pressure regulation. Compositional changes in the diel protein:carbohydrate ratios during buoyancy reversals suggest a complex relationship between light and nutrients (N:P) (Villareal and Carpenter 2003). It, however, seems that the regulation of gasvacuole synthesis is the most important. This almost unique feature of cyanobacteria gives these organisms a significant advantage over other phytoplankton. In turbulent waters cyanobacteria lose this advantage and often this characteristic is used to control their blooms (Grobbelaar 2004).

2.2.2 Chemical factors

Nitrogen and phosphorus ratios

Much has been made of the relationship between prevailing ratios of nitrogen and phosphorus, and the composition and density of phytoplankton assemblages that may occur. While certain broad categories generally and accurately support prediction of which algal division that may predominate, other biophysical features and attributes should not be excluded from the equation. It is becoming increasingly apparent that, notwithstanding the prevailing nitrogen and phosphorus ratio, the phytoplankton assemblage may be significantly altered through biomanipulation, and without any changes whatsoever to the ambient availability of nitrogen and phosphorus (Harding and Wright 1999). In 1986, Carmichael demonstrated that the omission of nitrogen causes approximately tenfold decrease in toxicity.

Iron and zinc

Certain metal ions such as Zn^{2+} and Fe^{2+} significantly influence toxin yield. Zn^{2+} is involved in the hydrolysis of phosphate esters, the replication and transcription of nucleic acids, and the hydration and dehydration of CO_2 (Sunda 1991). All cyanobacteria require Fe^{2+} for important physiological functions such as photosynthesis, nitrogen assimilation, respiration and chlorophyll synthesis (Boyer et al. 1987). It is not yet clear how Fe^{2+} deficiency modulates microcystin production, but it has been noted that as cyanobacteria experiences iron stress, they appear to compensate for some of the effects of iron loss by synthesizing new polypeptides (Lukač and Aegerter 1993).

2.3 The toxicology of microcystins in cyanobacteria

Cyanobacteria are capable of producing three kinds of toxins, the dermatotoxin, cyclic peptide hepatotoxin, and the alkaloid neurotoxin. Serious illnesses such as hepatoenteritis, a symptomatic pneumonia and dermatitis may result from consumption of, or contact with water contaminated with toxin producing cyanobacteria (Hawkins et al. 1985; Turner et al. 1990, for review see Briand et al. 2003). The dermatotoxins are mainly produced by marine cyanobacteria, but the dermatotoxins lyngbyatoxin A and aplysiatoxin are related to acute dermatitis, poisoning and animal death, especially in Japan and Hawaii (Briand et al. 2003). The neurotoxins include anatoxin-a, a depolarizing neuromuscular blocking agent; anatoxin-a [s], an anti-cholinesterase; and saxitoxin and neosaxitoxin that inhibit nerve conduction by blocking sodium channels (Carmicheal 1994, Briand et al. 2003).

Microcystins are a family of toxins produced by different species of freshwater Cyanobacteria, namely *Microcystis* [order Chroococcales], *Anabaena* [order Nostocales], and *Oscillatoria* [order Oscillatoriales]. Microcystins are monocyclic heptapeptides composed of D-alanine at position 1, two variable L-amino acids at positions 2 and 4, γ -linked D-glutamic acid at position 6, and 3 unusual amino acids; β -linked D-erythro- β -methylaspartic acid (MeAsp) at position 3; (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid (Adda) at position 5 and N-methyl dehydroalanine (MDha) at position 7. There are over 60 different microcystins that differ primarily in the two L-amino acids at positions 2 and 4, and methylation/demethylation on MeAsp and MDha. The unusual amino acid Adda is essential for the expression of biological activity. Other microcystins are characterized largely by variations in the degree of methylation; amino acid 3 has been found to be D-aspartic acid, replacing β -methylaspartic acid and amino acid 7 to be dehydroalanine, replacing N-

methyldehydroalanine (An and Carmichael 1994; Trogen et al. 1996). The most common microcystin, is microcystin-LR, where the variable L-amino acids are leucine (L) and arginine (R). Its structure is shown in Figure 2.2 (An and Carmichael 1994).

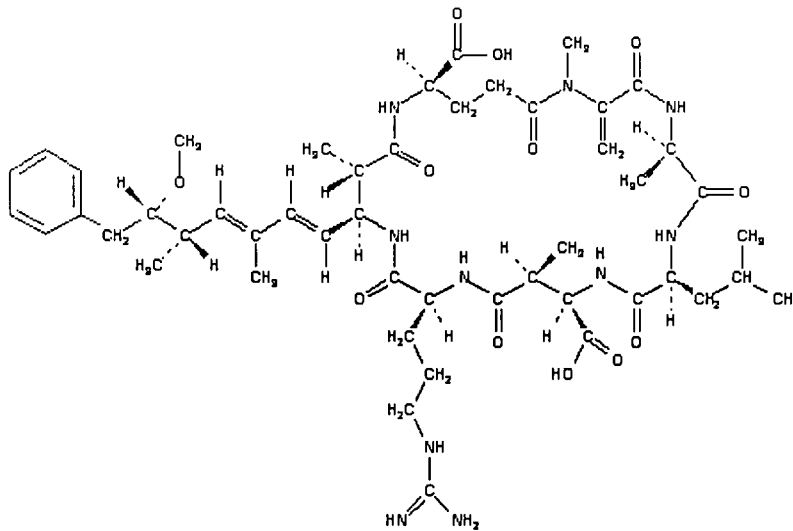


Figure 2.2 Chemical structure of microcystin-LR (An and Carmichael 1994).

Some esters of glutamic acid have been observed for amino acid 6 replacing γ -linked glutamic acid itself and N-methylserine sometimes replaces amino acid 7. Variations in the Adda subunit (amino acid 5) include 0-acetyl-0-demethyl-Adda and (6Z)-Adda (Rinehart et al. 1988).

The adda and D-glutamic acid portions of the microcystin-LR molecule play highly important roles in the hepatotoxicity of microcystins. Esterification of the free carboxyl group of glutamic acid results essentially in inactive compounds. Some of the Adda subunits assert little effect, especially the 0-dimethyl-0-acetyl analogs. However, the Adda molecules' overall shape seems to be critical since the (6Z0-Adda)(*cis*) isomer is inactive (Rinehart et al. 1988).

2.3.1 Synthesis of Microcystins

As stated before *Microcystis aeruginosa* is an organism that produces a vast number of peptides, some of which are highly toxic (Carmichael 1986). The most commonly occurring toxin is microcystin and to synthesize this complex peptide there obviously has to be genetic material present in the organism. Different possible localities of this genetic material have been investigated.

Chloroplast DNA

Shi et al. (1995) localised microcystins in a toxin-producing strain [PCC 7820] and non-toxin-producing strain [UTEX 2063] of *Microcystis aeruginosa* by using a polyclonal antibody against microcystins in conjunction with immuno-gold labeling. In the non-toxin-producing strain no specific labeling was found. In the toxin-producing strain specific labeling occurred in the region of the nucleoid in the thylakoid, to a lesser extent in the cell wall and sheath area. No specific labeling was found in cellular inclusions with storage functions. The reasons for this could not be determined, but Shi et al. (1995) suggested that microcystins are not compounds that the cell stores, but that they may be involved in specific cell activities.

Plasmids

Vakeria et al. (1985) investigated genetic control of toxin production by plasmids commonly found in some strains of *Microcystis aeruginosa*. Plasmid-curing agents were applied to toxin-producing strains, but no significant decrease in toxicity was observed. Schwabe et al. (1988) also supported this argument that toxin-producing strains do not contain plasmids. Apart from the reports of Vakeria et al. (1985) evidence has been presented of a South

African strain [WR 70] that shows a decrease in toxicity after treatment with plasmid-curing agents (Hauman 1981).

Thiotemplate Mechanism

Lipmann (1954) predicted a poly- or multienzymatic pathway of peptide synthesis and this mechanism has been verified for various types of peptides (Laland and Zimmer 1973). The first authors to propose the term thiotemplate mechanism and to distinguish this mechanism from other mechanisms of non-ribosomal peptide synthesis were Laland and Zimmer (1973). Many similarities are apparent when comparing ribosome-mediated protein synthesis with the thiotemplate mechanism. The most notable similarities are [1] the amino acids are activated through the formation of an amino acid adenylate, [2] the activated amino acyl residue is transferred to a receptor molecule, and [3] the peptide chain grows from the N-terminal end by insertion of the next amino acid at the activated C-terminal.

2.3.2 Analysis of microcystins

There are five basic methods to analyze microcystins namely; those based on reactions with a fluorescent probe; enzyme-linked immunosorbent assays [ELISA]; inhibition of protein phosphatase and mass spectrometry (Dawson 1998); and by polymerase chain reaction (PCR) (Baker et al. 2002, Pan et al. 2002). Shimizu and colleagues (1995, as cited by Dawson 1998) targeted conjugated dienes using a synthesized fluorogenic reagent called DMEQ-TAD. This reagent reacted with vitamin D metabolites and synthetic analogues, and the fluorescent products could be quantified linearly down to fmol quantities by HPLC. The reagent also reacted well with microcystin-LR, YR and RR at the conjugated diene moiety (Adda). An and Carmichael (1994) used a direct competitive ELISA to

examine the specificity of the rabbit anti-microcystin-LR polyclonal antibodies. Cross reactivity with some, but not all microcystin variants studied was observed and it became clear that Adda and arginine are essential for expressing the antibodies specificity. The inhibitor IC_{50} for microcystin-LR of the binding of microcystin-LR-horseradish peroxidase conjugate to the antibodies was 3 ng/ml. McDermott et al. (1995) described an ELISA potentially able to detect microcystins in water at a concentration as low as 100 pg/ml water.

Microcystins are inhibitors of protein phosphatase (Honkanen et al. 1996). An and Carmichael (1994) reported an IC_{50} of 6 ng/ml for microcystin-LR in their direct competitive ELISA, whilst ELISA microcystin-LR/YR/RR detection limits of 0.10, 0.12, 0.14 and 0.20 ng/ml were reported by Yu et al. (2002). A screening method for microcystins in cyanobacteria has been developed based on the formation of 3-methoxy-2-methyl-4-phenylbutyric acid by ozonolysis (Harada et al. 1996). The acid was detected by electron ionization-gas chromatography/mass spectrometry, using selected ion monitoring in a procedure that detected nanogram levels of microcystin in only 30 min.

Baker et al. (2002) determined the potential of microcystin production by PCR amplification of a gene involved in the microcystin biosynthetic pathway and the 16S rRNA gene of *Anabaena circinalis* strains. Pan et al. (2002) used primers deduced from the *mcy* gene to discriminate between toxic microcystin-producing and non-toxic strains. Cyanobacterial cells enriched from cultures, field samples, and sediment samples could successfully be used in the PCR assay.

2.3.3 Control and degradation of cyanobacterial blooms

Cousins et al. (1996) found in laboratory experiments with reservoir water using low levels of microcystin-LR [10mg/L], that degradation of the toxin occurred in less than one week. The toxin was stable for over 27 days in deionized water, and over 12 days in sterilized reservoir water, indicating that in normal reservoir water instability is due to biodegradation. Purified microcystins are also stable under irradiation by sunlight. However, significant decomposition of toxins by isomerization of a double bond in the Adda-side chain, occurs during sunlight irradiation in the presence of the pigments contained in cyanobacteria. The half-life for the whole process was estimated to be about ten days. Microcystin-LR and RR degraded much more rapidly when the toxins were exposed to UV light at wavelengths around their absorption maxima [238-254nm] (Tsuji et al. 1995).

It was found by Lam et al. (1995) that most of the microcystin-LR present in cells remains inside the cell until the cell is lysed. To control cyanobacteria blooms, cells are usually lysed in the presence of chemicals (e.g. Reglone A, NaOCl, KMnO₄, Simazine and CuSO₄) that inhibit new cell wall synthesis, enzymatic reactions or photosynthesis (Kenefick et al. 1993, Lam et al. 1995). A sudden release of microcystins into the surrounding waterbody can present a hazard to animals and humans using the water (Lam et al. 1995), as well as when used as potable water source.

Chemical control

Verhoeven and Eloff (1979) reported that copper is an effective algicide in natural waters for the control of cyanobacteria. *Microcystis aeruginosa* isolated from the Hartbeespoort Dam [UV-006], as well as *Microcystis aeruginosa* Berkeley strain 7005 [UV-007] were used to test the effects of copper on the ultrastructure of cells. Once cultures had been grown,

copper sulphate was added at different concentrations. It was found that toxicity of the copper was depended on cell concentration. At cell concentrations of 1.8×10^8 cells/ml [148 Klett units], 0.3 and 0.4 ppm Cu^{2+} decreased growth rates temporarily, whereas 0.5 ppm Cu^{2+} caused cell death. It was found that copper decreases the electron-density of the nucleoplasm, as well as cause aggregation of the DNA fibrils. Thykaloids were present as short membrane structures and membrane-bounded inclusions, while polyphosphate bodies disappeared.

Hoeger et al. (2002) tested the efficacy of ozonation coupled with various filtration steps to remove toxic cyanobacteria from raw water. They found that ozone concentrations of at least 1.5 mg/L were required to provide enough oxidation potential to destroy the toxin present in 5×10^5 *Microcystis aeruginosa* cells/ml (total organic carbon (TOC), 1.56 mg/L). High raw water with high cyanobacterial cell densities reduced the efficiency of the process, resulting in cell lysis and the liberation of intracellular toxins.

Biological control

Microcystins can be biodegraded by complex natural populations of micro-organisms from diverse ecosystems, such as sewage sludge (Lam et al. 1995), lake sediment, natural waters (Jones and Orr 1994; Jones 1990) and biofilms (Saitou et al. 2002). Jones (1990) demonstrated that microcystins extracted from *Microcystis aeruginosa* blooms were biodegraded in natural water bodies within 2-3 weeks. This time was reduced to a few days if the water body was previously exposed to microcystins.

Scott and Chutter (1981) suggest that viruses may be an important factor in controlling cyanobacteria. The first virus that was capable of lysing a filamentous cyanobacteria

Plectonema sp. was isolated from an oxidation pond. It was assumed by the authors that viruses were not important in controlling eukaryotic algae in large cultures. Thus on the basis of there being no apparent evidence to the contrary (e.g. reviews by Lemke 1976; Hoffman and Stanker 1976; Dodds 1979). Recently it was demonstrated that aqueous and methanolic extracts of cultured cyanobacteria of several genera, including *Microcystis*, expressed antiviral activity against the influenza virus (Zainuddin et al. 2002).

A myxobacterium capable of lysing freshwater algae was first reported by Stewart and Brown (1969, 1971). Scott and Chutter (1981) suggested that myxobacteria are a more important biological agent than viruses in controlling algae populations, since they are less host specific. Pioneering work was conducted by Canter (1950, 1951, 1957) on fungal parasites of freshwater algae in the English Lake District. Up to 70% of the individuals in an algae population could be infected by fungal parasites. A large proportion of fungal parasites were found to be host-specific, suggesting that in some cases, they may prevent cyanobacteria species from growing, while allowing environmental friendly species to proliferate.

Certain Pyrrophyta and Chrysophyta are capable of phagotrophic nutrition. In some instances, smaller algae such as *Chlorella* may be ingested. Cole and Wynne (1974) noted that when the chrysophyte *Ochromonas danica* was mixed into a culture with *Microcystis aeruginosa*, they declined 30-fold in 10 min, as a result of ingestion by *Ochromonas*.

Numerous reports exist in the literature documenting the success of using barley straw for the control of cyanobacteria. Newman and Barret (1993) demonstrated that decomposing barley straw effectively inhibits the growth rate of *Microcystis aeruginosa* to a sixth of that

achieved in control experiments. This inhibitory effect is presumably caused by the release of a chemical during aerobic microbial decomposition of the straw. This chemical, or chemicals, are so far unidentified, but there are several probabilities; firstly, antibiotics may be produced by the fungal flora active in the decomposition of the barley straw; secondly, during decomposition the release of modified cell wall components may have an effect on cyanobacterial growth; and thirdly, certain phenolic and aromatic compounds produced during cell wall biodegradation may also contribute to the declining of algal numbers. It seems that the inhibitory effect is rather algistatic than algicidal; therefore, the presence of decomposing barley straw can help prevent the development of cyanobacterial blooms.

Another report on the application of hay by a local municipality, to two small farm dams in Linfield Park near Pietermaritzburg, South Africa, suggested that hay may be useful in controlling cyanobacterial growth. The farm dams receive the bulk of their nutrient rich flow from a small sewage works, which caused the development of cyanobacterial scums. Reduction of algae populations in the upper of the two dams, closest to the sewage works, was total, with zero algae being detected within a few weeks of application of small quantities of hay in the water bodies (Harding and Plaxton 2001).

Water that had been treated with chlorine may have killed the algae, but the result will be the release of the toxins into the water. Very high concentrations of chlorine could, however, inactivate the microcystins. Conventional water treatment processes do not completely remove microcystins from raw water, even when activated carbon is included in the treatment (Lambert et al. 1996).

Blooms have been controlled with the treatment of lime without any significant increase in microcystin concentration in the surrounding water (Kenefick et al. 1993). Chemical control of *Microcystis* blooms appears to be the best solution, thus removing the source of the microcystins. It has been found that microcystins persist in the dried crust of lakes formed as water levels recede during dry seasons. Large quantities of microcystins leach from the dry materials upon re-wetting within 48 hours (Jones et al. 1995; Brunberg and Blomqvist 2002). This could present a significant problem with coagulation and sedimentation treatment as the water would not be suitable for consumption for up to three weeks before biodegradation commences (Jones 1990).

2.3.4 Toxicity

There have been many reports of the intoxication of birds, fish and other animals by cyanobacterial toxins (Vasconceles et al. 2001; Alonso-Andicoberry et al. 2002; Best et al. 2002; Romanowska-Duda et al. 2002; Krienitz et al. 2003). As stated before, blooms of cyanobacteria usually follow enrichment by nutrients such as phosphates and nitrates in the water. Most of these nutrients are derived from human wastes such as sewage and detergents, industrial pollution, run-off of fertilizers from agricultural land, and the input of animal or bird wastes from intensive farming (Bell and Codd 1994; Baker 2002). Illnesses caused by cyanobacterial toxins to humans fall into three categories; gastroenteritis and related diseases, allergic and irritation reaction, and liver diseases (Bell and Codd 1994). Microcystins have also been implicated as tumour-promoting substances (An and Carmichael 1994; Bell and Codd 1994; Rudolph-Böhner et al. 1994; Trogen et al. 1996; Zegura et al. 2003).

The LD₅₀ of microcystin-LR intraperitoneally (i.p) or intravenous (i.v.) in mice and rats is in the range 36-122 µg/kg, while the inhalation toxicity in mice is similar; LCT₅₀=180 mg/min/m³ or LD₅₀=43 µg/kg (Stoner et al. 1991). Therefore microcystin-LR has comparable toxicity to chemical organophosphate nerve agents. Symptoms associated with microcystin intoxication are diarrhea, vomiting, piloerection, weakness and pallor (Bell and Codd 1994). Microcystin targets the liver, causing cytoskeletal damage, necrosis and pooling of blood in the liver, with a consequent large increase in liver weight. Membrane blebbing and blistering of hepatocytes *in vitro* has been observed (Runnegar et al. 1991; Romanowska-Duda et al. 2002). High chromatin condensation and apoptotic bodies were observed in 90% of the cells of *Sirodela oligorrhizza* and rat hepatocytes after a treatment with microcystin-LR (MC-LR=500µg/dm) (Romanowska-Duda et al. 2002). Death appears to be the result of haemorrhagic shock (Hermansky et al. 1990) and can occur within a few hours after a high dose of microcystin-LR (Falconer et al. 1981; Bell and Codd 1994). The concentration of microcystin-LR in drinking water for humans as prescribed by the world health organization (WHO) is 1 µg/L (WHO 1998), however, Ueno et al. (1996) proposed a value of 0.01 µg/L, based on a possible correlation of primary liver cancer in certain areas of China with the presence of microcystins in water of ponds, rivers and shallow wells.

Mechanism of action of microcystins

It is known that microcystins mediate their toxicity by uptake into hepatocytes *via* a carrier-mediated transport system, followed by the inhibition of serine protein phosphatases 1 and 2A. The protein phosphorylation imbalance causes disruption of the liver cytoskeleton, which leads to massive hepatic haemorrhage that causes death (Honkanen et al. 1996; Eriksson et al. 1990a, b; Romanowska-Duda et al. 2002). The entry of toxin into the

hepatocytes of the liver and other targeted tissues is accomplished by the broad specificity anion transport bile acid carrier (Runnegar et al. 1991). In both cultured and *in vitro* hepatocytes, a rise in the amount of phosphorylated protein as a consequence of phosphatase inhibition was observed (Yoshizawa et al. 1990). The action of microcystin as a phosphatase inhibitor is not limited to mammalian cells, but also applies to plant phosphatases (MacKintosh et al. 1990; Siegl et al. 1990). It is, therefore, likely that the microcystins are general inhibitors of eukaryotic phosphatases of types 1 and 2A, limited only by the ability of the toxins to enter cells.

Phosphatase inhibition

The National Cancer Center Research Institute, Tokyo did discover the potency of microcystin-LR as an inhibitor of protein phosphatases types 1 and 2A (Yoshizawa et al. 1990; Matsushima et al. 1990) and this was also confirmed in other studies (MacKintosh et al. 1990; Honkanen et al. 1996; Eriksson et al. 1990a, b). The toxin-phosphatase interaction is extremely strong, and binding is essentially stoichiometric. Constant accurate inhibition can, therefore, only be obtained by extrapolation of the phosphatase concentration to zero. The value of k_i for protein phosphatase types 1 and 2A has been reported to be between 0.06–6 nM and 0.01–2 nM, respectively with microcystin-LR showing up to a 40-fold higher affinity of microcystin-LR for protein phosphatase type 2B. This is at least 1 000 fold lower than that for phosphatase type 1, while no interaction of microcystin-LR was observed with protein phosphatase type 2C or with a variety of other phosphatase or protein kinases (MacKintosh et al. 1990; Honkanen et al. 1996; Suganuma et al. 1992).

The correlation between inhibition of phosphatase activity and toxicity is indicated by the results of Runnegar et al. (1993), who administered microcystin–YM or LR to mice and observed that inhibition of liver protein phosphatase 1 and 2A activity preceded or accompanied clinical changes due to microcystin intoxication in all cases. Inhibition of protein phosphatases leads to phosphorylation of cytoskeletal protein and cytoskeletal associated protein and consequent redistribution of these proteins. Ghosh et al. (1995) showed that the collapse of cytoskeletal actin microfilaments occurs in rat hepatocytes prior to the dislocation of the associated proteins, α -actinin and talin rather than being caused by their dislocation.

Other effects of microcystins

Hermansky et al. (1991) observed a decrease in hepatic microsomal membrane fluidity, when they administered mice with microcystin–LR. These changes involved an indirect and secondary effect of the toxin, as no changes in membrane fluidity were observed when microcystin was incubated with control microsomes *in vitro*.

LeClaire et al. (1995) suggested a potential cardiogenic component in the pathogenesis of shock, in addition to the effects on the liver. The authors observed a sustained, rapid decline in cardiac output and stroke volume in rats intoxicated with microcystin–LR. The acute hypotension was responsive to volume expansion with the whole blood, and the acute drop in heart rate responded to both isoproterenol and dopamine. A peripheral vasoconstriction appeared to occur in response to hypotension.

2.4 Identification, diversity and population structure

The current cyanobacterial taxonomy does not provide an unequivocal system for the identification of toxigenic and bloom-forming genus *Microcystis* (Komárek 1991). The ambiguities that exist in the cyanobacterial taxonomy are due to the expressed variability, minor morphological and developmental characteristics used for identification, classification of the genus or species level (Doers and Parker 1988; Rippka 1988). Depending on the taxonomic parameters used for classification, which differs in their emphasis on the cell size, shape, buoyancy, toxicity of the planktonic, freshwater cyanobacteria, different generic assignments may be made (Rippka 1988; Rippka and Hardman 1992).

2.4.1 Molecular Tools for culture identification

rRNA and rDNA genes

The sequence signatures found in the 18S rDNA and 16S rRNA gene locus have been shown to be suitable for differentiation of bacteria at inter- and intrageneric taxonomic levels (Friedl and O'Kelly 2002; Lee and Bae 2002; Neiland et al. 1997; Fox et al. 1992; Woese 1987). In a study by Neiland et al. (1997) the 16S rRNA gene was applied to illustrate the evolutionary affiliations among *Microcystis* strains, other cyanobacteria, and related plastids and bacteria. It was concluded from the study that *Microcystis aeruginosa* was a monophyletic group, but the genus *Microcystis* was polyphyletic (Lee and Bae 2002; Neiland et al. 1997) and contained two strains that clustered with unicellular cyanobacteria belonging to the genus *Synechococcus*. The clustering of related *Microcystis* strains, including strains involved in the production of the cyclic peptide toxin microcystin, was consistent with cell morphology, gasvacuolation, and the low G+C contents of the genomes. The authors also found that the *Microcystis* lineage to be distinct from the lineage containing the unicellular genus *Synechocystis* and the filamentous, heterocyst-

forming genus *Nostoc*. It is interesting to note that Neiland et al. (1997) found no correlation between the evolution of the 16S rRNA gene and the toxicity of *Microcystis* strains. However, the major *Microcystis* taxonomic cluster exhibited a high incidence of toxic representatives and these were delineated from the non-toxic groups.

Polymerase chain reaction-restriction fragment length polymorphisms

Restriction fragment length polymorphisms (RFLPs) represents a DNA-based marker system that makes use of the detection of differences in the length of restriction fragments generated by the complete digestion of genomic DNA with restriction endo-nucleases (Sambrook et al. 1989). PCR-RFLPs is a modification of the above, as conventional RFLPs proved to laborious and require Southern hybridization and probes to detect the polymorphisms (Southern 1975). In the PCR-based system, a specific genomic sequence is amplified via PCR utilising primers designed to amplify the specific genomic region of interest. These fragments are then restricted with appropriate restriction enzymes. Fragment length polymorphisms are generated when a particular recognition site of a restriction enzyme is absent in one individual and present in another, resulting in differently sized restriction fragment at a locus (see Figure 4.1, Chapter IV). The polymorphic fragments are then visualized by resolving the DNA fragments using electrophoresis (Venter and Botha 2000).

Amplified Fragment Length Polymorphisms

Amplified fragment length polymorphisms (AFLPs), developed by Zabeau and Vos (1993), is a reproducible, multiplex assay with the ability to generate large numbers of polymorphic genomic fragments. The technology involves the restriction digestion of genomic DNA,

adapter ligation, which is followed by PCR rounds of pre-selective and selective amplification of restricted fragments (Vos et al. 1995) (Figure 2.3).

Comparative studies indicate that AFLPs offer a high level of utility compared with other marker systems (Powell et al, 1996, Venter and Botha 2000). However, AFLPs are technically more demanding, require more DNA (0.2 to 1 ug per reaction), and are more expensive than RAPDs. Because of their large genome coverage AFLP on average give 50-100 bands compared to 20 for RAPDs. Thus, AFLPs appear to be particularly useful for fingerprinting and can be used to assay genetic diversity within species (Powell et al. 1996).

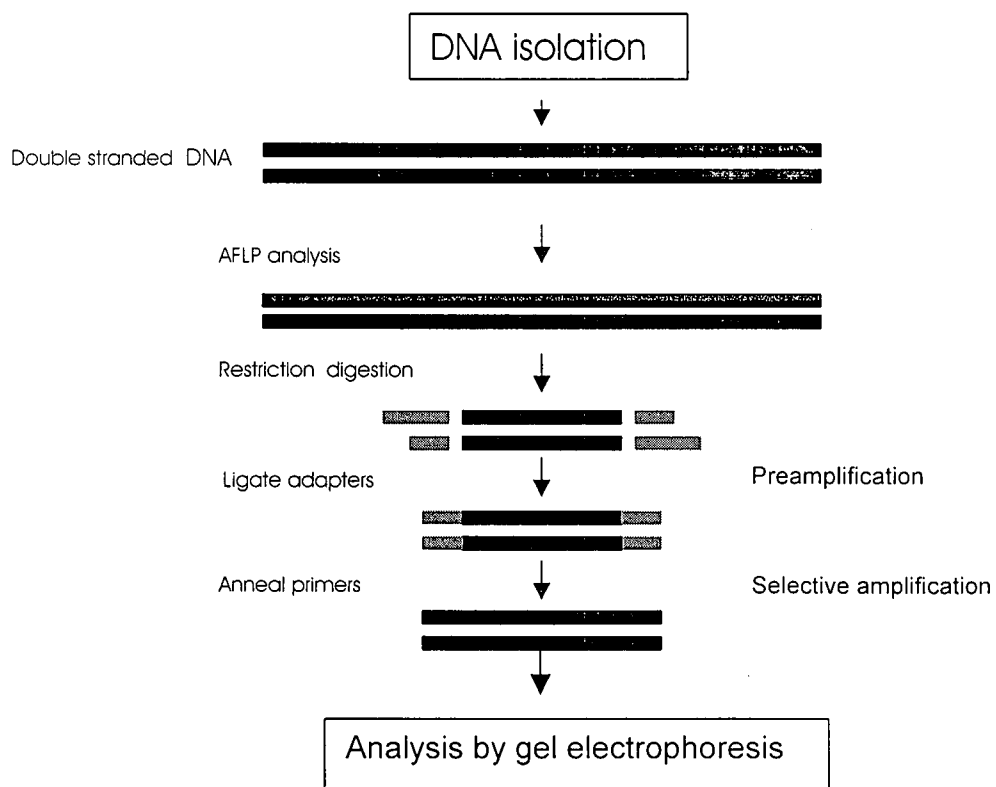


Figure 2.3 A schematic representation illustrating the process to generate amplified fragment length polymorphisms (AFLPs).

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Chapter III

Assessment of the genetic diversity of *Microcystis aeruginosa* strains using Amplified fragment length polymorphisms (AFLPs)

Introduction

Cyanobacteria are the dominant phytoplankton group in eutrophic freshwater bodies. Several of the common bloom-forming species are known to produce toxins. *Microcystis aeruginosa* is the most common toxic cyanobacterium, and the toxicity of the blooms seems to be mainly associated with this species (Carmichael 1986). The toxins produced by *Microcystis* species has been isolated and designated as microcystins, which are composed of cyclic heptapeptides (Falconer et al. 1983; Hawkins et al. 1985; Turner et al. 1990).

Due to the toxicity of these species, previous studies focused mainly on toxin production, although cyanobacterial classification has been problematic for a long time. Because of morphological simplicity of most prokaryotes, their classification was previously based largely on physiological properties, as expressed in pure laboratory cultures (Doers and Parker 1988). While field studies relied mostly on morphological analyses of natural populations, laboratory studies concentrated on culture characterisations. The principle of morphological studies includes the use of characteristics observable and measurable under a light microscope, such as shape of colony, presence of sheaths and envelopes, color of colonies, shape, differentiation and cell content, and the envelopes. Based on these criteria, a taxonomic classification is then devised. At the level of taxonomic distinction

between genera, the traditional systems of cyanophytes placed a high value on cell division patterns, colony formation and relationship to extracellular envelopes and sheaths. Cell shapes and dimensional differences were used largely to distinguish between species within each genus (Doers and Parker 1988). This method caused difficulties in their classification by introducing organisms with different cell organizations but similar cell arrangements to the same generic identity. The main problems met in applying morphological criteria in cyanophyte classification arise from the considerable variability in morphological features with environmental conditions (Komárek 1991).

AFLP markers have been used to scan genome-wide variations of strains, or closely related species, that have been impossible to resolve with morphological features or other molecular systematic characters. Therefore, AFLP has broad taxonomic applicability and have been used effectively in a variety of taxa including bacteria (Huys et al. 1996). AFLP analysis is based on selective amplification of DNA restriction fragments (Vos et al. 1995). It is technically similar to restriction fragment length polymorphism analysis, except that only a subset of the fragments are displayed and the number of fragments generated can be controlled by primer extensions. The advantage of AFLP over other techniques is that multiple bands are derived from all over the genome. This prevents over interpretation or misinterpretation due to point mutations or single-locus recombination, which may affect other genotypic characteristics. The main disadvantage of AFLP markers is that alleles are not easily recognized (Majer et al. 1998). PCR has proven to be successful in detecting genetic variation amongst plant-pathogenic fungi, as well as bacteria (Majer et al. 1996, Janssen et al. 1996). The utility, repeatability and efficiency of the AFLP technique are leading to broader application of this technique to the analysis of cyanobacteria populations (Janssen et al. 1996).

In an attempt to overcome problems with the current cyanobacterial taxonomy, which is based primarily on observed morphological characteristics, we have used amplified fragment length polymorphism (AFLP), a PCR-based fingerprinting method, which reveals variation around the whole genome by selectively amplifying a subset of restriction fragments for comparison.

Material and Methods

Chemicals, Strains and Culture Conditions

Analytical reagent grade chemicals were purchased from various commercial sources and were used without further purification. Unless otherwise stated, standard methods described in Sambrook et al. (1989) were used. *Microcystis aeruginosa* strains used in the study represented a wide variety of geographically unrelated strains (Table 3.1). Strains PCC7806 and PCC7813 were obtained from the Pasteur Institute Culture Collection, France; UV027 from the University of the Free State Culture Collection, South Africa; CCAP1450/1 was obtained from the Culture Collection of Algae and Protozoa, Institute of Freshwater Ecology, UK; NIES88, NIES89, NIES91, NIES99 from the National Institute for Environmental Studies, Japan; and SAG1 from the Pflanzen Physiologisches Institut, Universität Gottingen, Germany. All these strains were received as axenic, maintained as such and microscopically verified prior to further experiments. Unicellular strains UP01, UP03 and UP04 were collected by representatives of the Water Research Commission and Tswane Metro Council, respectively. Water samples were placed on ice in a darkened cooler during transport to the laboratory. Holding time for samples was less than 48 h in all cases. After the samples were vigorously spun with a vortex mixer to break the blooms, the

samples were diluted in sterilized, deionized and distilled water and placed in 100 ml of liquid BG-11 medium in 200-ml flasks.

Unicellular and axenic strains were maintained at a temperature of approximately 24 °C in liquid BG-11 nutrient medium containing 17.65 mM NaNO₃, 0.18 mM K₂HPO₄·3H₂O, 0.30 mM MgSO₄·7H₂O, 0.25 mM CaCl₂·2H₂O, 0.03 mM citric acid, 0.03 mM ferric ammonium citrate, 0.003 mM EDTA (ethylenediamine tetra-acetic acid, disodium magnesium), 0.19 mM Na₂CO₃, 0.05 mM H₃BO₃, 9.15 mM MnCl₂·4H₂O, 0.77 mM ZnSO₄·7H₂O, 1.61 mM Na₂MoO₄·2H₂O, 0.37 mM CuSO₄·5H₂O and 0.17 mM Co(NO₃)₂·6H₂O dissolved in 1L distilled water. Cultures were grown under constant light of approximately 60 μmol photons·m⁻²·s⁻¹ (PAR) at pH 8.0. The purity of cyanobacterial cultures was verified weekly by the absence of bacterial growth on TYG agar and in TYG broth (5.0 g tryptone (Difco); 2.5 g yeast extract (Difco); glucose, 1.0 g per liter) after incubation of two weeks at 26 °C.

Table 3.1 Different *Microcystis aeruginosa* strains used in the study and their origin.

Strain	Source	Origin
PCC7806	Pasteur Culture Collection, France	The Netherlands
PCC7813	Pasteur Culture Collection, France	Scotland
UV027	University of the Free State Culture Collection	ZA
NIES88	National Institute for Environmental Studies	Japan
NIES89	National Institute for Environmental Studies	Japan
NIES91	National Institute for Environmental Studies	Japan
NIES99	National Institute for Environmental Studies	Japan
NIES299	National Institute for Environmental Studies	Japan
SAG1	Pflanzen Physiologisches Institut, Universität Gottingen	Germany
CCAP 1450/1	Institute of Freshwater Ecology	UK
UP01	University of Pretoria Culture Collection	Rietvlei Dam, ZA
UP03	University of Pretoria Culture Collection	Rhoodeplaas Dam, ZA
UP04	University of Pretoria Culture Collection	Hartbeestpoort Dam, ZA

The final proof of purity was verified by microscopic examination. Cultures of *Microcystis aeruginosa* were harvested at the end of exponential growth phase (three weeks) by centrifugation at 6 000 g for 10 min at room temperature. The cultures were then freeze-dried and stored at -20°C . The strains isolated from the blooms were identified following the procedure as described by Komárek (1958).

DNA Extraction

Genomic DNA was extracted according to a modified method of Raeder and Broda (1985). The extraction buffer consisted of 200 mM Tris-HCl (pH 8.00), 150 mM NaCl, 25 mM EDTA, 0.5 % (w/v) SDS, 1 % (v/v) 2-mercaptoethanol, 1 % (w/v) Polyvinylpyrrolidone (PVP). A volume of extraction buffer were added to each 1 gram of freeze-dried culture, and homogenized in the presence of washed sand. The homogenate was placed at 60°C for 10 min. The homogenate was then centrifuged at 12 000 rpm for 15 min. The supernatant was removed and equal volumes of chloroform:phenol (1:1) was added, vortexed and centrifuged again at 12 000 rpm for 15 min. The upper layer was carefully removed. The DNA in the aqueous layer was precipitated with two volumes of ice-cold absolute ethanol and stored at -20°C for at least 1 h. Following a centrifugation step (12 000 rpm, 15 min), the resulting pellet was washed with 70 % ethanol (this step was repeated three times), and dried after removal of the liquid. The DNA was resuspended in distilled water and stored at -80°C .

DNA concentrations were determined by visualisation under UV light, on 1 % TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) agarose gels containing ethidium bromide (Sambrook et al., 1989), as well as through spectrophotometric measurements at absorbances of 260 and 280 nm, using a Beckman DU650 Spectrophotometer.

AFLP analysis

The AFLP procedure was carried out using the IRDyeTM Fluorescent AFLP® Kit (LI-COR Biosciences, Lincoln, USA) following the manufacturer's instructions. Two combinations of restriction endonucleases were used. For the combination *EcoR1/Mse1*, genomic DNA (75 ng) was incubated for 2 h at 37 °C with 1.25 U of *Mse1*, 1.25 U of *EcoR1*, 1 U of T4 DNA ligase, 40 pmol of *Mse1* adapters and 10 pmol *EcoR1* adapters. This reaction was done in a volume of 50 µl of restriction–ligase buffer containing 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 50 % (v/v) glycerol, 0.15 % (v/v) Triton X-100 and 200 ng/µl BSA. The reaction was terminated by heating at 70 °C for 15 min, and then placed on ice. For adaptor ligation, 25 µl of the Adapter mix, containing *Mse1* adapters and *EcoR1* adapters, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, and 50 mM K-acetate, and 1 U of T4 DNA ligase was added, and the reaction was incubated at 37 °C for 3 h. A 10-µl aliquot of the adapter-ligated DNA was diluted (1:10) with TE buffer (10 mM Tris-HCl pH 8.0, 1.0 mM EDTA) to serve as a template in the preselective amplification PCR. The remaining portion was used to verify that the digestion was complete.

The preselective PCR contained 2.5 µl of adapter-ligated DNA (diluted 1:10), 2.5 U of Taq DNA polymerase (Roche Molecular Biochemicals), 2.5 µl of 10 x PCR reaction buffer (Roche Molecular Biochemicals), 15 mM MgCl₂, 500 mM KCl and 100 µM of IRDye700TM-labeled *EcoR1* or IRDye800TM-labeled *EcoR1* and *Mse1* primers (containing dNTPs) with every selective nucleotide, in a total volume of 25.5 µl. The PCR program consisted of twenty cycles of 30s at 94 °C, 1 min at 56 °C, 1 min at 72 °C, and soaked at 4 °C. The selective PCR contained 2 µl of the diluted (1:10) product of the preselective PCR, 0.5 U of Taq DNA polymerase (Roche Molecular Biochemicals), 2 µl of 10 x Taq DNA polymerase

buffer (Roche Molecular Biochemicals), KCl and MgCl₂ as mentioned above, and 100 μM of IRDye700TM-labeled *EcoR1* or IRDye800TM-labeled *EcoR1* and *Mse1* primers (containing dNTPs) with every selective nucleotide, in a total volume of 11 μl. Eight primer pairs: *EcoR1*+*ACA*/*Mse1*+*CCA*, *EcoR1*+*ACA*/*Mse1*+*CCG*, *EcoR1*+*ACA*/*Mse1*+*CGG*, *EcoR1*+*ACA*/*Mse1*+*CAC*, *EcoR1*+*ACA*/*Mse1*+*CAG*, *EcoR1*+*ACA*/*Mse1*+*CTC*, *EcoR1*+*ACA*/*Mse1*+*CTG*, and *EcoR1*+*ACA*/*Mse1*+*CCT*, (LI-COR Biosciences, Lincoln, USA) were used for selective amplification. The first amplification cycle was carried out for 30s at 94 °C, 30s at 65 °C and 1 min at 72 °C. At each of the following 12 cycles, the annealing temperature was reduced by 0.7 °C per cycle. The last 23 cycles of annealing were carried out at 72 °C for 1 min; and then soaked at 4 °C. Each sample was diluted 1:1 with Blue Stop Solution (Li-COR Biosciences, Lincoln, USA), denatured for 3 min at 94 °C and placed on ice. AFLPs were fractionated on a 6.5 % KB^{PLUS} polyacrylamide gel in Tris-acetate-EDTA buffer (pH 7.5) using a LI-COR 4200 Automated DNA sequencer according to the manufacturers instructions.

Data analysis

AFLP fragments were manually scored as binary data with presence as "1" and absence as "0". Cluster analysis was performed on the similarity matrix employing the Unweighted Pair Group Method Using Arithmetic Means (UPGMA) algorithm (Sneath 1989) using the software programme PAUP 4.0.

Results

Fast screening of AFLP primer combinations

After screening 20 primer combinations on a subset of strains using either IRDye700TM-labeled *EcoR1* or IRDye800TM-labeled *EcoR1* primers, eight IRDye700TM-labeled *EcoR1* primer pairs were selected for analysis (Appendix A, Figure A1). The generated

fingerprints were evaluated for repeatability and overall clearness of the banding pattern. The number of informative fragments was also taken into account (Figure 3.1).

Genetic diversity as defined by AFLP fingerprinting

A total of 909 bands were amplified from the eight primer combinations, of which 665 were informative, 207 non-informative and 37 monomorphic (Appendix A, Tables A1 & A2), with an average of 83.12 polymorphic bands per primer combination. The genetic relationship among all the *Microcystis aeruginosa* strains based on the combination of data obtained with the eight primer combinations is represented in the dendrogram (Figure 3.2).

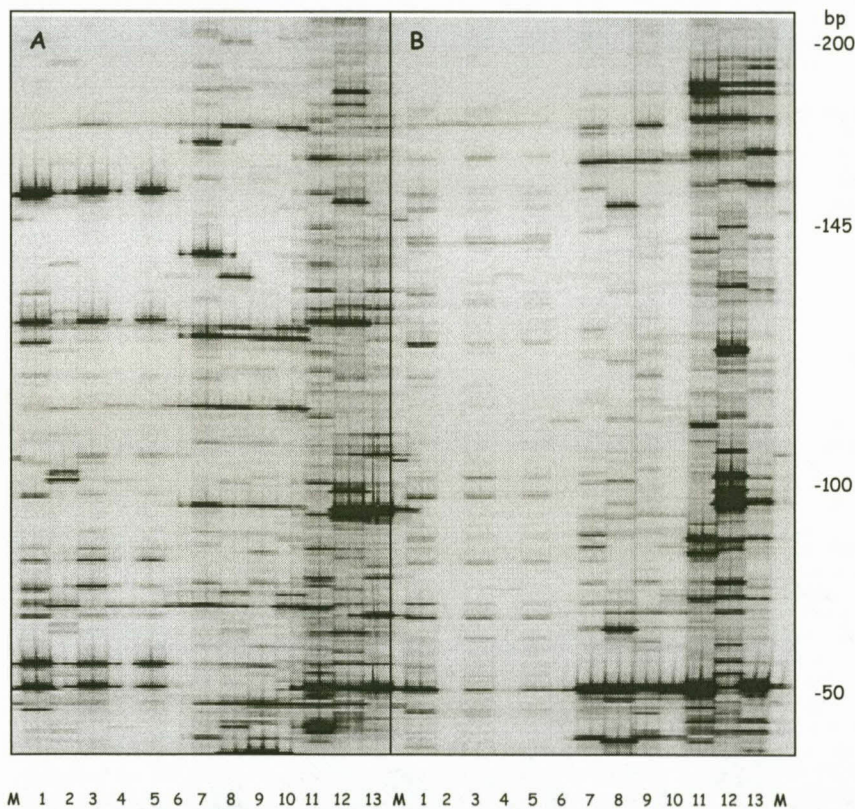


Figure 3.1 AFLP banding patterns generated using primer combinations *EcoR1+ACA/Mse1+CAC* (A) and *EcoR1+ACA/Mse1+CAG* (B). M = marker; 1 = NIES88; 2 = NIES89; 3 = NIES90; 4 = NIES99; 5 = NIES299; 6 = PC7806; 7 = CCAP1450/1; 8 = SAG1; 9 = UV027; 10 = PC7813; 11 = UP01; 12 = UP03; 13 = UP04.

The dendrogram consists of two clusters. The smaller cluster contains NIES90 and NIES299 in a grouping with NIES88 basal to this group. In the large cluster there are two groupings. The NIES strains group together, including PCC7806. The UP01, UP03 and UP04 strains fall into a group, and UV027, CCAP1450/1 and PCC7813 group together, with SAG1 basal to the group.

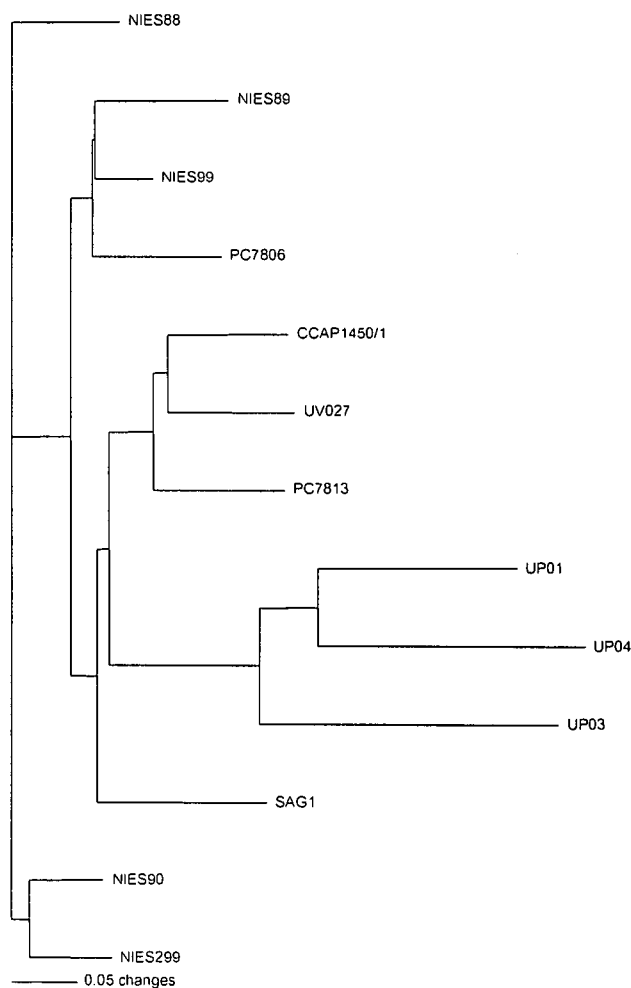


Figure 3.2 Combined cluster analysis derived from AFLP analysis of 13 *Microcystis aeruginosa* strains using eight AFLP primer combinations.

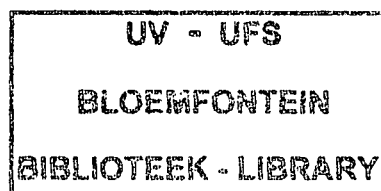
Discussion

AFLP fragments have been used to unravel cryptic genetic variation for a wide range of taxa, including plants (Mackill et al. 1996; Paul et al. 1997), fungi (Majer et al. 1996, 1998)

and bacteria (Huys et al. 1996), which have previously been impossible to resolve with morphological characters.

In the present study, complex AFLP banding patterns were obtained. Janssen et al. (1996) have showed that the choice of the restriction enzymes, and the length and composition of the selective nucleotide will determine the complexity of the final AFLP print. Primer selectivity is good for primers with one or two selective nucleotides in simple genomes such as fungi, bacteria and some plants, and still acceptable with primers having three selective nucleotides, but is lost with the addition of a fourth nucleotide (Vos et al. 1995). We used the EcoR1 (E) + 3 and Mse1 (E) +3 at the 3'-end of the primers on 13 *Microcystis* strains, and a total of 909 bands were amplified, constituting 73.2 % informative bands and 4.1 % monomorphic bands. The banding patterns of the UP-strains were also more complex than some of the other strains, which is quite expected, as these strains represent less cultured strains (e.g. "wild-type"), as it has been in culture for less than a year, unlike UV027, that has been in culture for decades.

In the dendrogram, the strains from Rietvlei (UP01) and Hartbeespoort Dams (UP04) group together and are thus genetically closer to each other, than to the strain from the Rhoodeplaat Dam (UP03). The Japanese strains (NIES88, NIES89, NIES90, NIES99, NIES299) also group separate from the other strains, with NIES90 and NIES299, genetically closest to each other. Interestingly, *Microcystis aeruginosa* strain PC7806 that originate from The Netherlands, also group within this group. *Microcystis aeruginosa* strains CCAP1450/1 (UK), UV027 (South Africa) and PC7813 group together, and are genetically closer to the UP-strains, than any of the other strains.



In view of the present study, AFLP analysis is useful for the identification of genetic diversity and analysis of population structure within *Microcystis aeruginosa*. The use of the AFLP fingerprinting method resulted in a high degree of discrimination and identification of *Microcystis aeruginosa* strains, and was found useful and practical. AFLPs seem to overcome the major pitfalls present in other PCR based methods, e.g. DAF or RAPD analysis, and appear to be as reproducible, heritable and intraspecific as RFLPs (Law et al. 1998). Additionally, AFLPs offer the opportunity to compare diversity of hyper-versus hypomethylated portions of the genome by comparing data from a restriction enzyme combination that is methylation-sensitive with a methylation-insensitive combination. Evidence indicates that DNA sequences are transcribed more readily when hypomethylated (Cedar 1988). Methylation may prove useful in future studies on the species.

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APPENDIX A

1. Amplified fragment length polymorphisms

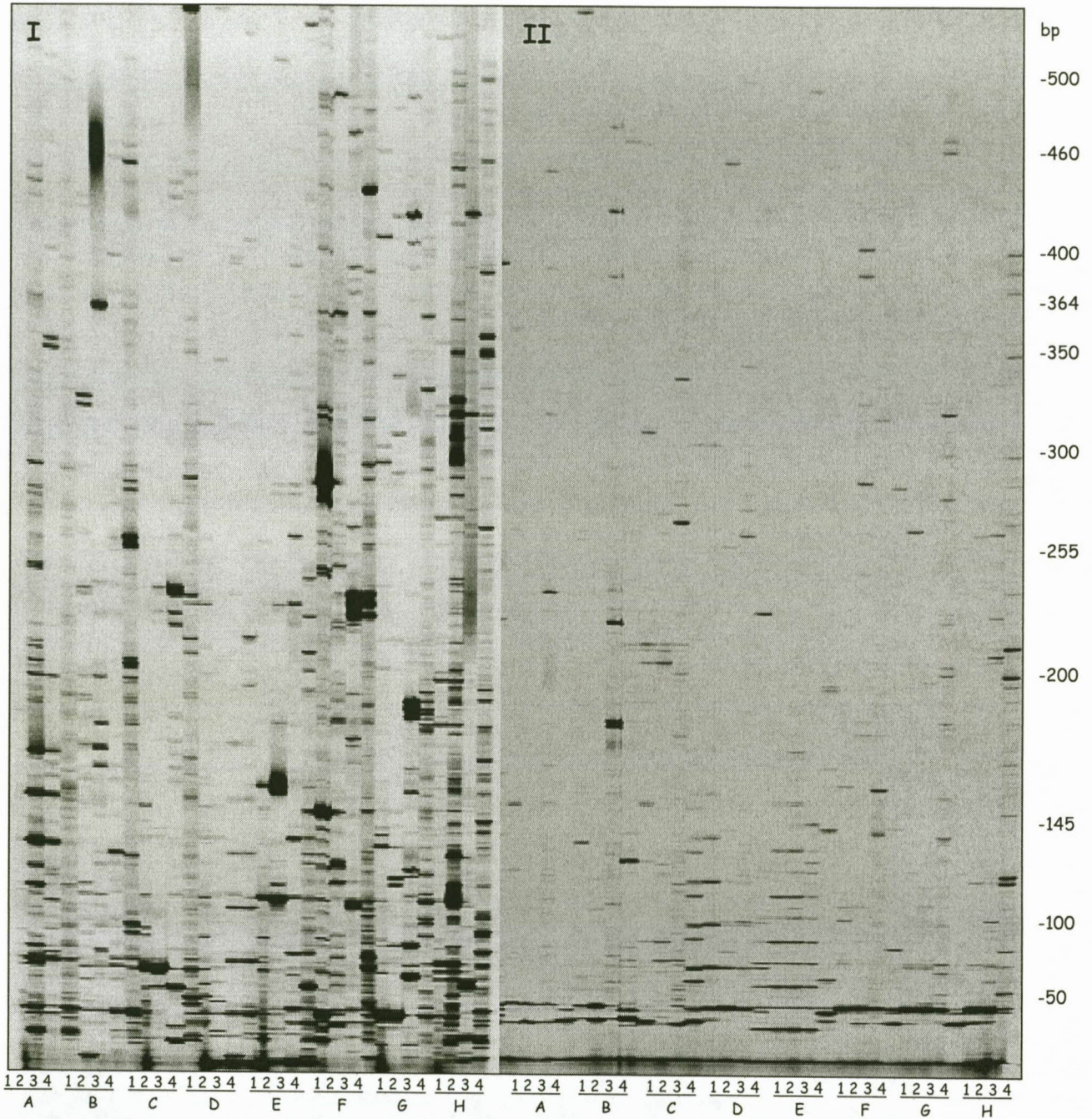


Figure A1. Primer screening with either IRDye700TM-labeled *EcoR1* (I) or IRDye800TM-labeled *EcoR1* (II) primers. (1) NIES90, (2) NIES99, (3) PCC7806, and (4) UP01; Primers Eco-ACA/Mse-CAC (A), Eco-ACA/Mse-CAG (B), Eco-ACA/Mse-CTC (C), Eco-ACA/Mse-CTG (D), Eco-ACA/Mse-CCT (E), Eco-ACA/Mse-CCA (F), Eco-ACA/Mse-CCG (G), and Eco-ACA/Mse-CGG (H).

Chapter IV

PCR-RFLP identification system for *Microcystis aeruginosa* utilising the *mcyB* gene sequence.

Introduction

The cyanobacterial division is worldwide immensely diverse with respect to form and habitat. Previously known as algae, over 2 000 species of cyanobacteria are classified using a few morphological characters like cell structure, photosynthetic pigment content and isozyme variation (Kato et al. 1992). Members of the coccoid genus *Microcystis* are toxigenic and are commonly presented in the mixed populations of cyanobacterial blooms. The cyclic heptapeptide hepatotoxin, microcystin, exhibits variable expression between strains of *Microcystis*, with over 60 isoforms possessing similar protein phosphatase inhibition activity. Economic losses related to freshwater cyanobacterial biotoxins are the result of contact with or consumption of water containing toxic cells, and these include the costs incurred from death of domestic animals, allergic and gastrointestinal problems in humans, and water treatment plants (Repavich et al. 1990).

Previously their taxonomy was based on the morphological characteristics of laboratory cultures, which is often considerably altered from the original morphology of environmental isolates. In an attempt to overcome analyses based on observed phenotypes, studies were undertaken making use of evolutionarily conserved genes in isolated cultures and field samples of the toxigenic and bloom-forming genus *Microcystis* (Komárek 1991). Several methods for the assessment of microcystins are available (Chorus and Bartram 1999), including HPLC, ELISA, or direct DNA testing to detect genetic sequences unique for the multigene cluster required for toxin synthesis (Tillett et al. 2000 & 2001, Nishizawa et al. 2000, Bittencourt-Oliveira 2003).

The *mcy* gene cluster assembly consists of 10 bidirectionally arranged genes, that reside in two operons (*mcyA-C* and *mcyD-J*) of *Microcystis aeruginosa*. The activities of these chromosomal gene products are primarily peptide synthetases (*mcyA-C, E, G*), polyketide synthases (*mcyD*, parts of *E* and *G*), and methylation (*mcyJ*), epimerization (*mcyF*),

dehydration (*mcyI*), and localization (*mcyH*), resulting in nonribosomal toxin synthesis. Disruption of some of these genes (*mcyA*, *B*, *D*, or *E*) resulted in no detectable toxin production (Nishiwaza et al. 2000). A *N*-methyltransferase (NMT) domain is usually associated with the *mcyA* and apparently with toxicity in strains. It was found that NMT-positive strains contained an open reading frame (OFR) of unknown function (*uma1*) at a conserved distance from *mcyC*. The results further suggested consistent linkage between *mcyC* and *uma1* in toxic and non-toxin strains. However, it was also found that *uma1* was not cotranscribed with the *mcyABC* cluster in non-toxic strains, suggesting that *mcyC* was also not transferred in non-toxic strains (Tillett et al. 2001).

The objective of this study was to determine the potential of using the *mcyB* gene sequence as means to differentiate taxonomically between a wide variety of geographically unrelated *Microcystis* strains.

Material and Methods

Chemicals

Reagent grade chemicals were purchased from various commercial sources and were used without further purification. Unless otherwise stated, standard methods described in Sambrook et al. (1989) were used.

Cyanobacterial strains, isolates, cultivation and lyophilization

Environmental samples

Waterbloom samples were collected from Rietvlei Dam, Gauteng, between September and December 2002, from Roodeplaat Dam, Gauteng, in September 2002; and from the Hartbeespoort Dam, North-West Province in January 2002 (Table 4.1). Within 24 h of collection, a 1 ml aliquot of each sample was examined microscopically at a magnification of 400x under phase contrast. The number of cells of each species was estimated, and a ratio of the component species was derived. Microscopically, the *Microcystis aeruginosa*

(UP01, UP03, UP04) or *M. wesenbergii* (UP02) cells were the most abundant in the blooms (Komárek et al. 1991; Steyn et al. 1975).

Axenic strains

Axenic *Microcystis aeruginosa* strains were obtained from the Culture Collections of the Institute Pasteur (PCC; Paris, France), the University of the Free State (UV, South Africa), the Algae and Protozoa, Institute of Freshwater Ecology (CCAP, United Kingdom), and the Pflanzen Physiologisches Institut (SAG; Universitat Gottingen, Germany) (Table 4.1).

Table 4.1 Table of *Microcystis aeruginosa* strains used in the study describing the origin of strains, as well as the reported toxicity.

Strain	Source	Toxicity
SAG1	Pflanzen Physiologisches Institut, Universität Gottingen, Germany	Toxin-producing
PCC7813	Pasteur Culture Collection, France	Toxin-producing
UV 027	University of the Free State Culture Collection, South Africa	Toxin-producing
CCAP1450/1	Institute of Freshwater Ecology, UK	Toxin-producing
UP01	University of Pretoria Culture Collection, Rietvlei Dam, ZA	Toxin-producing
UP02	University of Pretoria Culture Collection, Rietvlei Dam, ZA <i>Microcystis wesenbergii</i>	Unknown
UP03	University of Pretoria Culture Collection, Rhoodeplaas Dam, ZA	Toxin-producing
UP04	University of Pretoria Culture Collection, Hartbeestpoort Dam, ZA	Toxin-producing

Media and Culture

In the experiment liquid BG-11 culture medium was used and culture vessels were 200 ml Erlenmeyer flasks that contained 100 ml of medium. Cultivation took place in an incubation room with a temperature of 24 °C under continuous illumination of approximately 60 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at pH 8.0. The liquid BG-11 nutrient medium contained 17.65 mM NaNO_3 , 0.18 mM $\text{K}_2\text{HPO}_4\cdot 3\text{H}_2\text{O}$, 0.30 mM $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.25 mM $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 0.03 mM citric acid, 0.03 mM ferric ammonium citrate, 0.003 mM EDTA (ethylenediamine tetra-acetic acid, disodium magnesium), 0.19 mM Na_2CO_3 , 0.05 mM H_3BO_3 , 9.15 mM $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$, 0.77

mM ZnSO₄·7H₂O, 1.61 mM Na₂MoO₄·2H₂O, 0.37 mM CuSO₄·5H₂O and 0.17 mM Co(NO₃)₂·6H₂O made up to 1 L distilled water. All cultures were routinely screened for contamination by streaking samples on nutrient or yeast extract agar. Clonal cultures were established by picking apparently bacteria-free single colonies after growth from homogenized single cell suspensions. The strains isolated from the blooms were identified by the procedure of Komárek (1958).

DNA Extraction

The *Microcystis* cells in natural bloom samples were concentrated by centrifugation, washed, and subjected to a freeze-thaw treatment for PCR template preparation (Baker et al. 2002). All PCRs on natural strains described in this study were carried out after this treatment, by using approximately 1 000 cells per reaction. This method is simple and quick and has been proven effective with fresh bloom material, when most cells are intact. Genomic DNA was extracted according to a modified method of Raeder and Broda (1985). The extraction buffer consisted of 200 mM Tris-HCl (pH 8.00), 150 mM NaCl, 25 mM EDTA, 0.5 % (w/v) SDS, 1 % (v/v) 2-mercaptoethanol, 1 % (w/v) Polyvinylpyrrolidone (PVP). Extraction buffer (700 µl) was added to each 1 gram of freeze-dried culture, and homogenized in the presence of washed sand. Homogenisation was either done in a Bio 101 FastPrep machine at setting 2 for one minute or by hand in an eppendorf tube until the material was fully macerated. The homogenate was placed at 60 °C for 10 min. The homogenate was then centrifuged at 12 000 rpm for 15 min. The supernatant was removed and equal volumes of chloroform-phenol (1:1) were added, vortexed and centrifuged again at 12 000 rpm for 15 min. Phenol-chloroform purification were performed until no interface was visible. The upper layer was carefully removed. The DNA in the aqueous layer was precipitated with two volumes of ice-cold absolute ethanol and stored at

-20 °C for at least 1 h. Following a centrifugation step (12 000 rpm, 15 min), the resulting pellet was washed with 70 % ethanol (this step was repeated three times) to removed salts, and air dried after removal of the liquid. The DNA was resuspended in sterile, double-distilled water and stored at -80 °C.

DNA was separated in 1 % (w/v) agarose gels dissolved in 1x TAE buffer (50x TAE buffer: 2 M Tris-acetate and 0.05 M EDTA, pH 8) at 5 V/cm for 60 min (Sambrook et al. 1989). DNA fragments were visualised by the addition of ethidium bromide at 0.5 µg/ml to the melted gel. The DNA, with the chelated ethidium bromide, was viewed under UV light and photographed (Sambrook et al. 1989). As standard protocol, DNA samples were loaded with 6x loading buffer (15 % (w/v) ficoll and 0.25 % (w/v) bromophenol blue indicator dye). Bromophenol blue migrates though agarose gels at approximately the same rate as linear doublestranded DNA of 300 base pair in length (Sambrook et al. 1989).

Polymerase Chain Reaction (PCR)

The PCR reaction was optimized using the Taguchi method as described by Cobb and Clarkson (1994). The reactions were performed in a total volume of 12.5 µl containing 1.5 µl of DNA template (approximately 250 ng), 10 mM Tris-HCl (pH 9), 50 mM KCl, 0.1 % (v/v) Triton®X-100, 0.2 mM of each dATP, dTTP, dGTP and dCTP, 2 mM MgCl₂, 2.5 U Taq DNA Polymerase, all from Promega, and 0.8 pmol of any of two of the appropriate primers (Roche Molecular Biochemicals) (Table 4.2).

The PCR-reactions were performed on a GeneAmp PCR System 2400 (PE Biosystems) thermal cycler. The cycle consisted of an initial denaturation step of 5 minutes at 94 °C. Four subsequent 'touchdown' cycles of 5 cycles each, consisted of denaturation at 94 °C for 30 seconds, primer annealing at 45 °C, 42.5 °C, 40 °C and 38.5 °C for 30 seconds, and

strand elongation at 72 °C for 45 seconds. An additional 35 similar cycles were performed with an annealing temperature of 45 °C. To complete all strands, the reactions were incubated at 72 °C for 7 minutes.

The products were analysed by agarose gel electrophoresis through horizontal slab gels of 1 % agarose (Techcomp Ltd.) dissolved in 1x TAE buffer (Tris-acetate-EDTA buffer (pH 7.5) containing 0.15 µg/ml ethidium bromide (Sigma). The generated fragments were separated at 85 mV for 1 h, visualized under UV-light and photographed.

PCR Cleanup

Fragments generated by the various PCR-reactions were isolated with the High Pure PCR Product Purification Kit (Roche) for further experiments. The total volume of the PCR reaction was adjusted to 100 µl with 1x TE buffer (pH 8.0). Binding Buffer (3 M guanidine-thiocyanite, 10 mM Tris-HCl, 5 % EtOH (v/v), pH 6.6) up to a volume of 600 µl was added, thoroughly mixed, applied to a High Pure Filter Tube (Roche) and then centrifuged at 10 000 rpm for 1 minute. The flow-through was discarded, 500 µl Wash Buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5, 80 % EtOH (v/v)) added and centrifuged as above. The washing step was repeated with 200 µl Wash Buffer and the flow-through discarded. The tube was centrifuged for an additional 1 minute at 10 000 rpm to remove residual ethanol. The High Pure Filter Tube was transferred to a clean centrifuge tube, 50 µl Elution Buffer (1 mM Tris-HCl, pH 8.5) added, and centrifuged as above.

Table 4.2 Primers used in the study describing sequence, orientation and melting temperatures.

Primer	Sequence (5'–3')	Orientation	T _m
Tox 1P	CGATTGTTACTGATACTCGCC	Forward	57.9 °C
Tox 3P	GGAGAATCTTTCATGGCAGAC	Forward	62.4 °C
Tox 7P	CCTCAGACAATCAACGGTTAG	Forward	53.7 °C
Tox 10P	GCCTAATATAGAGCCATTGCC	Forward	59.8 °C
Tox 1M	TAAGCGGGCAGTTCCTGC	Reverse	58.2 °C
Tox 2M	CCAATCCCTATCTAACACAGTACCTCGG	Reverse	65.1 °C
Tox 3M	CGTGGATAATAGTACGGGTTTC	Reverse	58.4 °C
Tox 4M	CCAGTGGGTTAATTGAGTCAG	Reverse	57.9 °C

Fragments generated with primer pairs Tox 3P/2M, Tox 1P/1M, Tox 7P/3M and Tox 10P/4M from PCC7813 and UV027 were subsequently cloned into the pGem®T-Easy vector (Promega), transformed into *E. coli* cells (JM 109; > 10⁸ cfu/μl) and blue/white screening was carried out in order to determine transformation efficiency. The cells were resuspended in 100 μl LB-media (Luria Bertrani; 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L Difco agar, pH 7.0, Sambrook et al. 1989), plated out on LB/IPTG (0.5 mM isopropylthio-β-D-galactoside)/X-gal (80 μg/μl 5-bromo-4-chloro-3-indolyl-β-D-galactoside) plates and incubated overnight at 37 °C.

Single white colonies were used to inoculate 5 ml LB-media containing 2.5 mg ampicillin and incubated at 37 °C overnight with shaking. The cells were centrifuged at 10 000 rpm for 2 minutes and the supernatant discarded. The pellet was resuspended in 300 μl STET buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 5 % (v/v) Triton X-100). Lysozyme (0.15 mg) was added and the cells incubated at room temperature for 5 minutes. To facilitate lyses the cells were then incubated at 95 °C for 1 minute and centrifuged at 14 000 rpm for 15 minutes at 4 °C. The pellet was removed, 5 % (w/v) CTAB (N-cetyl-N-N-N-

trimethylammonium bromide) was added to the supernatant and centrifuged at 14 000 rpm for 5 minutes.

The supernatant was discarded, the pellet resuspended in 300 μ l 1.2 M NaCl and 750 μ l cold absolute ethanol added. This mixture was then centrifuged at 14 000 rpm for 10 minutes at 4 °C. The supernatant was discarded, 1 ml cold 70 % ethanol added and centrifuged at 14 000 rpm for 2 minutes at 4 °C. The supernatant was removed, the pellet vacuum-dried in a SpeedVac Concentrator SVC 100H (Savant) and resuspended in 30 – 50 μ l ddH₂O.

The inserts were verified by restriction analysis with approximately 1 μ g plasmid DNA, 5 U *Eco*RI, 50 mM Tris-HCl, 10 mM MgAc₂, 10 mM MgCl₂, 66 mM KAc, 100 mM NaCl and 0.5 mM DDT at pH 7.5 all from Roche. The entire reaction was loaded onto a 1 % TAE agarose gel (Techcomp Ltd.) containing 0.15 mg ethidium bromide (Sigma), separated at 85 mV and visualized under UV-light.

Sequencing

Sequencing of the fragments were performed using the ABI Prism® Big Dye® Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). Sequencing reactions were performed according to the manufacturers' instructions and contained 200 – 500 ng plasmid template and 3.2 – 5 pmol of the appropriate primer. Reactions were cycled on a GeneAmp PCR System 2400 (PE Biosystems) thermal cycler and the products precipitated with NaOAc and EtOH according to the manufacturers' instructions. Samples were dried in

a SpeedVac Concentrator SVC 100H (Savant) and resuspended in formamide and 25 mM EDTA buffer.

Approximately 30 – 50 % of each reaction were loaded onto a 4 % acrylamide gel, separated at 1.6 kV for 7 h at 51°C and data collected on an ABI Prism 377 DNA Sequencer (PE Biosystems). The data were analysed using Sequencing Analysis V 3.3. Sequences were reverse-complemented and compared by using Sequence Navigator V 1.0.1 and assembled using AutoAssembler V 1.4.0 and DNAssist V 1.02. Analyzed sequences were used to search the Genbank Database (<http://www.ncbi.nlm.nih.gov/>).

Composition of the genetic map

The obtained sequences (Appendix B, Figure B1) were utilized and imported into the Webcutter 2.0 software programme (<http://www.firstmarket.com/cgi-bin/cutter>) to obtain potential nucleotide restriction sites that can be used to differentiate between the strains (Table 4.3; Appendix B, Figure B2).

PCR of *mcyB* fragments for restriction analyses.

Amplification reactions were carried out on a Perkin Elmer GeneAmp® PCR system 9700 (PE Applied Biosystems) using touchdown PCR reactions in 50 µl. The reactions consist of 50 ng/µl DNA template, 50 mM MgCl₂, 2 mM dNTPs, 10 pmol forward primer (Tox 1P, Tox 3P, Tox 7P or Tox 10P, respectively), 10 pmol reverse primer (Tox 1M, Tox 2M, Tox 3M or Tox 4M, respectively), 1 U Promega DNA Taq polymerase enzyme and 10x PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1 % (v/v) Triton®X-100 — magnesium free). The PCR cycles were as follows: five cycles of 30s at 94°C, 30s at 65 °C and 45s at 72 °C; five cycles of 30s at 94°C, 30s at 65 °C and 45s at 72 °C; five cycles of 30s at 94°C, 30s at 62.5 °C and

45s at 72 °C; five cycles of 30s at 94°C, 30s at 60 °C and 45s at 72 °C; twenty cycles of 30s at 94°C, 30s at 58 °C and 45s at 72 °C, and a final elongation step of 7 min at 72 °C.

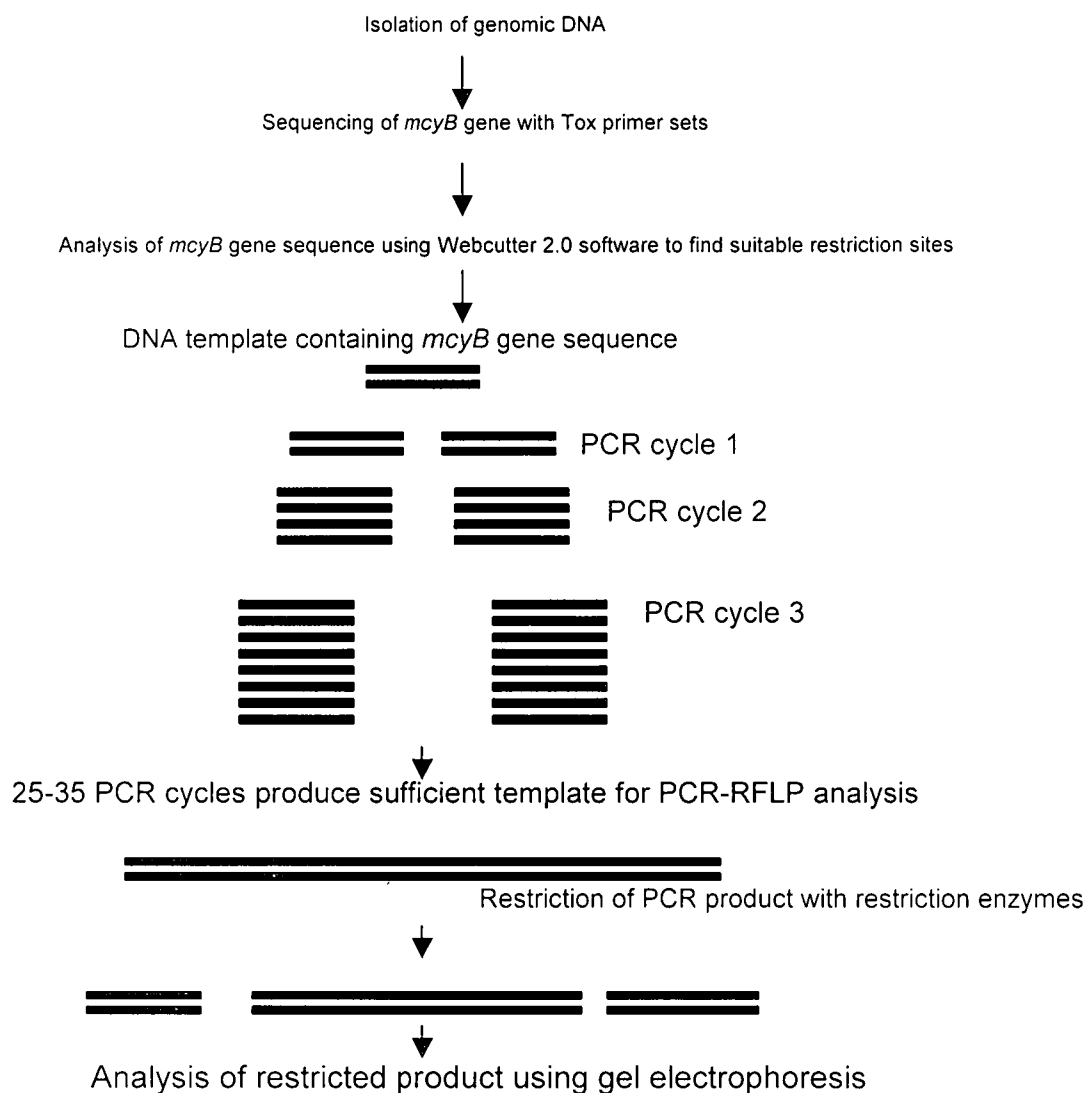


Figure 4.1 Representation to demonstrate the process involved in the regeneration of PCR-RFLP polymorphic fragments using the *mcyB* gene sequence.

Restriction of PCR fragments.

The obtained cloned fragments were restricted with approximately 1 µg plasmid DNA, restriction enzyme (5 U *EcoRI*, Buffer H; 5 U *AluI*, Buffer A; 5 U *RsaI*, Buffer L; 5 U *Sau3AI*,

Buffer A), 10x Buffer (as recommended by the supplier, Buffer H, Buffer L or Buffer A) at 37°C for 1-3 h until completion of the reaction. The entire reaction was loaded onto a 2 % TAE agarose gel (Techcomp Ltd.) containing 0.15 mg ethidium bromide (Sigma), separated at 85 mV and visualized under UV-light (Herolab UVT-28 M), or separated on 7.5 % non-denaturing polyacrylamide gels (3.75 % (v/v) FMC® Long Ranger Gel solution, 1x TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0), 1 % (w/v) APS, 0.004 % (v/v) TEMED) in 1x TBE buffer at 26.6 V/cm for 1-1.5h. After separation the resulting products were detected by staining with (1:10 000) Sybr®GreenI Nucleic Acid Gel Stain (Roche). The resulting products were visualized by using UV illumination. Marker III (λ DNA restricted with *EcoRI* and *HindIII*) was included as the molecular size marker in all electrophoresis profiles.

Results

Using the primer pairs Tox 3P/2M, Tox 1P/1M, Tox 7P/3M and Tox 10P/4M, the *mcvB* gene from PCC7813 and UV027 were sequenced, resulting in fragments of 2174 and 2170 base pairs in size, respectively (Appendix B, Figure B1). The obtained sequences were analyzed using nucleotide BLASTN annotation of the Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990) at <http://www.ncbi.nlm.nih.gov/BLAST>. The sequence alignment indicated high homology to other published sequences in GenBank (AY034601 for PCC7813 and AY034602 for UV027; e-value = 0.0). Upon analysis of the sequences it was obvious that there are several base differences between the sequences of the two strains, which led us to investigate the potential of using differences in restriction sites, and thus insertions/deletions (indels) in nucleotide sequence to discriminate between the strains.

The sequences were submitted to the Webcutter 2.0 software programme (<http://www.firstmarket.com/cgi-bin/cutter>) to obtain all the available restriction sites present in the strains (Appendix B, Figure B2.a and B2.b). A vast number of restriction sites were identified with differences, for example *Acil* (6; 26, 550, 800, 928, 1459, 1947 bp) in PCC7813 vs *Acil* (5; 26, 799, 927, 1458 bp) in UV027. Unique restriction sites have also been identified, e.g. *Mval* (1 fragment, 276 bp) and *Eco130I* (1, 1101 bp) in UV027; and *Aocl* (1, 652 bp), *Bse21I* (1, 652 bp) and *BspHI* (1, 873 bp) in PCC7813, to name a few (Table 4.3, Appendix B).

Table 4.3 List of unique restriction enzyme sites obtained after analysis of the *mcyB* gene sequences from strains PCC7813 and UV027.

Enzyme name	No. cuts	Position of sites	Recognition sequence
PCC7813			
<i>Acil</i>	6	26 550 800 928 1459 1947	ccgc
<i>AclWI</i>	1	1661	ggatc
<i>AcsI</i>	6	367 427 998 1158 1781 1859	r/aatty
<i>AfaI</i>	2	791 1649	gt/ac
<i>AluI</i>	5	1444 1531 1642 1737 2064	ag/ct
<i>Alw26I</i>	2	938 2036	gtctc
<i>AlwI</i>	1	1661	ggatc
<i>AocI</i>	1	652	cc/tnagg
<i>AsnI</i>	2	914 1836	at/taat
<i>AspLEI</i>	3	296 392 759	gcg/c
<i>AspS9I</i>	4	414 1558 1696 1960	g/gncc
<i>AsuI</i>	4	414 1558 1696 1960	g/gncc
<i>AvaII</i>	2	414 1558	g/gwcc
<i>BbvI</i>	6	396 619 931 1277 1448 1945	gcagc
<i>BfaI</i>	5	787 1103 1238 1597 1920	c/tag
<i>Bme18I</i>	2	414 1558	g/gwcc
<i>BpmI</i>	2	141 672	ctggag
<i>BsaJI</i>	5	274 1057 1391 1515 1634	c/cnngg
<i>BselI</i>	3	673 1696 2039	actgg
<i>Bse21I</i>	1	652	cc/tnagg
<i>BseDI</i>	5	274 1057 1391 1515 1634	c/cnngg
<i>BseNI</i>	3	673 1696 2039	actgg
<i>BsmAI</i>	2	938 2036	gtctc
<i>BsmFI</i>	1	555	gggac
<i>Bsp143I</i>	1	1656	/gatc
<i>BspHI</i>	1	873	t/catga
<i>BspMI</i>	3	516 748 1979	acctgc
<i>BsrDI</i>	1	925	gcaatg
<i>BsrI</i>	3	673 1696 2039	actgg

BsrSI	3	673 1696 2039	actgg
BssT1I	1	274	c/cwwgg
Bst71I	6	396 619 931 1277 1448 1945	gcagc
BstF5I	4	75 463 1153 1272	ggatg
BstX2I	1	1656	r/gatcy
BstXI	1	33	ccannnnn/ntgg
BstYI	1	1656	r/gatcy
Bsu36I	1	652	cc/tnagg
BsuRI	3	796 1698 1961	gg/cc
CfoI	3	296 392 759	gcg/c
Cfr13I	4	414 1558 1696 1960	g/gncc
CviJI	16	693 727 796 1027 1241 1444 1531 1633 1642 1698 1737 1747 1788 1843 1961 2064	rg/cy
CvnI	1	652	cc/tnagg
DpnI	1	1658	ga/tc
DpnII	1	1656	/gatc
Eco47I	2	414 1558	g/gwcc
Eco57I	1	688	ctgaag
Eco81I	1	652	cc/tnagg
EcoT14I	1	274	c/cwwgg
ErhI	1	274	c/cwwgg
FokI	4	75 463 1153 1272	ggatg
GsuI	2	141 672	ctggag
HaeIII	3	796 1698 1961	gg/cc
HgaI	5	196 355 553 1885 1942	gacgc
HgiEI	2	414 1558	g/gwcc
HhaI	3	296 392 759	gcg/c
Hin6I	3	294 390 757	g/cgc
HinPII	3	294 390 757	g/cgc
HincII	1	355	gty/rac
HindII	1	355	gty/rac
HinfI	17	37 100 210 402 639 665 807 832 932 946 1123 1218 1452 1665 1772 1927 1982	g/antc
HphI	2	548 2059	ggtga
HspAI	3	294 390 757	g/cgc
Kzo9I	1	1656	/gatc
MaeI	5	787 1103 1238 1597 1920	c/tag
MaeIII	9	193 338 575 852 968 1211 1305 1326 1702	/gtnac
MboI	1	1656	/gatc
MboII	4	541 814 1484 1686	gaaga
MflI	1	1656	r/gatcy
MnlI	10	52 142 244 435 511 657 874 1168 1396 1438	cctc
MspR9I	3	1058 1516 1635	cc/ngg
MwoI	4	252 802 1639 1929	gcnnnnn/nngc
NdeII	1	1656	/gatc
PalI	3	796 1698 1961	gg/cc
PleI	8	41 214 643 669 836 936 1776 1986	gagtc
RcaI	1	873	t/catga
Sau3AI	1	1656	/gatc
Sau96I	4	414 1558 1696 1960	g/gncc
ScrFI	3	1058 1516 1635	cc/ngg
SfaNI	3	76 391 1878	gcac
SinI	2	414 1558	g/gwcc
Sse9I	20	128 146 248 367 427 976 998 1095 1158 1173 1333 1426 1489 1781 1837 1859 1864 1891 1909 2041	/aatt
StyI	1	274	c/cwwgg

Tsp509I	20	128 146 248 367 427 976 998 1095	/aatt
		1158 1173 1333 1426 1489 1781	
		1837 1859 1864 1891 1909 2041	
TspEI	20	128 146 248 367 427 976 998 1095	/aatt
		1158 1173 1333 1426 1489 1781	
		1837 1859 1864 1891 1909 2041	
XhoII	1	1656	r/gatcy
UV027			
AciI	5	26 550 799 927 1458	ccgc
AclWI	2	1315 1660	ggatc
AfeI	2	1590 1677	agc/gct
AluI	4	1443 1530 1641 1800	ag/ct
Alw26I	1	937	gtctc
AlwI	2	1315 1660	ggatc
Aor51HI	2	1590 1677	agc/gct
AsnI	2	673 913	at/taat
AspLEI	5	296 392 758 1591 1678	gcg/c
AspS9I	2	414 1759	g/gncc
AsuI	2	414 1759	g/gncc
AvaII	1	414	g/gwcc
AvrII	1	1101	c/ctagg
BbsI	1	1484	gaagac
BbvI	5	396 586 619 930 1447	gcagc
BfaI	7	786 1102 1201 1237 1560 1596	c/tag
		2049	
BglII	1	740	a/gatct
BlnI	1	1101	c/ctagg
Bme18I	1	414	g/gwcc
BpmI	1	141	ctggag
BsaJI	6	274 1056 1101 1314 1514 1633	c/cnngg
BsaMI	1	1977	gaatgc
BseI	1	1759	actgg
BseDI	6	274 1056 1101 1314 1514 1633	c/cnngg
BseNI	1	1759	actgg
BsmAI	1	937	gtctc
BsmI	1	1977	gaatgc
Bsp143I	4	740 1310 1655 1967	/gatc
BspMI	1	516	acctgc
BsrDI	1	924	gcaatg
BsrI	1	1759	actgg
BsrSI	1	1759	actgg
BssT1I	1	1101	c/cwgg
Bst2UI	1	276	cc/wgg
Bst71I	5	396 586 619 930 1447	gcagc
BstF5I	5	75 463 1152 1271 1707	ggatg
BstH2I	2	1592 1679	rgcgc/y
BstNI	1	276	cc/wgg
BstOI	1	276	cc/wgg
BstX2I	2	740 1655	r/gatcy
BstXI	2	33 657	ccannnnn/ntgg
BstYI	2	740 1655	r/gatcy
BsuRI	2	795 1761	gg/cc
CfoI	5	296 392 758 1591 1678	gcg/c
Cfr13I	2	414 1759	g/gncc
CviJI	14	693 726 795 1026 1240 1395 1443	rg/cy
		1530 1632 1641 1761 1800 1851	
		2058	
DpnI	4	742 1312 1657 1969	ga/tc

DpnII	4	740 1310 1655 1967	/gatc
Eco32I	1	1688	gat/atc
Eco47I	1	414	g/gwcc
Eco47III	2	1590 1677	agc/gct
Eco57I	2	688 1968	ctgaag
EcoRII	1	274	/ccwgg
EcoRV	1	1688	gat/atc
EcoT14I	1	1101	c/cwwgg
ErhI	1	1101	c/cwwgg
FauNDI	1	1934	ca/tatg
FokI	5	75 463 1152 1271 1707	ggatg
GsuI	1	141	ctggag
HaeII	2	1592 1679	rgcgc/y
HaeIII	2	795 1761	gg/cc
HgaI	3	196 355 553	gacgc
HgiEI	1	414	g/gwcc
HhaI	5	296 392 758 1591 1678	gcg/c
Hin6I	5	294 390 756 1589 1676	g/cgc
HinPII	5	294 390 756 1589 1676	g/cgc
HincII	2	355 1720	gty/rac
HindII	2	355 1720	gty/rac
HinfI	16	37 100 210 402 639 665 806 831 931 945 1122 1217 1451 1698 1728 1835	g/antc
HpaI	1	1720	gtt/aac
HspAI	5	294 390 756 1589 1676	g/cgc
Kzo9I	4	740 1310 1655 1967	/gatc
MaeI	7	786 1102 1201 1237 1560 1596 2049	c/tag
MaeIII	10	193 338 575 851 967 1210 1304 1325 1765 2117	/gtnac
MboI	4	740 1310 1655 1967	/gatc
MboII	6	541 813 1438 1483 1749 1969	gaaga
MflI	2	740 1655	r/gatcy
MnlI	12	52 142 435 511 657 1167 1317 1395 1694 1915 1963 2143	cctc
MslI	1	649	caynn/nnrtg
MspR9I	4	276 1057 1515 1634	cc/ngg
Mval269I	1	1977	gaatgc
MwoI	3	252 801 1638	gcnnnnn/nngc
NdeI	1	1934	ca/tatg
NdeII	4	740 1310 1655 1967	/gatc
PalI	2	795 1761	gg/cc
PleI	7	41 214 643 669 835 935 1839	gagtc
Sau3AI	4	740 1310 1655 1967	/gatc
Sau96I	2	414 1759	g/gncc
ScrFI	4	276 1057 1515 1634	cc/ngg
SfaNI	1	76	gcate
SinI	1	414	g/gwcc
Sse9I	19	128 146 248 367 427 670 975 997 1094 1157 1172 1300 1332 1425 1488 1844 1915 1981 2101	/aatt
SspI	3	1904 1955 2146	aat/att
StyI	1	1101	c/cwwgg
Tsp509I	19	128 146 248 367 427 670 975 997 1094 1157 1172 1300 1332 1425 1488 1844 1915 1981 2101	/aatt
TspEI	19	128 146 248 367 427 670 975 997 1094 1157 1172 1300 1332 1425 1488 1844 1915 1981 2101	/aatt
XbaI	1	1559	t/ctaga

PCR fragments were then amplified to verify differences using available restriction enzymes (Figure 4.2).

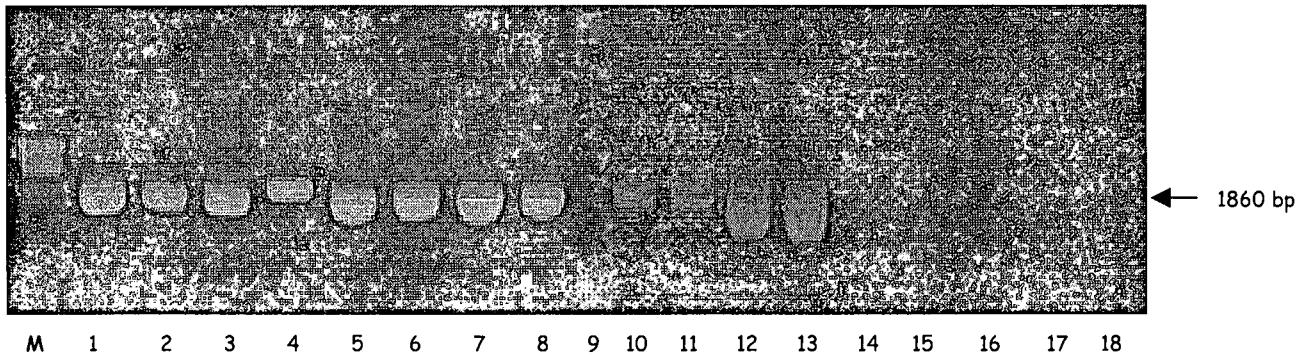


Figure 4.2 PCR fragments obtained after amplification of *Microcystis aeruginosa* strains with primer pair Tox 10P/Tox 4M. M = Marker III (lambda DNA restricted with *EcoRI* and *HindIII*), 1 = PCC7813 (0.2 μ l DNA); 2 = SAG1 (0.2 μ l DNA); 3 = CCAP1450/1 (0.2 μ l DNA), 4 = UV027 (0.2 μ l DNA), 5 = PCC7813 (0.4 μ l DNA); 6 = PCC7813 (0.5 μ l DNA); 7 = SAG1 (1 μ l DNA), 8 = SAG1 (0.8 μ l DNA); 9 = water control; 10 = UP01 (0.2 μ l DNA); 11 = UP03 (0.2 μ l DNA); 12 = UP03 (1 μ l DNA); 13 = UP04 (1 μ l DNA); 14 = UP02 (0.2 μ l); 15 = UP02 (0.2 μ l); 16 = UP02 (1 μ l); 17 = UP02 (1 μ l); 18 = water control.

PCR amplification with primer pair Tox3P/Tox 2M resulted in a fragment with approximate size of 1850 bp in all the *M. aeruginosa* strains, but not in *M. wesenbergii* (Figure 4.2).

These fragments were then restricted with *EcoRI*, *RsaI*, *Sau3AI*, and *AluI*.

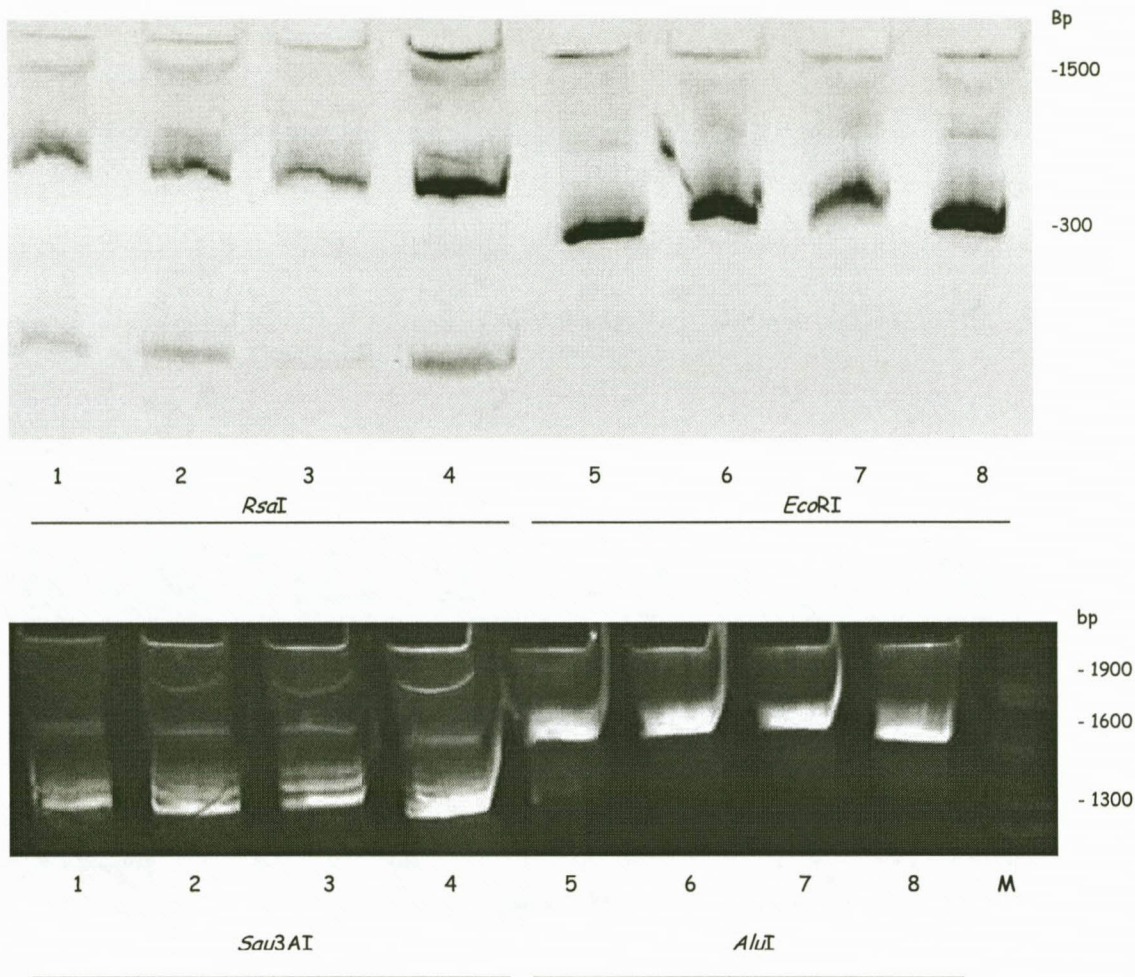


Figure 4.3 Polymorphic loci obtained after digestion of the *mcyB* fragments with different restriction enzymes (*RsaI*, *EcoRI*, *Sau3AI*, *AluI*). The *mcyB* gene fragments were obtained after amplification with primer pair Tox 3P/Tox 2M. 1 & 5 = PCC7813; 2 & 6 = SAG1; 3 & 7 = CCAP1450/1; 4 & 8 = UV027; M = 100 bp ladder.

Restriction with *EcoRI* and *AluI* resulted in one fragment in all the strains, while *RsaI* gave two fragments (Table 4.4, Figure 4.3). Restriction with *Sau3AI* gave four bands in strain PCC7813, but five in strains SAG1, CCAP1450/1, and UV027, respectively (Table 4.4, Figure 4.3).

Table 4.4 Number of indels observed after restriction of the *mcyB* gene with selected enzymes of *Microcystis aeruginosa* strains.

<i>Microcystis</i> strains	<i>RsaI</i>	<i>EcoRI</i>	<i>Sau3AI</i>	<i>AluI</i>
PCC7813	2	1	4	1 ^a
SAG1	2	1	5	1 ^b
CCAP1450/1	2	1	5	1
UV027	2	1	5	1
UP01	2	1	5	1
UP02	x*	x	x	x
UP03	2	1	5	1
UP04	2	1	5	1

*= no *mcyB* fragment present for endo-nuclease restriction; a = 5 indels expected; b = 4 indels expected.

Discussion

Ambiguities exist in the cyanobacterial taxonomy and these are due to variability in expression, minimal morphological and developmental characteristics that could be used for identification, making the classification to the genus or species level difficult (Doers and Parker 1988, Rippka 1988). Cyanobacterial identification depends largely on the taxonomic parameters that are applied. These parameters can differ in emphasis relating to cell size, shape, buoyancy and toxicity (Rippka and Herdman 1992). Among botanical taxonomists, there is a suspicion that too many species have been described over the years as many descriptions are based on a single character difference (e.g. such as the presence or absence of sheath or slight deviation in cell dimensions or cell forms) (Anagnostidis and Komárek 1990). The problem of the species morphological variability has prompted Drouet (1968) to revise the taxonomy profoundly. His basic idea was that there existed ecophenes, where organisms sharing the same genotype but expressing distinct morphologies under the influence of environmental factors. He drastically reduced the number of species down to 62. Later, DNA-DNA hybridizations showed that taxa placed by Drouet (1968) in the same species were genotypically different (Stam and Venema 1975). Komárek and

Anagnostidis (1989) stated that the features of more than 50 % of the strains in collections do not correspond to the diagnoses of the taxa to which they are assigned. Thus there is a real need for further characterization of the numerous cyanobacterial cultures available worldwide.

Pan et al. (2002) and do Carmo Bittencourt-Olivera (2003) proposed to use a PCR-based method utilising the *mcyB* gene to confirm the presence or absence of biotoxin producing organisms in raw water. They aimed at improving water management strategies, working on the premise that the detection of the genus will alert water purification facilities to scale-up on purification procedures. When PCR-based methods are used for diagnostic purposes, only small amounts of DNA are required for the analysis (Venter and Botha 2000). Here we used PCR based technology and the *mcyB* gene sequence not only to confirm the presence of *Microcystis aeruginosa*, but also to identify specific species and strains of *Microcystis*, by making use of the uniqueness of genomic DNA with regard to their specific restriction endo-nuclease restriction sites.

In the present study, the gene sequence of *mcyB* was confirmed and the sequence homology was verified through alignment with published sequences using the BLASTN algorithm (Altschul et al. 1997) in GenBank. The *mcyB* gene proved useful to discern between *M. aeruginosa* and *M. wesenbergii*. The *mcy* gene cluster present in *M. aeruginosa* had been shown to be responsible for toxin production, as disruption of some of the genes within the cluster, including *mcyB*, resulted in no detectable toxin production (Nishiwaza et al. 2000). The absence of the *mcyB* fragment in the *M. wesenbergii* sample may be indicative of non-toxicity. The unique restriction sites were obtained using Webcutter 2.0 software programme to obtain unique restriction sites. More than 70 differences in the restriction sites were obtained between the two strains, namely PCC7813

and UV027, clearly indicating the potential of using PCR-RFLPs as a low-cost and effective way of identifying *Microcystis aeruginosa* strains.

From the results obtained in the present study, it is evident that PCR-based technology (e.g. PCR amplifying the *mcy* gene cluster or PCR-PFLP thereof) has great potential for fast screening and detection of *Microcystis* toxic strains in waterbodies. This will potentially ease current water purification management strategies through early detection prior to occurrence of undesirable 'blooms', as well as proven genetic information that can be used in attempts to reconstruct the evolution of organisms and improve their taxonomy. Most DNA tests have relied on Southern blots of cyanobacterial samples (Meißner et al. 1996, Dittman et al. 1997, Nishizawa et al. 1999, 2000) or degenerate PCR amplification of rRNA (Neiland et al. 1999). In the work presented here, the amplification of the *mcyB* gene by PCR from DNA isolated from axenic cultures and field samples has proven to be a sensitive means to differentiate taxonomically between a wide variety of geographically unrelated *Microcystis aeruginosa* axenic and environmental strains, as well as the detection of toxic strains.

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Appendix B

PCC7813	1	ATGGCAGACACAAAAAATCAACCCGCCAAAAATGTGGAGTCTATTTATCCTCTTTCCCCCATGC 64
UV027	1	ATGGCAGACACAAAAAATCAACCCGCCAAAAATGTGGAGTCTATTTATCCTCTTTCCCCCATGC 64
<i>mcyB</i>	1	ATGGCAGACACAAAAAATCAACCCGCCAAAAATGTGGAGTCTATTTATCCTCTTTCCCCCATGC 64
<i>dnaN</i>	1	ATGGCAGACACAAAAAATCAACCCGCCAAAAATGTGGAGTCTATTTATCCTCTTTCCCCCATGC 64
PCC7813	65	AGGAAGGGATGCTCTTTCATAGTCTTTATACTCCTGATTCAGGGATTATTGTAGTCAAACCTCT 128
UV027	65	AGGAAGGGATGCTCTTTCATAGTCTTTATACTCCTGATTCAGGGATTATTGTAGTCAAACCTCT 128
<i>mcyB</i>	65	AGGAAGGGATGCTCTTTCATAGTCTTTATACTCCTGATTCAGGGATTATTGTAGTCAAACCTCT 128
<i>dnaN</i>	65	AGGAAGGGATGCTCTTTCATAGTCTTTATACTCCTGATTCAGGGATTATTGTAGTCAAACCTCT 128
PCC7813	129	AATTACTCTGGAGGGAGAAATTAACCTTGCAGTTTTTAGGCAAGCGTGGGAAAAAGTTGTAGAG 192
UV027	129	AATTACTCTGGAGGGAGAAATTAACCTTGCAGTTTTTAGGCAAGCGTGGGAAAAAGTTGTAGAG 192
<i>mcyB</i>	129	AATTACTCTGGAGGGAGAAATTAACCTTGCAGTTTTTAGGCAAGCGTGGGAAAAAGTTGTAGAG 192
<i>dnaN</i>	129	AATTACTCTGGAGGGAGAAATTAACCTTGCAGTTTTTAGGCAAGCGTGGGAAAAAGTTGTAGAG 192
PCC7813	193	CGTCACTCGGTATTAAGGACTCTATTTCTTTGGGAAAAACGGGAAAAACCTCTGCAAATTGTGC 256
UV027	193	CGTCACTCGGTATTAAGGACTCTATTTCTTTGGGAAAAACGGGAAAAACCTCTGCAAATTGTGC 256
<i>mcyB</i>	193	CGTCACTCGGTATTAAGGACTCTATTTCTTTGGGAAAAACGGGAAAAACCTCTGCAAATTGTGC 256
<i>dnaN</i>	193	CGTCACTCGGTATTAAGGACTCTATTTCTTTGGGAAAAACGGGAAAAACCTCTGCAAATTGTGC 256
PCC7813	257	GAAAAAAGGTTGATTTGCCCTGGGATTATCAGGATTGGCGCAATCTTTCCCCACAGAACAACA 320
UV027	257	GAAAAAAGGTTGATTTGCCCTGGGATTATCAGGATTGGCGCAATCTTTCCCCACAGAACAACA 320
<i>mcyB</i>	257	GAAAAAAGGTTGATTTGCCCTGGGATTATCAGGATTGGCGCAATCTTTCCCCACAGAACAACA 320
<i>dnaN</i>	257	GAAAAAAGGTTGATTTGCCCTGGGATTATCAGGATTGGCGCAATCTTTCCCCACAGAACAACA 320
PCC7813	321	ACAGCGTTTAGATTTATTGTTACAAACAGAGCGTCAACAAGGGTTTGAATTCAAAGTTGCTCCT 384
UV027	321	ACAGCGTTTAGATTTATTGTTACAAACAGAGCGTCAACAAGGGTTTGAATTCAAAGTTGCTCCT 384
<i>mcyB</i>	321	ACAGCGTTTAGATTTATTGTTAGAAACAGAGCGTCAACAAGGGTTTGAATTCAAAGTTGCTCCT 384
<i>dnaN</i>	321	ACAGCGTTTAGATTTATTGTTACAAACAGAGCGTCAACAAGGGTTTGAATTCAAAGTTGCTCCT 384
PCC7813	385	TTGATGCGCTGCTTGATGATTCAACTATCGGACCAAACCTTATAAATTCCTCTGCAATCATCATC 448
UV027	385	TTAATGCGCTGCTTGATGATTCAACTATCGGACCAAACCTTATAAATTCCTCTGCAATCATCATC 448
<i>mcyB</i>	385	TTAATGCGTTGCTTGATGATTCAACTATCGGACCAAACCTTATAAATTCCTCTGCAATCATCATC 448
<i>dnaN</i>	385	TTAATGCGCTGCTTGATGATTCAACTATCGAGACCAAACCTTATAAATTCCTCTGCAATCATCATC 448
PCC7813	449	ATATTATTCTGGATGGTTGGAGTATGCCTATTATTTATCAAGAAGTTTTAGGGTTTTATGAGGC 512
UV027	449	ATATTATTCTGGATGGTTGGAGTATGCCTATTATTTATCAAGAAGTTTTAGGGTTTTATGAGGC 512
<i>mcyB</i>	449	ATATTATTCTGGATGGTTGGAGTATGCCTATTATTTATCAAGAAGTTTTAGGGTTTTATGAGGC 512
<i>dnaN</i>	449	ATATTATTCTGGATGGTTGGAGTATGCCTATTATTTATCAAGAAGTTTTAGGGTTTTATGAGGC 512
PCC7813	513	AGGTATTCAAGGGAAAAGTTATCATCTTCCCTTACC CGCGTCCCTATCAAGATTATATTGTTTGG 576
UV027	513	AGGTATTCAAGGGAAAAGTTATCATCTTCCCTTACC CGCGTCCCTATCAAGATTATATTGTTTGG 576
<i>mcyB</i>	513	AGGTATTCAAGGGAAAAGTTATCATCTTCCCTTACC CGCGTCCCTATCAAGATTATATTGTTTGG 576
<i>dnaN</i>	513	AGGTATTCAAGGGAAAAGTTATCATCTTCCCTTACC CGCGTCCCTATCAAGATTATATTGTTTGG 576
PCC7813	577	TTACAGGAGCAAACCCATCTATTGCTGAGAGTTTTTGGCAGCGAACTCTTGAAGGGTTTATGA 640
UV027	577	TTACAGGAGCAAACCCATCTATTGCTGAGAGTTTTTGGCAGCGAACTCTTGAAGGGTTTATGA 640
<i>mcyB</i>	577	TTACAGGAGCAAACCCATCTATTGCTGAGAGTTTTTGGCAGCGAACTCTTGAAGGGTTTATGA 640
<i>dnaN</i>	577	TTACAGGAGCAAACCCATCTGTTGCTGAGAGTTATTGGCAGCGAACTCTTGAAGGGTTTATGA 640
PCC7813	641	CTCCCACCCCTGAGGGTGGACAGACTCCAGTTAATGAAATCTGAAGGTAAGCCAACCTTATAA 704
UV027	641	CTCCCACCCCTGAGGGTGGACAGACTCCAAATTAATGAAATCTGAAGGTAAGCCGACTTATAA 704
<i>mcyB</i>	641	CTCCCACCCCTGAGGGTGGACAGACTCCAGTTAATGAAATCTGAAGGTAAGCCAACCTTATAA 704
<i>dnaN</i>	641	CTCCCACCCCTGAGGGTGGACAGACTCCAGTTAATGAAATCTGAAGGTAAGCCGACTTATAA 704
PCC7813	705	AGAGTAACTGTCATTTATCGGCTTCTCACTCCAAAGACCTGCAATCTTTGGCGCAAAGCAT 768
UV027	705	AGAGCATAACTGTCATTTATCGGCTTCTCACTCCAAAGATCTGCAATCTTTGGCGCAAAGCAT 768
<i>mcyB</i>	705	AGAGTAACTGTCATTTATCGGCTTCTCACTCCAAAGACCTGCAATCTTTGGCGCAAAGCAT 768
<i>dnaN</i>	705	AGAGTAACTGTCATTTATCGGCTTCTCTCTCCAAAGACCTGCAATCTTTGGCGCAAAGCAT 768
PCC7813	769	AATCTGACCTTATCTACTCTAGTACAGGCCGCTTGGGCGATTCTTCTCAGTCGCTATAGTGGGG 832
UV027	769	AATCTGACCTTATCTACTCTAGTACAGGCCGCTTGGGCGATTCTTCTCAGTCGCTATAGTGGGG 832
<i>mcyB</i>	769	AATCTGACCTTATCTACTCTAGTACAGGCCGCTTGGGCGATTCTTCTCAGTCGCTATAGTGGGG 832
<i>dnaN</i>	769	AATCTGACCTTATCTACTCTAGTACAGGCCGCTTGGGCGATTCTTCTCAGTCGCTATAGTGGGG 832
PCC7813	833	AGTCAGAAGTTTTATTTGGGGTTACGGTTTCTGGTCGCCCTCATGATTTATCAGGGGTAGAACA 896
UV027	833	AGTCAGAAGTTTTATTTGGGGTTACGGTTTCTGGTCGCCCTCATGATTTATCAGGGGTAGAACA 896
<i>mcyB</i>	833	AGTCAGAAGTTTTATTTGGGGTTACGGTTTCTGGTCGCCCTCATGATTTATCAGGGGTAGAACA 896
<i>dnaN</i>	833	AATCAGAAGTTTTATTTGGGGTTACGGTTTCTGGTCGCCCTCATGATTTATCAGGGGTAGAACC 896

PCC7813	897	TAGGGTAGGATTATTTATTAATACATTGCCGCTGCGAGTCTCCATCAGAGAATCAGATTTATTG	960
UV027	897	TAGGGTAGGATTATTTATTAATACATTGCCGCTGCGAGTCTCCATCAGAGAATCAGATTTATTG	960
<i>mcyB</i>	897	TAGGGTAGGATTATTTATTAATACATTGCCGCTGCGAGTCTCCATCAGAGAATCAGATTTATTG	960
<i>dnaN</i>	897	TAGGGTAGGATTATTTATTAATACATTGCCGCTGCGAGTCTCCATCAGAGAATCAGATTTATTG	960
PCC7813	961	CTATCTTGGTTACAGGAATTACAGCAAAAGCAAGCAGAAATTCAGGATTATGCTTATGTTTCTC	1024
UV027	961	CTATCTTGGTTACAGGAATTACAGCAAAAGCAAGCAGAAATTCAGGATTATGCTTATGTTTCTC	1024
<i>mcyB</i>	961	CTATCTTGGTTACAGGAATTACAGCAAAAGCAAGCAGAAATTCAGGATTATGCTTATGTTTCTC	1024
<i>dnaN</i>	961	CTATCTTGGTTACAGGAATTACAGCAAAAGCAAGCAGAAATTCAGGATTATGCTTATGTTTCTC	1024
PCC7813	1025	TGGCTGAAATACAGAGATTAAGTGATATTCACCGGGGGTCCCCTGTTTGAGAGTTTGGTCGT	1088
UV027	1025	TGGCTGAAATACAGAGATTAAGTGATATTCACCGGGGGTCCCCTGTTTGAGAGTTTGGTCGT	1088
<i>mcyB</i>	1025	TGGCTGAAATACAGAGATTAAGTGATATTCACCGGGGGTCCCCTGTTTGAGAGTTTGGTCGT	1088
<i>dnaN</i>	1025	TGGCTGAAATACAGAGATTAAGTGATATTCACCGGGGGTCCCCTGTTTGAGAGTTTGGTCGT	1088
PCC7813	1089	TTTTGAGAATTATCCTAGAGAAGCGTTATCGAGAGATTCTCGTCAATCCTTAAGGGTTAAGGAT	1152
UV027	1089	TTTTGAGAATTATCCTAGGGAAGCGTTATCGAGAGATTCTCGTCAATCCTTAAGGGTTAAGGAT	1152
<i>mcyB</i>	1089	TTTTGAGAATTATCCTAGAGAAGCGTTATCGAGAGATTCTCGTCAATCCTTAAGGGTTAAGGAT	1152
<i>dnaN</i>	1089	TTTTGAGAATTATCCTAGGGAAGCGTTATCGAGAGATTCTCGTCAATCCTTAAGGGTTAAGGAT	1152
PCC7813	1153	GTGGAGAATTTTGAGGAAACTAATTATCCTTTGACGGTGGTTGCTATTCTAAACAGGAGTTAC	1216
UV027	1153	GTGGAGAATTTTGAGGAAACTAATTATCCTTTGACGGTGGTTGCTATTCTAGACAAGAGTTAC	1216
<i>mcyB</i>	1153	GTGGAGAATTTTGAGGAAACTAATTATCCTTTGACGGTGGTTGCTATTCTAGACAAGAGTTAC	1216
<i>dnaN</i>	1153	GTGGAGAATTTTGAGGAAACTAATTATCCTTTAACGGTGGTTGCTATTCTAGACAAGAGTTAC	1216
PCC7813	1217	TGATTCAGTTAGTCTATGATACTAGCCGTTTTACTCAGGATACGATTGAACGGATGGCAGCACA	1280
UV027	1217	TGATTCAGATAATCTATGATACTAGCCGTTTTACTCAGGATACGATTGAACGGATGGCAGGACA	1280
<i>mcyB</i>	1217	TGATTCAGTTAGTCTATGATACTAGCCGTTTTACTCAGGATACGATTGAACGGATGGCAGCACA	1280
<i>dnaN</i>	1217	TGATTCAGTTAATCTATGATACTAGCCGTTTTACTCAGGATACGATTGAACGGATGGCAGGACA	1280
PCC7813	1281	TTTACAGACTATTTTAACAGGGATTGTTACTGATACTCGGCAACGGGTAACACAATTACCTATA	1344
UV027	1281	TTTACAGACTATTTTAACAGGAATTGTTACTGATCCTCGGCAACGGGTAACACAATTACCTATA	1344
<i>mcyB</i>	1281	TTTACAGACTATTTTAACAGGGATTGTTACTGATACTCGGCAACGGGTAACACAATTACCTATA	1344
<i>dnaN</i>	1281	TTTACAGACTATTTTAACAGGAATTGTTACTGATCCTCGGCAACGGGTAACACAATTACCTATA	1344
PCC7813	1345	TTGACAACACAAGAGCAACATCAGTTATTAGTAGAGTGGAACAATACCGAGGCAGATTATCCTT	1408
UV027	1345	TTGACAACACAAGAGCAACATCAGTTATTAGTAGAGTGGAACAATAGGGAGGCTGATTATCCTT	1408
<i>mcyB</i>	1345	TTGACAACACAAGAGCAACATCAGTTATTAGTAGAGTGGAACAATACCGAGGCAGATTATCCTT	1408
<i>dnaN</i>	1345	TTGACAACCCAAGAGCAACATCAGTTATTAGTAGAGTGGAACAATAGGGAGGCTGATTATCCTT	1408
PCC7813	1409	TAGATAAGTCTTTACATCAATTATTTGAGGAACAAGCTGCACAGAATCCGCAGGGAATAGTGGT	1472
UV027	1409	TAGATAAGTCTTTACATCAATTATTTGAAGAACAAGCTGCACAGAATCCGCAGGGAATAGTGGT	1472
<i>mcyB</i>	1409	TAGATAAGTCTTTACATCAATTATTTGAGGAACAAGTTGCACAGAATCCGCAGGGAATAGCGGT	1472
<i>dnaN</i>	1409	TAGATAAGTCTTTACATCAATTATTTGAAGAACAAGCTGCACAGAATCCGCAGGGAATAGCGGT	1472
PCC7813	1473	TATTTTTGAAGACCAAAAATTAACCTATCAACAGTTAAATAACCGGGGCAATCAGTTAGCTCAC	1536
UV027	1473	TATTTTTGAAGACCAAAAATTAACCTATCAACAGTTAAATAACCGGGGCAATCAGTTAGCTCAC	1536
<i>mcyB</i>	1473	TATTTTTGAAGGACCAAAAATTAACCTATCAACAGTTAAATAACCGGGGCAATCAGTTAGCTCAC	1536
<i>dnaN</i>	1473	TATTTTTGAAGACCAAAAATTAACCTATCAACAGTTAAATAACCGGGGCAATCAGTTAGCTCAC	1536
PCC7813	1537	TGTTTACGAGATAAGGGTGTAGGTCCAGAAAGTTTGGTCGGGATTTTTATGGAGCGTCCCTAG	1600
UV027	1537	TGTTTACGAGATAAGGGTGTAGGTCTAGAAAGTTTGGTCGGGATTTTTATGGAGCGTCCCTAG	1600
<i>mcyB</i>	1537	TGTTTACGAGATAAGGGTGTAGGTCCAGAAAGTTTGGTCGGGATTTTTATGGAGCGTCCCTAG	1600
<i>dnaN</i>	1537	TGTTTACGAGATAAGGGTGTAGGTCCAGAAAGTTTGGTCGGGATTTTTATGGAGCGTCCCTAG	1600
PCC7813	1601	AGATGGTCATCGGTTTATTAGGGATATTAAGCCGGGGGAGCTTATGTACCTTTAGATCCGGA	1664
UV027	1601	AGATGGTCATCGGTTTATTAGGGATATTAAGCCGGGGGAGCTTATGTACCTTTAGATCCGGA	1664
<i>mcyB</i>	1601	AGATGGTCATCGGTTTATTAGGGATATTAAGCCGGGGGAGCTTATGTACCTTTAGATCCGGA	1664
<i>dnaN</i>	1601	AGATGGTCATCGGTTTATTAGGGATATTAAGCCGGGGGAGCTTATGTACCTTTAGATCCGGA	1664
PCC7813	1665	TTATCCTACCGAGCGCTTGGGGGATATCCTCTCAGATTCCGGGGTTTCTTTGGTGTTAACTCAG	1728
UV027	1665	TTATCCTACCGAGCGCTTGGGGGATATCCTCTCAGATTCCGGATGTTTCTTTGGTGTTAACTCAG	1728
<i>mcyB</i>	1665	TTATCCTACCGAGCGCTTGGGGGATATCCTCTCAGATTCCGGGTGTTTCTTTGGTGTTAACTCAG	1728
<i>dnaN</i>	1665	TTATCCACCGAGCGCTTGGGGGATATCCTCTCAGATTCCGGGGTTTCTTTGGTGTTAACTCAG	1728
PCC7813	1729	GAATCTTTAGGGGATTTTCTTCCCAAACCTGGGGCCGAGTTACTGTGTTTAGATAGGGATTGGG	1792
UV027	1729	GAATCTTTAGGGGATTTTCTTCCCAAACCTGGGGCCGAGTTACTGTGTTTAGATAGGGATTGGG	1792
<i>mcyB</i>	1729	GAATCTTTAGGGGATTTTCTTCCCAAACCTGGGGCCGAGTTACTGTGTTTAGATAGGGATTGGG	1792
<i>dnaN</i>	1729	GAATCTTTAGGGGATTTTCTTCCCAAACCTGGTCCGGAATCACTGTGTTTAGATAGGGATTGGG	1792
PCC7813	1793	AAAAGATAGCTACCTATAGCCAGAAAATCCCTTCAATCTAACGACTCCTGAGAATTTAGCCTA	1856
UV027	1793	AAAAGATAGCTACCTATAGTCCAGAAAATCCCTTCAATCTAACGACTCCTGAGAATTTAGCCTA	1856
<i>mcyB</i>	1793	AAAAGATAGCTACCTATAGTCCAGAAAATCCCTTCAATCTAACGACTCCTGAGAATTTAGCCTA	1856

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dnaN      1793  AAAAGATAGCTACCTATAGCCCAGAAAATCACTTCAATCTAACGACTCCTGAGAATTTAGCCTA 1856
          *
PCC7813   1857  TGTTATTTATACATCAGGTTCAACGGGAAAACCCAAAGGAGTATTAATTAGCCATCGGGGGTTT 1920
UV027     1857  TGTTATTTATACATCAGGTTCAACGGGAAAACCCAAAGGCGTGATGAATATTCATAGAGGAATT 1920
mcyB      1857  TGTTATTTATACATCAGGTTCAACGGGAAAACCCAAAGGAGTATTAATTAGCCATCGGGGGTTA 1920
dnaN      1857  TGTTATTTATACATCAGGTTCAACGGGAAAACCCAAAGGGGTATTAATTAGCCATCGGGGGTTA 1920
          * * * * *
PCC7813   1921  ATGAATTTAATTTGTTGGCATCAAGACGCTTTTGAATACGCCTTTAGACAAAATTAICTCAAC 1984
UV027     1921  TGTAATACTCTGACATATGCTATTGGTCATTATAATATTACCTCTGAAGATCGCATTCTCCAAA 1984
mcyB      1921  ATGAATTTAATTTGTTGGCATCAAGACGCTTTTGAATACGCCTTTAGACAAAATTAICTCAAC 1984
dnaN      1921  ATGAATTTAATTTGTTGGCATCAAGACGCTTTTGAATACGCCTTTAGACAAAATTAICTCAAC 1984
          * * * * *
PCC7813   1985  TAGCAAGAATCGCTTTTGACGCTGCGGTTTGGGAGTTATGGCCCTGTTAACAGCAGGTGCGAG 2048
UV027     1985  TTACTTCCTTGAGTTTTGATGTTTCAGTTTGGGAAGTTTTCTCGTCTTTAATATCTGGTGCTTC 2048
mcyB      1985  TAGCAAGAAGTGCTTTTGACGCTGCGGTTTGGGAGTTATGGCCCTGTTAACAGCAGGTGCGAG 2048
dnaN      1985  TAGCAAGAATCGCTTTTGACGCTGCGGTTTGGGAGTTATGGCCCTGTTAACAGCAGGTGCGAG 2048
          * * * * *
PCC7813   2049  TCTTGCTTAGTTAAACCTGAAATCATGCAATCTCCCCAGACTTGCGAGACTGGTTAATTGCC 2112
UV027     2049  TCTAGTCGTGGCTAAACCTGACGGGTATAAA-----GATATAGATTATTTAATAGATTTAATTGTG 2109
mcyB      2049  TCTTGCTTAGTTAAACCTGAAATCATGCAATCTCCCCGACTTGCGAGACTGGTTAATTGCC 2112
dnaN      2049  TCTTGCTTAGTTAAACCTGAAATCATGCAATCTCCCCAGACTTGCGAGACTGGTTAATTGCC 2112
          * * * * *
PCC7813   2113  CAAGAAATCACCGTCAGCTTTTACCAACTCCCCTAGTTGAGAAGATTTTATCTTTAAAATG 2174
UV027     2113  CAAGAA--CAA-GTAACTTGTTTCACTTGTGTTCCCTCAATATTGCGAGTTTTTCTGCAACATC 2170
mcyB      2113  TTTTACCAA-----CTC--CCCTAGTTGAGAAGATTTTATCTTTAAAATG 2174
dnaN      2113  CAAGAAATCACCGTCAGCTTTTACCAACTC-CCCTAGTTGAGAAGATTTTATCTTTAGAATG 2174
          * * * * *

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Figure B1. Sequence alignment of the *mcyB* genes from *Microcystis aeruginosa* strains PC7813 and UV027 to published sequences on GenBank (<http://www.ncbi.nlm.nih.gov/BLAST>, Altschul et al. 1990). * = differences in sequence; - = gaps.

(a) PCC7813

PCC7813

2078 base pairs

Graphic map

BsII
AciI BstXI PleI MnlI Hsp92II Bsc4I
atggcagacacaaaaaatcaaccgcgcaaaaaatgtggagtcctttatcctctttcccccatgcaggaaggatg base pairs
taccgtctgtgttttttagttggcggtttttacacctcagataaataggagaaaaggggtacgtccttccctac 1 to 75
FauI HinfI NlaIII BsiYI

SfaNI BsiYI EcoNI Tsp509I MnlI MseI
BstF5I TfiI Sse9I GsuI TruII
ctctttcatagtcctttatactcctgattcagggattttattgtagtcaaaactctaattactctggaggagaaatt base pairs
gagaaagtatcagaaatagaggactaagtccttaataacatcagtttgagattaatgagacctcctctttaa 76 to 150
FokI HinfI TspEI BpmI TspEI
Bsc4I Tsp5
BslI Tru9I

Cac8I HgaI Tru9I
MaeIII TruII PleI
aaccttgcagtttttaggcaagcgtgggaaaaagttgtagagcgtcactcgggtattaaggactctatttctttgg base pairs
ttggaacgtcaaaaaatccggttcgcacctttttcaacatctcgcagtgagccataattcctgagataaagaacc 151 to 225
Tsp45I MseI HinfI

09I

BseDI AspLEI
TspEI BssT1I Hin6I
MnlI MwoI Eco130I HinP1I
gaaaaacgggaaaaacctctgcaaattgtgcaaaaaaggttgatttgccttgggattatcaggattggcgcaat base pairs
ctttttgcccttttttgagacgtttaacacgctttttccaactaaacggaaccctaataagtcctaaccgcgta 226 to 300
Sse9I StyI EcoT14I HspAI
Tsp509I ErhI CfoI
BsaJI HhaI

MaeIII HincII ApoI
HindII TspEI
EcoRI
ctttccccacagaacaacaacagcgttagattttattgttacaacagagcgtcaacaagggtttgaattcaaa base pairs
gaaagggggtgtcctgttgttgcgaaatctaataacaatgtttgtctcgcagttgttcccaacttaagttt 301 to 375
HgaI Sse9I
AcsI
Tsp509I

CfoI Bst71I SinI AvaII Tsp509I
Hin6I ItaI HinfI Sau96I AcsI
HinP1I Fsp4HI Bme18I HgiEI Sse9I MnlI
gttgctcctttgatgctgctgtgatgattcaactatcggaacaaacttataaattcctctgcaatcatcatcat base pairs
caacgaggaaactacgcgacgaactactaagttgatagcctggtttgaatatttaaggagacgttagtagtagta 376 to 450
HspAI HhaI TfiI Eco47I TspEI
SfaNI BsoFI AsuI AspS9I ApoI
AspLEI BbvI Cfr13I

BstF5I MnlI BspMI
attattctggatgggttgagatgcctattttatcaagaagtttttagggttttatgaggcaggtattcaaggg base pairs
taataagacctaccaacctcatcggataataaatagttcttcaaaatcccaaaataactccgtccataagttccc 451 to 525
FokI

MvnI
ThaI HgaI
MboII HphI AciI MaeIII
aaaagtatcatcttcttcaccgctccctatcaagattatattgtttggttacaggagcaaaacccatctatt base pairs
ttttcaatagtagaaggaagtggcgaggatagttctaataatacaaaacaaatgtcctcgtttttgggttagataa 526 to 600
AccII BsmFI
BstUI

MaeIII TfiI CviJI BsoFI
BfaI DdeI ItaI
BstF5I
cctaaacaggagttactgattcagttagtctatgatactagccgttttactcaggatgacgattgaacggatggca base pairs
ggattgtcctcaatgactaagtcaatcagatactatgatcggaacaaatgagtcctatgctaacttgccctaccgt 1201 to 1275
HinFI MaeI BstDEI FokI
Fsp4HI

Tru9I Tsp509I
TruI MaeIII MaeIII Sse9I
gcacatttacagactattttaacagggattgtactgatactcggaacgggtaacacaattacctatatgaca base pairs
cgtgtaaatgtctgataaaattgtccctaacaatgactatgagccgttgcccattgtgttaatggatataactgt 1276 to 1350
Bst71I MseI TspEI

BbvI

MnlI
BseDI
acacaagagcaacatcagttattagtagagtgaacaataccgaggcagattatcctttagataagtctttacat base pairs
tgtgttctcgtgtagtaataatcatctcacctgttatggctccgtctaataaggaaatctattcagaaatgta 1351 to 1425
BsaJI

BsoFI BpiI MseI
Tsp509I ItaI TfiI BpuAI TruI
Sse9I MnlI AluI BbvI AciI MboII TspEI
caattatttgaggaacaagctgcacagaatccgcaggggaatagtggttattttgaagacaaaaattaacctat base pairs
gttaataaactccttgttcgacgtgtcttaggcgtcccttatccaataaaaaacttctggtttttaattggata 1426 to 1500
TspEI CviJI BsgI Bbv16II Tru9I
Fsp4HI HinFI BbsI Tsp509I
Bst71I Sse9I

BsaJI SinI AvaII
Tru9I BsiSI MspR9I Sau96I
TruI MspI ScrFI AluI TspRI Bme18I HgiEI
caacagttaaataaaccggggcaatcagttagctcactgtttacagataaaggggtgtaggtccagaagaagtttggtc base pairs
gttgtcaatttattggccccgttagtcaatcgagtgcacaaatgctctattcccacatccaggtctttcaaccag 1501 to 1575
MseI HapII NciI CviJI Eco47I
BseDI BcnI AsuI AspS9I
HpaII Cfr13I

Bsh1365I BseDI BcnI RsaI
Bsc4I BsaBI Tru9I HapII NciI Csp6I
BfaI BsiYI TruI CviJI HpaII AluI
gggattttatggagcgttccctagagatggctatcggtttattagggatattaaaagccgggggagcttatgta base pairs
ccctaaaaataccctcgcaagggatctctaccagtagcacaataatccctataattttcgccccctcgaatacat 1576 to 1650
MaeI BslI Bse8I MseI MspI ScrFI CviJI
BsrBRI BsiSI MspR9I AfaI
MamI BsaJI MwoI

XhoII MflI BseAI Kpn2I TfiI AsuI BseI BsuRI
MboI NdeII Bsp13I HapII HinFI BslI BsrI BsrSI Pali
Kzo9I DpnII BsiMI MspI AlwI MboII BsiYI BseNI MaeIII
ccttttagatccggagaatctttaggggattttcttccccaaactggggccgagttactgtgttttagatagggatt base pairs
ggaaatctaggcctcttagaaatccccaaaaagaaggggtttgaccocggctcaatgacacaaatctatccctaa 1651 to 1725
BstX2I BstYI BspEI HpaII Bsc4I AspS9I CviJI
Bsp143I MroI AccIII AclWI Sau96I NlaIV MaeIII
Sau3AI DpnI BsaWI BsiSI Cfr13I PspN4I

AcsI
DdeI Tsp509I
SfcI HinFI Sse9I CviJI
AluI BstSFI
gggaaaagatagctacctatagcccagaaaatcccttcaatctaacgactcctgagaatttagcctatgttttt base pairs
cccttttctatcgatggatcgggcttttaggaagtttagattgctgaggactcttaaatcggatacaataaa 1726 to 1800
CviJI CviJI PleI TspEI
BstDEI
ApoI

atacatcaggttcaacgggaaaacccaaaggagtatttaattagccatcgggggtttatgaatttaattggttgc base pairs
 tagttagtccaagttgccccttttgggtttcctcataattaatcggtagcccccataacttaaattaacaaccg 1801 to 1875
 MseI TspEI Tsp509I Tsp509I
 TruI CviJI AcsI MseI
 VspI AsnI Sse9I Sse9I
 PshBI Tsp509I TspEI TspEI
 AseI Sse9I ApoI Tru9I
 Tru9I TruI

atcaagacgcttttgaaattacgccttttagacaaaattactcaactgaagaatcgcttttgacgctgcggttt base pairs
 tagttctgcgaaaactttaatgcggaatctgttttaagtgtgatcgttcttagcgaactgacgacgcaaaa 1876 to 1950
 Tsp509I Tsp509I MwoI Bst71I
 SfaNI HgaI Sse9I Sse9I BfaI TfiI Fsp4HI
 ItaI AciI
 TspEI TspEI MaeI HinfI HgaI
 BsoFI
 BbvI

gggagttatggccctgtttaacagcaggtgagcttctgtcttagttaaacctgaaatcatgcaatctccccag base pairs
 ccctcaataaccgggacaaaatgtcgtccacgctcagaacagaatcaatttgacttttagtacgttagaggggtc 1951 to 2025
 HaeIII Tru9I MseI
 Cfr13I TruI
 Sau96I Pali BspMI PleI DdeI TruI NlaIII
 AsuI BsuRI HinfI BstDEI Hsp92II
 AspS9I MseI Tru9I
 CviJI

attgcgagactggttaattgccaagaaatcacctcagctttttaccaact base pairs
 tgaacgctctgaccaattaacggttcttttagtggcagtcgaaaaatggttgag 2026 to 2078
 BseI TspEI
 BsrI Tru9I
 BsmAI TruI HphI AluI
 Alw26I MseI CviJI
 BseNI Sse9I
 BsrSI Tsp509I

(b) UV027

UV027

2169 base pairs
Graphic map

atggcagacacaaaaaatcaaccgccaaaaatgtggagtctatcttcttccccatgcaggaaggatg base pairs
 taccgtctgtgttttttagttggcggtttttacacctcagataaataggagaaggggtacgtccttcctac 1 to 75
 BslI
 Hsp92II
 Bsc4I
 AciI BstXI PleI MnlI Bsc4I
 FauI HinfI NlaIII
 BsiYI
 SfaNI BsiYI MseI
 BstF5I EcoNI Tsp509I MnlI TruI
 TfiI Sse9I GsuI Sse9I
 ctctttcatagtctttatactcctgattcagggatttattgtagtcaaactcctaattactctggagggagaaatt base pairs
 gagaaagtatcagaaatgataggactaagtcctaaataacatcagtttgagattaatgagacctccctcttaa 76 to 150
 FokI HinfI TspEI BpmI TspEI
 Bsc4I Tsp5
 BslI Tru9I
 Cac8I HgaI Tru9I
 MaeIII TruI PleI
 aacctgacgttttttaggcaagcgtgggaaaagggttgtagagcgtcactcgggtattaaggactctatttcttgg base pairs
 ttggaacgtcaaaaatccggttcgaccctttccaacatctcgcagtgaccataattcctgagataagaaacc 151 to 225
 Tsp45I MseI HinfI09I

Tsp509I MvaI AspLEI
 BsaJI Bst2UI Hin6I
 Sse9I EcoRII BstOI HinPII
 gaaaaacgggaaaaaaccttgcaaatgtgtcgaaaaaagggttgatttgccctgggattatcaggattggcgcaat base pairs
 ctttttgccttttttggaaacgtttaacacgcttttttccaactaaacgggaccctaatagtcctaaccggtta 226 to 300
 TspEI BseDI MspR9I HspAI
 MwoI ScrFI CfoI
 BstNI HhaI
 ApoI
 HincII TspEI
 MaeIII HindII EcoRI
 ctttccccacagaacaacaacagcgcttagattttggttcaaacagagcgctcaacaagggttgattcaaaa base pairs
 gaaaggggtgtcttgttgtgtcgcaaatctaaataacaatggttgtctcgagttgttcccaacttaagttt 301 to 375
 HgaI Sse9I
 AcsI
 Tsp509I
 Hin6I Fsp4HI SinI AvaII Tsp509I
 Tru9I AspLEI TfiI Sau96I AcsI
 TruII CfoI BsoFI Bme18I HgiEI Sse9I MnlI
 gttgctcctttaatgctgctgttgatgattcaactatcggaccaaacttataaattcctctgcaatcatcatcat base pairs
 caacgaggaattacgcgacgaactactaagttgatagcctgggttgatatttaaggagacgttagtagtagta 376 to 450
 MseI HinPII Bst7II Eco47I TspEI
 HspAI ItaI HinfI AsuI AspS9I ApoI
 HhaI BbvI Cfr13I
 BstF5I MnlI BspMI
 attattctggatggttggagtatgcctattttatcaagaagttttagggttttatgaggcaggtattcaaggg base pairs
 taataagacctaccacctcatcaggataataaatagttcttcaaaatcccaaaataactcgtccataagttccc 451 to 525
 FokI
 ThaI HgaI BbvI
 BsoFI Bsh1236I BsoFI
 MboII ItaI MvnI MaeIII ItaI
 aaaagttatcatcttcttgcgcgctccttatcaagattatattggttgggttacagcagcaaaacctctatt base pairs
 ttttcaatagtagaaggaacggcgaggaatagttctaataatacaaaaccaatgtctcgttttgggtagataa 526 to 600
 Fsp4HI AciI Fsp4HI
 AccII Bst7II
 BstUI
 BbvI Hsp92II Tsp509I
 BsoFI BsiYI Sse9I
 DdeI ItaI HinfI MslI NlaIII HinfI VspI
 gctgagagtttttggcagcgaactcttgaagggtttatgactcccaccatgagggtggacagactccaatta base pairs
 cgactctcaaaaaccgtcgcttgagaacttcccaaatgagggtgggtactcccacctgtctgaggttaat 601 to 675
 BstDEI Fsp4HI PleI Bsc4I BstXI PleI TruII
 Bst7II BslI TspEI
 MnlI PshBI
 AsnI Bsp143I DpnI
 AseI EglII DpnII
 Tru9I Eco57I CviJI Kzo9I NdeII
 atgaaatctgaaggttaagccgacttataagagcataactgtcatttatcggcttctcactccaaagatctgcaat base pairs
 tactttagacttccattcggctgaatattctcgtattgacagtaaatagccgaagagtgaggttctagacgta 676 to 750
 CviJI BstX2I BstYI
 MseI MboI Sau3AI
 XhoII MflI
 AspLEI RsaI ItaI BglI
 Hin6I Csp6I BsuRI MwoI BstDEI
 HinPII BfaI HaeIII TfiI MboII BstSFI
 ctttggcgcaaaagcataatctgaccttactctactgtacagcgcttggcgattcttctcagtcgctata base pairs
 gaaaccgcttttctgattagactggaatagatgatcatgtccggcgcaaccgctaagaagagtcagcgatat 751 to 825
 HspAI MaeI CviJI AciI HinfI DdeI SfcI
 CfoI AfaI Pali BsoFI
 HhaI Fsp4HI

HinfI MaeIII NlaIII
gtggggagtcagaaggtttatttgggggttacgggtttctggtcgccccatgatttatcggggtagaacataggg base pairs
caccctcagtcctccaataaacccaatgccaagaccagcggggtactaaatagccccatcttgtatccc 826 to 900
PleI Hsp92II

MseI NspBII
TruII BsoFI Bst71I Alw26I
VspI AsnI ItaI Acil PleI TfiI MaeIII
taggattatttattaatacattgcccgtgagctccatcagagaatcagatttattgctatcttgggttacagg base pairs
atcctaataaataattatgtaacggcgacgctcagaggtagtctcttagtctaaataacgatagaaccaatgtcc 901 to 975
PshBI Fsp4HI HinfI BsmAI HinfI
AseI BsrDI BbvI
Tru9I MspAII

Tsp509I
Tsp509I AcsI Tru9I
Sse9I Cac8I Sse9I CviJI TruII
aattacagcaaaggcaagcagaattcaggattatgcttatgtttctctggctgaaatacagagattaagtgata base pairs
ttaatgtcgtttccgcttctttaaagtcctaatacgaatacaaaagagaccgactttatgtctctaattcactat 976 to 1050
TspEI TspEI MseI
ApoI

BsaJI NlaIV BssTII MaeI
BsiSI MspR9I Tsp509I ErhI EcoT14I HinfI
MspI ScrFI Sse9I Ecol30I TthHB8I
ttccaccgggggttccctgtttgagagtttggctggttttggagaattatccttagggaagcgttatcgagagatt base pairs
aaggtggcccccaggggacaaaactctcaaacagcaaaaactcttaataggatcccttcgcaatagctctctaa 1051 to 1125
HapII NciI TspEI BlnI BseDI TaqI TfiI
BseDI BcnI StyI BsaJI
HpaII PspN4I AvrII BfaI

Bst98I MseI ApoI
MspCI Tru9I TspEI Tsp509I
BspTI MseI BstF5I MnlI Sse9I
ctcgtcaatccttaagggttaaggatgtggagaattttgaggaaactaatatcctttgacgggtggttctattc base pairs
gagcagtttaggaattccaattcctacacctcttaaaactcctttgattaataggaaactgccaccaacgataag 1126 to 1200
BfrI TruII FokI Sse9I TspEI
Vha464I TruII AcsI
AflII Tru9I Tsp509I

CviJI
BfaI MaeIII TfiI BfaI DdeI BstF5I
ctagacaagagtactgattcagataatctatgatactagccgttttactcaggatacagattgaacggatggcag base pairs
gatctgttctcaatgactaagtctattagatactatgatcgcaaatgagtcctatgctaacttgccctaccgtc 1201 to 1275
MaeI HinfI MaeI BstDEI FokI

Sau3AI AlwI
Tru9I Tsp509I Bsp143I MnlI Tsp509I
TruII Sse9I Kzo9I BseDI MaeIII Sse9I
gacatttacagactattttaacaggaattgttactgatcctcggaacgggtaacacaattacctatattgacaa base pairs
ctgtaaatgtctgataaaaattgtccttaacaatgactaggagccggttcccattgtgttaaatggatataactggt 1276 to 1350
MseI TspEI MboI DpnI TspEI
MaeIII DpnII AclWI
NdeII BsaJI

MnlI
cacaagcaacatcagttattagtagagtggaacaatagggaggctgattatccttttagataagtctttacatc base pairs
gtgttctcggttagtcaataatcatctcaccttggtatccctccgactaataggaaatctattcagaaatgtag 1351 to 1425
CviJI

BsoFI BpiI MseI
Tsp509I CviJI TfiI BpuAI TruII
Sse9I MboII ItaI BsgI AciI MboII TspEI
aattatttgaagaacaagctgcacagaatccgcagggatagtggttatttttgaagacaaaaattaacctatc base pairs
ttaataaacttctgttcgacgtgtcttaggcgtcccttatccaataaaaacttctggttttaattggatag 1426 to 1500
TspEI AluI BbvI Bbv16II Tru9I
Fsp4HI HinfI BbsI Tsp509I
Bst71I Sse9I

BsaJI
Tru9I BsiSI MspR9I MaeI
TruII MspI ScrFI AluI TspRI XbaI
aacagttaaataaccggggcaatcagtttagctcactgtttacgagataaggggtgtaggtctagaaagtgtggtcg base pairs
ttgtcaattttattggccccgttagtcaatcgagtgacaaaatgctctattccccacatccagatctttcaaacagc 1501 to 1575
MseI HapII NciI CviJI BfaI
BseDI BcnI
HpaII

AfeI HhaI MaeI Bsh1365I BseDI BcnI RsaI
Hin6I AspLEI Bsc4I MamI Tru9I HapII NciI Csp6I
HinP1I BstH2I BsiYI BsaBI TruII CviJI HpaII AluI
ggatttttatggagcgctccctagagatggtcatcggtttattagggatattaaaagccgggggagcttatgtac base pairs
cctaaaaatacctcgcgagggatctctaccagtagccaaataatccctataattttcggccccctcgaatacatg 1576 to 1650
HspAI CfoI BfaI BsrBRI MseI MspI ScrFI CviJI
Aor51HI HaeII BslI BsiSI MspR9I AfaI
Eco47III Bsp143II Bse8I BsaJI MwoI

XhoII MflI BseAI Kpn2I HinP1I AspLEI BstDEI HpaI
MboI NdeII Bsp13I HapII HspAI CfoI Bsp143II Tru9I
Kzo9I DpnII BsiMI MspI AlwI Eco47III Eco32I HinfI BstF5I TruII
ctttagatccggattatcctaccgagcgcttgggggatatcctctcagattcggatgtttctttgggtgtaactc base pairs
gaaatctaggcctaataagggatggtcggaacccctataggagagcttaagcctacaagaaccacaattgag 1651 to 1725
BstX2I BstYI BspEI HpaII Aor51HI HaeII DdeI FokI MseI DdeI
Bsp143I MroI AccIII AclWI AfeI BstH2I MnlI HindII
Sau3AI DpnI BsaWI BsiSI Hin6I HhaI EcoRV TfiI HincII

AsuI BseII BsuRI
TfiI BslI BsrI BsrSI Pali
BstDEI MboII BsiYI BseNI HaeIII
aggaatcttttagggattttcttccccaaactgggcccaggttactgtgttttagatagggattgggaaaagatag base pairs
tccttagaaatcccctaaaagaaggggtttgacccccggtcaatgacacaaaatctatccctaacccttttctatc 1726 to 1800
HinfI Bsc4I AspS9I CviJI
Sau96I NlaIV MaeIII
Cfr13I PspN4I

AcsI
SfcI DdeI Tsp509I
AluI BstSFI HinfI Sse9I CviJI
ctacctatagtcagaaaatcccttcaatctaacgactcctgagaatttagcctatgttattatacatcaggtt base pairs
gatggatcaggtcttttagggaggttagattgctgaggactcttaaatcggatacaataaatatgtagtccaa 1801 to 1875
CviJI PleI TspEI
BstDEI
ApoI

ApoI
MnlI
SspI Sse9I FauNDI
caacgggaaaacccaaaggcgtgatgaatattcatagaggaatttgaataactctgacatatgctattggtcatt base pairs
gttgccccctttgggtttccgcactacttataagtatctccttaaacattatgagactgtatacgataaccagtaa 1876 to 1950
TspEI NdeI
AcsI
Tsp509I

Sau3AI Mva1269I
MboI Eco57I TspEI
SspI MnlI DpnII BsaMI
ataatattacctctgaagatcgattctccaaattacttctctgagttttgatgtttcagtttgggaagttttct base pairs
tattataatggagacttctagcgttaagaggtttaatgaaggaactcaaaactcaaaagtcaaaccttcaaaaga 1951 to 2025
Kzo9I MboII Sse9I
Bsp143I BsmI
NdeII DpnI Tsp509I

Tru9I Tru9I
TruII BfaI CviJI TruII
cgtctttaatatctgggtcttctctagtcggtgtaaacctgacgggtataaagatatagattttaaagatt base pairs
gcagaaattatagaccgaagatcagcaccgatttggactgccccatatttctatatctataaattatctaa 2026 to 2100
MseI MaeI MseI

SUMMARY

There are 150 cyanobacterial genera and approximately 2 000 species known in the world. More than 40 of these have toxin producing strains. Cyanobacteria, commonly known as blue-green algae, are often present in small numbers together with a diverse assemblage of other photosynthetic algae that naturally occur in surface water worldwide. However, under conditions of warm temperatures, minimal water movement and elevated concentrations of phosphorus in a water body, cyanobacteria may frequently become dominant and form thick scums of floating algal cells. These dense aggregations of floating cells, termed 'blooms', presents a number of water quality problems; most often offensive odours and tastes, and sometimes biotoxins that can be divided into alkaloid neurotoxins and cyclic peptide hepatotoxins, commonly from the genus *Microcystis* and released in waterbodies. The neurotoxins act chiefly at neuromuscular junctions and cause rapid death because of respiratory paralysis. The hepatotoxins act on the hepatocyte cytoskeleton and cause intrahepatic haemorrhage and centrilobular necrosis. Clinically the hepatotoxin most often causes peracute or acute death, or subacute poisoning with signs such as icterus and hepatogenous photosensitivity.

Currently cyanobacterial taxonomy does not provide an unequivocal system for the identification of toxigenic and bloom-forming genus *Microcystis*. The ambiguities that exist in the cyanobacterial taxonomy are due to the expressed variability, minor morphological and developmental characteristics that are used for identification. In this study geographically unrelated axenic strains of *Microcystis aeruginosa* were obtained from the Pasteur Institute, France (PCC); the National Institute for Environmental Studies, Japan (NIES); the Institute of Freshwater Ecology, UK (CCAP); the Pflanzen Physiologisches

Institut, Universität Göttingen, Germany (SAG) and the University of the Free State, South Africa (UV) culture collections. Nonaxenic strains were collected from Hartbeespoort, Rietvlei and Roodeplaat Dams in South Africa. After screening 20 primer combinations on a subset of strains eight IRDye700TM-labeled *EcoR*I primer pairs were selected for amplified fragment length polymorphism (AFLP) analysis to determine the genetic relationship of these geographically unrelated strains. A total of 909 bands were amplified from the eight primer combinations, of which 665 were informative, 207 non-informative and 37 monomorphic, with an average of 83.12 polymorphic bands per primer combination. The genetic relationship among all the *Microcystis aeruginosa* strains based on the combination of data obtained with the eight primer combinations was analysed employing the Unweighted Pair Group Method using Arithmetic Means (UPGMA) algorithm and presented as a dendrogram. In the dendrogram, the strains from Rietvlei (UP01) and Hartbeespoort Dams (UP04) grouped together and were thus genetically closer to each other, than to the strain from the Rhodeplaat Dam (UP03). The Japanese strains (NIES88, NIES89, NIES90, NIES99, NIES299) also grouped separate from the other strains, with NIES90 and NIES299, genetically closest to each other. Interestingly, *Microcystis aeruginosa* strain PC7806 that originated from The Netherlands, also grouped within this group. *Microcystis aeruginosa* strains CCAP1450/1 (UK), UV027 (South Africa) and PC7813 grouped together, and are genetically closer to the UP-strains, than any of the other strains. In the present study, AFLP analysis proved useful for the identification of genetic diversity and analysis of population structure within *Microcystis aeruginosa*.

In order to link the identification of strains with toxicity, the utility of the *mcyB* gene sequence for identification of strains was tested. Based on conserved motifs present in known sequences of *mcyB* four primer pairs were designed. Using the primer pairs Tox 3P/2M, Tox 1P/1M, Tox 7P/3M and Tox 10P/4M, the *mcyB* gene from PCC7813 and

UV027 were sequenced, resulting in fragments of 2174 and 2170 base pairs in size, respectively. The obtained sequences were analyzed using nucleotide BLASTN annotation of the Basic Local Alignment Search Tool (BLAST). The sequence alignment indicated high homology to other published sequences in GenBank (AY034601 for PCC7813 and AY034602 for UV027; e-value = 0.0). Upon further analysis of the sequences it was obvious that there are several base differences between the sequences of the two strains, which led us to investigate the potential of using differences in restriction sites, and thus insertions/deletions (indels) in nucleotide sequence to discriminate between the other *M. aeruginosa* strains, as well as using the *mcyB* gene to discern between *M. aeruginosa* and *M. wesenbergii* in raw water samples. A vast number of restriction sites were identified with differences followed by restriction digest of the specific polymerase chain reaction (PCR) *mcyB* gene fragment. This work demonstrates that PCR assays provide a useful indicator of toxicity as well as the identification of taxonomical characteristics between laboratory cultures and environmental isolates.

A number of questions arise from the present study and future research therefore needs to address the following issues:

- Are there more than one *Microcystis aeruginosa* strain / "population" present at a given time in a specific water reservoir? Do these populations change through the season? What role do the individual populations play in a cyanobacterial bloom? Thus, the dynamics and structure of populations need to be clarified.
- Which *mcy* gene in the cluster is mostly responsible for toxin production? Does the expression of the genes correlate with gene structure/sequence? What role does the environment play in determining the level of expression, and thus toxin production?

OPSOMMING

Daar is tans 150 sianobakteriële genera en ongeveer 2 000 spesies in die wêreld bekend, waarvan 40 toksiene produseer. Sianobakteriële staan algemeen bekend as blou-groen alge en kom in klein hoeveelhede saam met ander fotosinterende algspesies in oppervlakwaters reg oor die wêreld voor. Wanneer omgewingstoestande ideal vir sianobakteriële is, bv. warm temperature, minimum waterbeweging en hoë konsentrasies voedingstowwe kan dit lei tot die dominansie van die spesie in eutrofiëse waterliggame. As gevolg van die hoë konsentrasie van groeiperende selle ontstaan 'n sianobakteriese opbloeï, wat waterkwaliteit en waterbestuur bemoeilik deurdat dit slegte smake en reuke aan die water gee. In sommige gevalle kan die ontbindende sianobakterie-selle biologiese toksiene in die vorm van neurotoksiene en hepatotoksiene in die water vrystel. Hierdie toksiene word algemeen met die genus *Microcystis* geassosieer.

Die huidige sianobakteriële taksonomie voldoen nie aan die behoeftes vir die klassifikasies van die genera *Microcystis* nie, aangesien dit gefundeer is op ontwikkeling en morfologiese eienskappe soos selgrootte, vorm, dryfbaarheid deur gasvakuole en toksisiteit. Geografiese onverwante suiwer enkel laboratorium kultuurstamme van *Microcystis aeruginosa* is bekom vanaf die Pasteur Instituut, Frankryk (PCC), die National Institute for Environmental Studies, Japan (NIES); die Institute for Freshwater Ecology, Verenigde Koninkryk (CCAP); die Pflanzen Physiologisches Institut, Universität Göttingen, Duitsland (SAG) en die Universiteit van die Vrystaat, Suid-Afrika (UV) kultuurversamelinge. Natuurlike sianobakteriese monsters is versamel in die Rietvlei-, Hartbeespoort- en Roodeplaatdamme. Na die sifting van 20 inleier-kombinasies op 'n kleiner groep stamme is agt IRDye700TM-gemarkte *EcoR1* inleier-pare gekies vir geamplifiseerde fragment lengte polimorfisme (AFLP)-analise om die genetiese verwantskap tussen die geografies

onverwante stamme te bepaal. 'n Totaal van 909 bande is geamplifiseer deur die agt inleier kombinasies, waarvan 665 beduidende inligting bevat het, 207 onbeduidend en 37 monomorfies was, met 'n gemiddeld van 83.12 polimorfiese bande per inleier-kombinasie. Die genetiese verwantskap tussen al die *Microcystis aeruginosa*-stamme gebasseer op die kombinasie van inligting is geanaliseer deur gebruik te maak van die "Unweighted Pair Group Method using Arithmetic Means (UPGMA)"-algoritme en is voorgestel in 'n dendrogram. In die dendrogram, het die drie stamme afkomstig van Rietvlei- (UP01) en Hartbeespoortdamme (UP04) saam gegropeer, en is dus geneties nader aan mekaar as aan die stam afkomstig van die Rhooedeplaatdam (UP03). Die Japanese stamme (NIES88, NIES89, NIES90, NIES99, NIES299) het apart van die ander stamme gegropeer, met NIES90 en NIES299, geneties die naaste aan mekaar. Interessant, is dat *Microcystis aeruginosa* stam PC7806 wat van Nederland afkomstig is, ook in die groep gegropeer is. *Microcystis aeruginosa*-stamme CCAP1450/1 (VK), UV027 (Suid-Afrika) en PC7813 het saamgroepeer, en is geneties die naaste verwant aan die UP-stamme. Uit die huidige studie het dit geblyk dat AFLP-analise bruikbaar is in die identifikasie van die genetiese diversiteit en analiese van die populasiestruktuur van *Microcystis aeruginosa*.

Om die identifikasie van die stamme met toksisiteit in verband te bring is die bruikbaarheid van die *mcyB*-geen se basisvolgorde vir die identifikasie van stamme getoets. Gebasseer op die gekonserveerde motiewe teenwoordig in bekende basisvolgordes van *mcyB* is vier inleier-pare ontwerp. Deur gebruik te maak van inleier- pare Tox 3P/2M, Tox 1P/1M, Tox 7P/3M en Tox 10P/4M, is die basisvolgordes van die *mcyB*-gene vanaf PCC7813 en UV027 bepaal en fragmentlengtes van 2174 en 2170 basispare in lengte is respektiewelik verkry. Die bepaalde basisvolgordes is geanaliseer deur middel van die nukleotied BLASTN-annotasie van die Basic Local Alignment Search Tool (BLAST). Die basisvolgorde afparing het groot ooreenkomste met ander gepubliseerde basisvolgordes in

GenBank (AY034601 vir PCC7813 en AY034602 vir UV027; e-waarde = 0.0) vertoon. Na verdere analiese van die basisvolgordes het dit geblyk dat daar verskeie nukleotied verskille tussen die twee stamme se basisvolgordes teenwoordig is. Dit het daartoe aanleiding gegee dat die potensiaal vir die gebruik van die basisvolgorde van *mcyB*-geen om tussen die ander *M. aeruginosa* stamme, so wel as tussen *M. aeruginosa* en *M. wesenbergii* te onderskei, ondersoek is. Polimerase kettingreaksie (PKR)-analise met verskeie inleier-pare het getoon dat *mcyB*-geen in *M. aeruginosa* teenwoordig en waarskynlik in die *M. wesenbergii*-isolaat afwesig is. 'n Groot aantal beperkingsnypunt-verskille is geïdentifiseer, met verskille na beperkingsnyding van die spesifieke *mcyB*-geen-PKR-fragment. Die studie demonstreer dat PKR-metodes 'n bruikbare indikator van toksisiteit, sowel as 'n identifikasie-karakter tussen laboratorium en natuurlike stamme kan voorsien.

'n Aantal vrae het uit die studie na vore gekom, en die vrae behoort in toekomstige navorsing aangespreuk te word. Die vrae sluit die volgende in:

- Is daar meer as een *Microcystis aeruginosa*-stam / "populasie" op 'n bepaalde tyd in 'n water opgaardam teenwoordig? Verander hierdie populasies gedurende die seisoen? Watter bydrae maak die individuele populasies tot opbloeie? Met ander woorde vrae oor die dinamieka en struktuur van populasies moet beantwoord word.
- Watter een van die *mcy* gene in die geenkompleks is meestal vir toksienproduksie verantwoordelik? Bestaan daar enige verband tussen geenuiting en geenstruktuur/basisvolgorde? Het omgewingsfaktore enige bepalende rol in die vlakke van geenuiting, and dus toksienproduksie?