The Cooperative Research Centre for
Water Quality and Treatment

Genetics of Microcystis Toxicity – Identification of the Genetic Pathway for Microcystin Production in *Microcystis aeruginosa*

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FOREWORD

Genetics of *Microcystis* toxicity – Identification of the genetic pathway for microcystin production in *Microcystis aeruginosa*

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CRC for Water Quality and Treatment Project No. 2.3.2.2 – Genetics of *Microcystis* Toxicity

EXECUTIVE SUMMARY

Of the known toxins produced by cyanobacteria, the microcystins are the most significant threat to human and animal health. These cyclic peptides are potent inhibitors of eukaryotic protein phosphatases (PP) type 1 and 2A. Synthesised nonribosomally, the microcystins contain a number of unusual amino acid residues including the ß-amino polyketide moiety Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid). We have identified and characterised the locus responsible for microcystin synthesis in *Microcystis aeruginosa*. A cluster spanning 55 kb, composed of 10 bidirectionally transcribed open reading frames arranged in two putative operons *(mcyA-C* and *mcyD-J)*, has been correlated with microcystin formation by gene disruption and mutant analysis. This is the first complete description of the biosynthesis pathway of a complex cyanobacterial metabolite. The enzymatic organization of the microcystin assembly represents an integrated polyketide-peptide biosynthetic pathway with a number of unusual structural and enzymatic features. These include the integrated synthesis of a ß-amino-pentaketide precursor and the formation of ß- and □-carboxyl-peptide bonds, respectively.

In this report we present an examination of the evolution and global distribution of microcystin synthetase within the genus *Microcystis*. The taxonomic structure of this genus is examined by 16S rRNA gene sequencing and correlated with both microcystin production and toxigenicity. Specific probes have been designed and tested to identify microcystin-producing *Microcystis* strains. In addition, the acquisition and evolution of this locus in members of the genus *Microcystis* is examined.

We also present results from an investigation into the effects different environmental parameters, particularly light and stress, have on *M. aeruginosa* and specifically the levels mcy gene transcription. Cells were grown either under continuous light of varying intensities, or under low light with subsequent short term exposure to different light intensities and qualities, and various stress factors. RNase protection assays were employed to observe the level of *mcyB* and *mcyD* transcription under each condition. Both *mcyB* and *mcyD* transcript levels were increased under high light intensities and red light. Blue light and certain artificial stress factors led to reduced levels of transcript. Our findings show that the effect of light on microcystin synthetase production is due to light quality and is initiated at certain threshold intensities, which are not necessarily reflected by observed intracellular toxin bioactivity.

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1. INTRODUCTION

1.1 Project Objectives

The objective of this research is an understanding of the mechanism for microcystin production in a cyanobacterial genus which is present (often abundantly) in almost all Australian water storages, namely *Microcystis aeruginosa*.

1.2 Specific Goals

1. Isolate and characterise the gene(s) responsible for microcystin production in *M. aeruginosa*.
2. Design molecular probes, based on these "toxin" genes, to discriminate toxigenic and non-toxigenic strains.
3. Establish if the occurrence of "toxic" blooms is due to genetic or environmental factors.
4. Investigate the influence of selected environmental factors on the regulation of microcystin production within natural *Microcystis* populations.

1.3 Background to Microcystin: Biosynthesis, Gene Organisation and Regulation

Of the known toxins produced by cyanobacteria, the microcystins are the most significant threat to human and animal health. Microcystins are a remarkable family of more than 65 cyclic heptapeptides (Fig. 1) produced by a diverse range of cyanobacteria, including species of the genera *Microcystis, Anabaena, Nostoc* and *Oscillatoria* (Rinehart 1994, Sivonen 1996). These cyclic peptides are potent inhibitors of eukaryotic protein phosphatases (PP) type 1 and 2A. Synthesised nonribosomally, the microcystins contain a number of unusual amino acid residues including the ß-amino polyketide moiety Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid).

Toxicity in vertebrates is mediated through the active transport of microcystin into hepatocytes by the bile acid organic anion transport system (Eriksson 1990). Acute poisoning leading to death, from massive hepatic hemorrhage, has been reported in both animals and humans (Beasley 1989, Jochimsen 1998, Pouria 1998). Chronic ingestion of sub-lethal doses has been demonstrated to induce primary hepatocellular carcinoma in rodents (Nishiwaki-Matsushima 1992) and has been epidemiologically linked to primary liver cancer in humans (Yu 1989, Yu 1995).

Biochemical and genetic studies have suggested a mixed polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS) origin for the microcystins (Arment and Carmichael 1996, Dittmann 1997, Moore 1991). While polyketides and nonribosomal peptides appear structurally unrelated, they are assembled in a remarkably similar manner. Both groups of metabolites are biosynthesised by large, multifunctional protein complexes that are organized into coordinate clusters of enzymatic sites termed modules, in which each module is responsible for one cycle of polyketide or polypeptide chain elongation (Cane 1998, Marahiel 1997, von Döhren 1999). The order of these modules, together with the number and type of catalytic domains within each module, determines the structure of the resulting polyketide or peptide product.

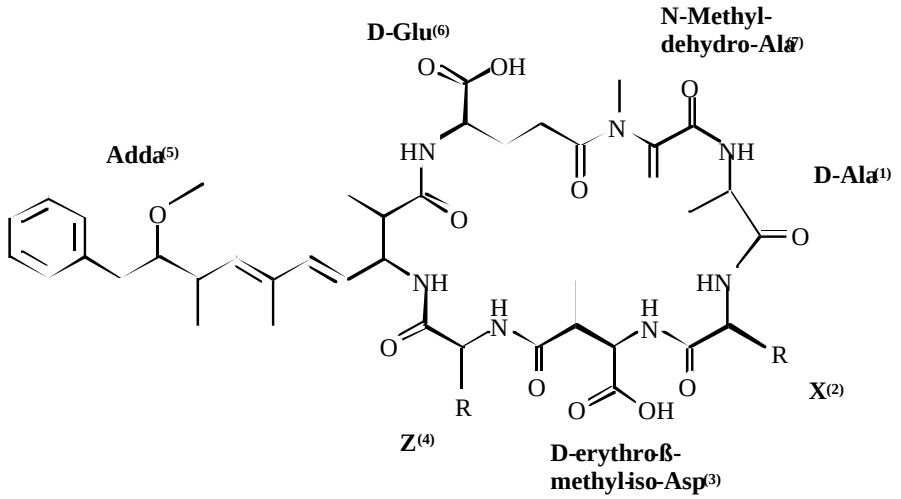

Figure 1. General structure of the microcystins. Variable L-amino acid residues are found at positions X and Z.

The recent identification of the locus responsible for microcystin synthesis in *M. aeruginosa* (see results sections below) allows the question of toxigenicity to be re-examined (Dittmann 1997, Tillett 2000). The traditional taxonomy of *Microcystis* (based on cell size, arrangement and mucilage characteristics) is unable to discriminate toxin producing strains from non-toxic strains (Komarek 1991). This is not surprising given the simple and often plastic morphology of *Microcystis*. This inability of the classical taxonomic approach to differentiate toxic from non-toxic strains has important management implications, as it has not been possible prior to this work to predict if a particular *Microcystis* bloom is toxic without directly testing for the presence of the toxin. In addition, it is not possible to determine if a bloom which is found to be non-toxic is potentially toxic if exposed to the appropriate environmental conditions.

A large number of molecular based studies have attempted to resolve the ambiguous nature of *Microcystis* taxonomy and toxigenicity. These have been based on allozyme polymorphisms (Kato 1991) 16S rRNA genes (Neilan 1997, Otsuka 1998, Rudi 1997, Rudi 1998) the phycocyanin spacer region (Neilan 1995), DNA-DNA hybridisation (Wilmotte 1994), nucleotide base composition (Fahrenkrug 1992), random amplified polymorphic DNA (RAPD) (Neilan 1995, Nishihara 1997), 16-23S rRNA internal transcribed spacer region (Neilan 1997, Otsuka 1999), *rbcL* gene (Rudi 1998), repetitive DNA elements (Asayama 1996, Rouhiainen 1995), and *rpoD* homologs (Sakamoto 1993). Unfortunately, these studies, while demonstrating the heterogenous nature of the genus *Microcystis*, have failed to identify consistent toxigenic populations, or in many cases, even consistent genotypes. The taxonomic structure of this genus was examined by 16S rRNA gene sequencing and correlated with both microcystin production and toxigenicity. Specific probes have been designed and tested to identify microcystin-producing *Microcystis* strains. In addition, the acquisition and evolution of this locus in members of the genus *Microcystis* was examined. Finally, the importance of genetic verses environmental factors in mediating toxicity was examined.

The effect of environmental or laboratory conditions on toxin quantities is usually lower than observed differences in toxin levels between different strains of a given species, or that observed in natural blooms of *M. aeruginosa* (Sivonen and Jones 1999). Nevertheless, several environmental factors have been described as influencing the biosynthesis of cyanotoxins for several defined isolates. The identification of the *mcy* genes in the production of microcystin synthetase provides an avenue to

study the regulation of microcystin production at a genetic level. In this study, *M. aeruginosa* PCC7806 was grown either under continuous light at various intensities, or under low light with subsequent short term exposure to different light intensities and qualities, and various stress factors. RNase protection assays were employed to observe the level of *mcyB* and *mcyD* transcription under each condition.

A variety of studies have focused on the effects of nutrients, such as nitrogen and phosphorous (Codd and Poon 1988, Oh 2000, Orr and Jones 1998, Rapala 1997, Sivonen 1990, Watanabe and Oishi 1985), trace metals (Lukac and Aegerter 1993, Utkilen and Gjolme 1992), temperature (Sivonen 1990, van der Westhuizen and Eloff 1985, Watanabe and Oishi 1985), pH (Gleason and Wood 1987, van der Westhuizen 1988) and light (Rapala 1997, Sivonen 1990, Utkilen and Gjolme 1992, Watanabe and Oishi 1985) on microcystin production. Several, but not all, studies have suggested that toxin production is highest under optimal growth conditions (Sivonen and Jones 1999). Orr and Jones (Orr and Jones 1998) concluded that the rate of microcystin production is directly proportional to the growth rate of the cyanobacterial population regardless of the environmental parameter tested. Increasing toxicity has been observed when light intensities were raised from approximately 7 to 40 μmol photons/m^2/s, depending on the study, with no further increases observed at higher light (Utkilen and Gjolme 1992, van der Westhuizen and Eloff 1985, Watanabe and Oishi 1985). In contrast, microcystin concentrations in *Anabaena* and *Oscillatoria* strains, were reduced at high light intensities (Rapala 1997, Sivonen 1990). Unfortunately, however, these studies are not readily comparable due to the various growth and toxicity assessment techniques employed.

More precise investigations of potential regulatory mechanisms of cyanotoxin biosynthesis require knowledge of the genes and enzymes involved. For the first time in the case of a cyanobacterial toxin, such studies are possible with the recent discovery of the genes and biosynthetic pathway required for the production of microcystins in *M. aeruginosa* (Dittmann 1997).

2. MATERIALS AND METHODS

2.1 Bacterial Strains

Cyanobacterial strains with designations PCC or UWOCC were obtained from the Pasteur Culture Collection (Rippka and Herdman 1992), or the University of Wisconsin at Oshkosh Culture Collection, parts of which have subsequently been deposited in the University of Texas Collection of Algae and Cyanobacteria (http://bluebonnet.pai.utexas.edu/infores/utex/) as accession numbers L2661-L2679 (Starr and Zeikus 1993). The cyanobacterial strains were maintained in either J (Corbett and Parker 1976) or BG-11 (Castenholz and Waterbury 1989) media at 25˚C with a light intensity of approximately 1500 lux (20 mol photons/m^2/s). Cultures were harvested after 21 days and 2 ml of each culture lyophilised for 48 hr. Samples were stored under vacuum until DNA extraction.

2.2 Cloning and Sequencing of the Microcystin Synthetase Operon

Chromosomal DNA was isolated from *Microcystis aeruginosa* PCC7806 as described (Tillett and Neilan 2000). Lambda Zap II library (Stratagene, La Jolla, CA) constructions and screenings were performed using the supplied protocol. PCR gene walking was performed using the hemidegenerate PCR technique to flanking peptide synthease domains (Neilan 1999) and a modified version of the suppression PCR approach (Siebert 1995). DNA sequencing was performed for both strands as described (Tillett and Neilan 1999).

2.3 Insertional Inactivation of *mcyA* and *mcyD*

The plasmid pMCYA5 was constructed by cloning a PCR amplified 5 kb fragment of *mcyA* into the pGEM-T vector (Promega, Madison, WI). The 1.4 kb *BsaA*1 fragment from pACYC184 containing the chloramphenicol resistance cassette was inserted into the *Bal*I site of pMCYA5. The plasmid pMCYD7 was constructed by cloning a PCR amplified 7.6 kb fragment of *mcyD* into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). The 0.8 kb *HinC*II fragment from pUCBM20 containing the chloramphenicol resistance cassette was inserted into the *Xmn*I site of pMCYD7. The plasmids pMCYA5C and pMCYD7C were used to perform homologous recombinational inactivation of *mcyA* and *mcyD* in *M. aeruginosa* PCC7806 via natural transformation (Dittmann 1997) and electroporation (14 kV/cm, 25 µF, 200 Ω), respectively.

2.4 Partial Purification of Microcystin Synthetase and Western Blotting

Partial purification of microcystin synthetase was performed essentially as described previously (Dittmann 1997). The western blot was performed using standard procedures as described previously with the nitrocellulose membrane blocked with BSA for one hour at 37˚C and the GS2 antibody as the primary antibody (Wessels 1996).

2.5 Microcystin Analysis

Analysis of microcystin content was performed as described previously by the protein phosphatase inhibition (PPi) assay (An and Carmicheal 1994) and MALDI-TOF mass spectrometry (Dittmann 1997). The PPi assay has an IC_{50} of 6.72 µg/l for the microcystin-LR standard, using 0.5 mg/ml of PP-2a, and a limit of detection of 0.033 µg/l (=33 pM). Prior to the assay, 1 ml of cell suspension in water (supernatant removed) was freeze/thawed three times and diluted 1:100 to 1:8000 times. Samples were measured in duplicate in the same assay, and also in repeated assays. After adjusting each assay to percentages of protein phosphatase activities, the concentration of microcystin (pmol/ml) was calculated from an average standard curve of all assays. PP-2a inhibitor (microcystin) content was calculated with respect to cells/ml of sample, resulting in values reported as pmol microcystin/cell.

2.6 Amplification and Sequencing of *mcyA* and 16S rDNA Genes

Amplification of the N-methyl transferase (NMT) region of *mcyA* and the 16S rRNA gene were performed by PCR. The former PCR reaction mixture contained 5 µl of 10x PCR buffer (Biotech

International, Perth, Australia), 5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM of each deoxynucleotide triphosphate (dNTP), 2 pmol of the NMT primers MSF and MSR, 10 ng of genomic DNA, 1 unit of *Taq* DNA polymerase (Biotech International), and water to a final volume of 50 µl. PCR conditions were as follows, 30 cycles of 94˚C for 10 sec, 60˚C for 20 sec, and 72˚C for 60 sec.

The 16S rRNA gene PCR amplification was performed as described previously (Neilan 1997), except that only 2 pmol of primer 27F1 and 1494Rc were used with 30 cycles of 94˚C for 10 sec, 50˚C for 20 sec, and 72˚C for 60 sec.

Automated BigDye terminator sequencing (PE Applied Biosystems, Foster City, CA) reactions were performed using 2 µl (~100 ng) of each PCR product and 10 pmol of each appropriate primer in a half-scale reaction. Three sequencing reactions were performed for each NMT PCR product using the MSF, MSR and MSI primers, while five sequencing reactions were performed for each 16S rDNA product using the primers 27F1, 530F, 929R, 942F, and 1494Rc. Sequencing reactions were purified and analysed as described previously (Tillett and Neilan 1999).

2.7 Gene Walking

DNA sequence flanking *uma1* in *Microcystis flos-aquae* UWOCC N was obtained using a modified version of the suppression PCR method (Siebert 1995). The resulting PCR products were sequenced as described previously using 10 pmol of the UMF and AP1 primers.

2.8 Phylogenetic and Sequence Analysis

DNA and protein sequences were aligned using the programs Pileup (GCG 1994) and CLUSTAL W (Thompson 1994). The aligned sequences were studied using the Phylip package of phylogenetic programs (Felsenstein 1989). All sequence manipulation and phylogeny programs were accessed through the Australian National Genomic Information Service (Sydney, Australia).

2.9 Data Deposition

The sequence of the *mcy* gene cluster has been deposited in GenBank under accession no. AF183408. The nucleotide sequences for 16S rRNA and toxin gene sequences have been deposited in GenBank under accession numbers AF139292 to AF139348.

2.10 Culture Conditions for Varying Light Intensities and Stress Inducers

M. aeruginosa PCC7806 was grown in Z8 media (NIVA, 1985) as batch cultures, in glass vessels with a diameter of 4 cm, with continuous aeration at 23˚C. The effect of light on *mcyB* and *mcyD* transcripts was tested in a series of experiments. In the first set, batch cultures (250 ml) were grown at low (16 µmol photons/m^2/s), medium (31 µmol photons/m^2/s) and high (68 µmol photons/m^2/s) light, from an optical density of 0.15 to ~1.6 at 750 nm (OD_{750}). During the growth period, OD_{750}, chlorophyll-*a* (chl-*a*) and total protein was measured every day, starting on day 3. Samples for RNase protection assays (RPA) (50 ml) were taken three times during; at early (OD_{750} = 0.4-0.7), mid (OD_{750} = 0.84-0.87) and late (OD_{750} = 1.6-2.0) growth phase, at which time cells were counted using a haemocytometer. In the second set of experiments, a pre-culture of one litre was grown under low light (16 µmol photons/m^2/s) to an OD_{750} of 0.66. Thereafter, the culture was divided (100 ml/sample) and exposed to different light or stress conditions.

A light intensity of 68 µmol photons/m^2/s (high light) was provided by cool fluorescence globes. Low (16 µmol photons/m^2/s) and medium (31 µmol photons/m^2/s) light intensities were obtained by shading cultures with two and one grey filter (Lee Filters, Hampshire, England), respectively. Very high light (400 µmol photons/m^2/s) and low light (16 µmol photons/m^2/s), for light quality (red and blue light) experiments, was produced by a Fibre Illuminator (FL-440 WALZ, Effeltrich, Germany) in the dark. Red and blue light environments were generated using cut-off filter foils (Lee Filters) wrapped around the culture vessels. Blue light filters allowed light transmission between 400-510 nm peaking at 475 nm, and red light filters at 620 nm with ~1/2 at 666 nm. In all cases, light intensity was set and measured using a spherical (SPQA) light meter (LI-250, WALZ) placed inside an empty culture vessel, surrounded with the appropriate filter foils, at the position of the test culture. Oxidative stress was induced by the addition of 1 mM methylviologen (MV) ($C_{12}H_{12}N_2$.2HCL) (SERVA,

Feinbiochemica, Heidelberg) to low and high light cultures. A second stress factor was introduced by the addition of 250 mM sodium chloride to low light cultures.

2.11 Sampling and RNA Extraction

During sampling, extreme care was taken to maintain light conditions similar to those tested. Twenty-five millilitres of culture were placed into 50 ml tubes and the remaining volume was filled with ice, prior to centrifugation at 5500 x g for 10 min, at 4˚C. Cell pellets were frozen in liquid nitrogen and kept at –20˚C until RNA extraction. Total RNA was extracted using Trizol Reagent (Gibco BRL, Life Technologies, Rockville, USA), following a pretreatment of the cells. The frozen pellet was crushed in a pre-cooled (with liquid nitrogen) mortar. This was repeatedly filled (3x) with liquid nitrogen in between crushing the cells. Still in a frozen state, Trizol Reagent was added onto the cells and combined with the cell suspension until the mixture was completely defrosted. Phenol extraction and precipitation was performed as per manufacturers instructions. RNA was further purified via columns (High Pure RNA isolation Kit, Boehringer, Mannheim, Germany). Concentrations of purified RNA were measured in duplicate at OD_{260} (GeneQuant II, RNA/DNA calculator Pharmacia Biotech, Uppsala, Sweden).

2.12 Northern Analysis

One microgram of RNA from each sample was separated by electrophoresis on a 1.3% formaldehyde gel, and blotted onto a charged nylon membrane (GeneScreen Plus, Hybridization Transfer Membrane, NEN Life Science Products, Boston, MA) as described by Sambrook *et al*. (1989). Blots were probed with radioactively labelled (readyprime™, Amersham, Braunschweig, Germany) 16S rRNA gene PCR product. 16S rRNA gene amplification was carried out as described previously (Neilan 1997). Hybridisation and washing conditions were performed using standard procedures (Sambrook 1989).

2.13 RNase Protection Assays

Oligonucleotides tox2+ and tox2- were used to amplify a 200 bp fragment within *mcyB* (peptide synthetase gene). Similarly, a 297 bp fragment of *mcyD* (microcystin polyketide synthase gene) was amplified with the oligonucleotide set *mcyD*F2 and *mcyD*R2. These fragments were ligated into the cloning vector pGEM (Promega) and checked for directional insertion via PCR and sequencing. After linearisation of the vector, the probes for the RPAs were prepared via *in vitro* transcription and labelled with [α^{32}P]UTP (MaxiScript, Ambion, Austin, USA). The resulting probes were gel purified and eluted (0.5 M NH_4OAc, 1 mM EDTA, 0.1% SDS) overnight. RPAs were carried out according to the manufacturers instruction (Boehringer), with the co-precipitation of probe and RNA (exactly 8 μg RNA used per sample) as the first step. Digestion products were subjected to PAGE (5%, 19:1 crosslinking with 7 M urea in TBE) at 3-5 mA for 3 hours (Sambrook 1989). Gels were exposed to X-ray film for various time periods to obtain autoradiograms in the appropriate exposure ranges for photography.

3. RESULTS AND DISCUSSION

3.1 Isolation and Characterisation of the Microcystin Gene(s)

A 758 bp fragment of the microcystin synthetase gene, *mcyB*, was amplified using primers FAA and RAA (Neilan 1999). This fragment was used to screen a lambda Zap library of *M. aeruginosa* PCC7806 and isolate a clone containing a 7 kb fragment of the *mcy* cluster. As further attempts to isolate flanking clones from this library proved unsuccessful the remaining *mcy* sequence was obtained by various PCR gene walking approaches (Neilan 1999, Siebert 1995). In total, 63.6 kb of the *mcy* gene cluster and flanking regions was isolated from *M. aeruginosa* PCC7806. The G+C content of the nucleotide sequence *mcy* region is 39.2%. This is similar to the total G+C content of *M. aeruginosa* PCC7806 at 41.6% (Neilan 1997).

A region spanning 55 kb was revealed, composed of 10 bidirectionally transcribed open reading frames arranged in two putative operons *(mcyA-C* and *mcyD-J*, Fig. 2). Gene disruption studies provided evidence for the involvement of *mcyA* and *mcyD* in microcystin biosynthesis. Additionally, western blot analysis of partially purified microcystin synthetase revealed homology to other characterised NRPS. Of the 48 sequential catalytic reactions involved in microcystin synthesis, 45 have been assigned to catalytic domains within six large multienzyme synthases/synthetases (McyA-E, G). The additional four monofunctional proteins are putatively involved in O-methylation (McyJ), epimerisation (McyF), dehydration (McyI), and localisation (McyH). The unusual polyketide amino acid Adda is formed by transamination of a polyketide precursor as enzyme-bound intermediate, and not released during the process.

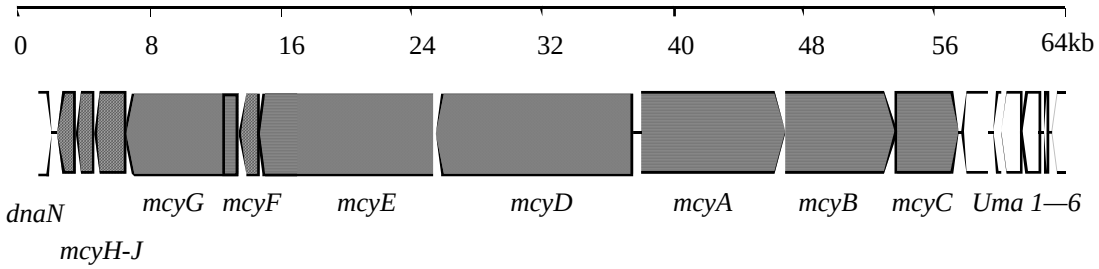

Figure 2. Organisation of the gene cluster for microcystin biosynthesis. The direction of transcription and relative sizes of the ORFs deduced from the analysis of the nucleotide sequence are indicated. ORFs containing regions homologous to nonribosomal peptide synthetases or polyketide syntheses are indicated horizontal stripes and shaded gray, respectively. Additional ORFs of putative microcystin tailoring function are indicated by diagonal stripes. Non-microcystin synthetase ORFs are shown in white.

This report is the first complete description of the biosynthesis pathway of a complex cyanobacterial metabolite. The enzymatic organization of the microcystin assembly represents an integrated polyketide-peptide biosynthetic pathway with a number of unusual structural and enzymatic features. These include the integrated synthesis of a ß-amino-pentaketide precursor and the formation of ß- and □-carboxyl-peptide bonds, respectively. Other features of this complex system also observed in diverse related biosynthetic clusters are integrated C- and N-methyltransferases, an integrated aminotransferase, and an associated O-methyltransferase and a racemase acting on acidic amino acids. The ORFs identified in this study are flanked on both sides by genes not involved in microcystin biosynthesis, and which show high similarity to genes localised on the chromosome of *Synechocystis* sp. PCC6803. This strongly suggests the *mcy* gene cluster is located on the chromosome and not on a large plasmid as previously proposed (Bolch 1997).

3.2 16S rDNA Phylogeny of *Microcystis*

The 16S rDNA gene sequence of 37 microcystin- and non-microcystin-producing *Microcystis* strains from several continents was determined and a phylogeny inferred (Fig. 3). Also included in the phylogenetic analysis were the 16S rDNA sequences previously obtained from 10 east Asian isolates

of *M. aeruginosa, M. ichthyoblabe, M. novacekii, M. wesenbergii* and *M. viridis* (Otsuka 1998) as well as that from *Synechocystis* sp. PCC6803 (Kaneko 1996).

3.3 Distribution of the N-methyl Transferase Domain Within *Microcystis*

Analysis of the first gene in the microcystin synthetase operon, *mcyA*, revealed two large peptide synthetase modules. In addition to the adenylation and thiolation domains, the first module contained an approximate 420 amino acid insertion between activation motifs A8 and A9 (Marahiel 1997). This region revealed a significant similarity of between 42% and 49% to known integrated peptide synthetase N-methyl transferase (NMT) domains (Haese 1993). These NMT regions contribute to the biological activity of the peptide product and provide protection of the peptide against proteolytic cleavage (Marahiel 1997).

Oligonucleotide primers MSF and MSR were designed to PCR amplify the single NMT region of microcystin synthetase. These primers allowed the specific amplification of this region from a range of *Microcystis* strains. A 1.3 kb PCR product was obtained from all 18 microcystin-producing strains tested. In addition, DNA from two strains that do not produce microcystin, UWOCC CBS and MR-C, also amplified this 1.3 kb PCR fragment.

The NMT-specific primers (MSF and MSR) proved to be reliable in identifying toxigenic *Microcystis* cultures. Of the 18 laboratory strains that gave a positive reaction in the PPi assays for microcystin, none lacked the NMT region of *mcyA*. Therefore, primers MSF and MSR may prove useful as genetic probes enabling the identification and management of toxigenic *Microcystis* blooms, although further work will be required to ensure their reliability under field conditions.

Two strains (CBS and MR-C) contained the NMT module of *mcyA*, but did not make detectable levels of microcystin. The absence of microcystin production by *M. aeruginosa* MR-C is particularly interesting because this strain was originally toxic by mouse assay (M. Runnegar, personal communication). Strain MR-C was derived from the same toxic field isolate as were strains MR-A, -B, and –D (Jackson 1984), all of which still produce microcystin. All four MR strains exhibit identical 16S rDNA and NMT DNA sequences (Fig. 3). It is therefore probable that strain MR-C mutated to lose toxicity during 25 years of laboratory passage. This is, to the best of our knowledge, the first report of the loss of *Microcystis* toxicity through a natural mutation event. Further study of these strains may provide insight into the environmental function of microcystin production.

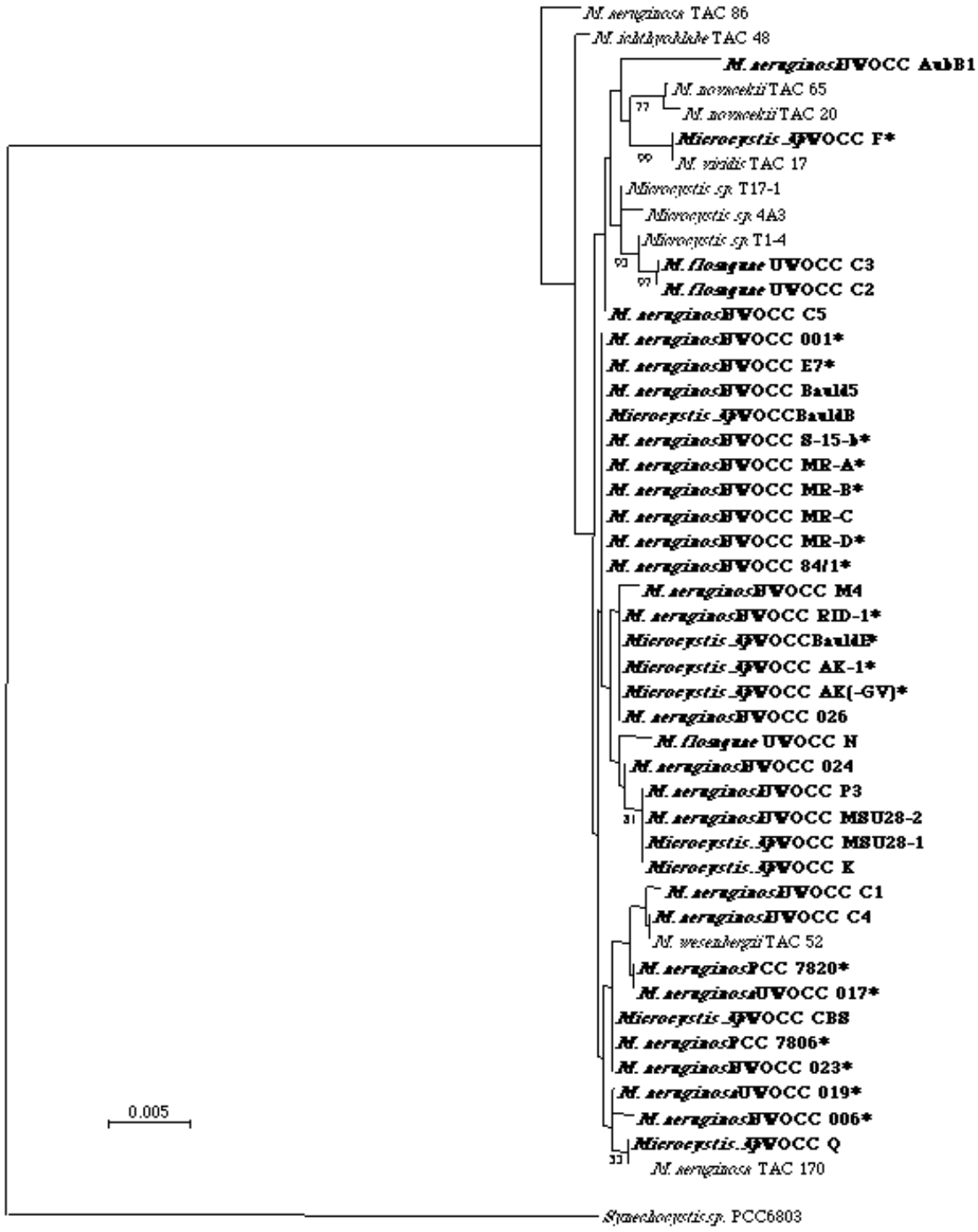

Figure 3. *Microcystis* 16S rDNA distance tree. Strains in bold were sequenced during this study, with strains additionally known to produce microcystin indicated with an asterisk.

3.4 Sequencing of the N-methyl transferase PCR products.

The DNA and the deduced amino acid sequences of the N-methyl transferase region were aligned. This region proved far more divergent than the 16S rRNA locus, containing 111 polymorphic sites across 20 *Microcystis* strains, as compared to 18 polymorphic sites within the 16S rDNA gene obtained from 47 *Microcystis* strains. Phylogenetic analysis of the NMT region showed a consistent, robust, bifurcated topology. This bifurcated topology was independent of the approach used in tree reconstruction, with both the genetic distance and maximum likelihood methods providing similar trees. In addition, this topology was maintained using either the protein or DNA sequence (data not shown).

No consistent relationship was found between the NMT genotype and the geographical region of isolation or species identification. An example of geographically dispersed strains containing identical NMT DNA sequence is the cluster of strains 017 (South Africa), 001 (Canada) and 7806 (The Netherlands). This widespread dispersal of each of the two NMT groups could be explained in several ways, including: (a) ancient separation of the two types, each of which has been relatively conserved since, or (b) long-distance transport of genetically related strains, perhaps on the feet of migrating waterfowl.

While the 16S rDNA sequence data suggest that the genus *Microcystis* forms a robust phylogenetic cluster, in agreement with previous investigations (Neilan 1997, Otsuka 1998, Rudi 1997), no consistent division between *Microcystis* strains could be made (Fig. 3). This absence of a reliable tree topology was independent of the method used, with both the genetic distance and maximum likelihood approaches giving topologies with insignificant bootstrap values (data not shown). We interpret the low bootstrap values and the high degree of sequence similarity (99%) among the strains to indicate that the 16S rDNA sequence is too highly conserved to pinpoint reliable differences among these closely related strains. In contrast, the NMT, phycocyanin spacer, 16-23S rRNA spacer, and other more variable regions give quite deeply branched phylogenetic trees with the same strains.

3.5 Gene organisation near the microcystin synthetase region

Analysis of the DNA sequence immediately downstream of *mcyC* revealed the presence of a large 684 amino acid ORF, *uma1* (Fig. 2). This ORF is of unknown function and is transcribed in a direction opposite to that of the *mcy* operon. BLAST analysis of *uma1* revealed a high identity (60%) to a 684 amino acid ORF (sll0471) also of unknown function encoded by *Synechocystis sp.* PCC 6803 (Kaneko 1996). Since *Synechocystis* sp. PCC 6803 does not produce microcystin, *uma1* is unlikely to be involved in microcystin production. Conserved primers designed to this region (UMF and UMR) PCR amplified a single 867 bp product from all 37 toxic and non-toxic *Microcystis* strains.

An oligonucleotide primer, MCYCF, was designed to allow PCR amplification from the 3' end of *mcyC* to *uma1*. PCR reactions were performed using primers MCYCF and UMF on 37 toxic and non-toxic *Microcystis* strains. All strain**s** containing the N-methyl transferase region produced a 1.5 kb PCR product, except for UWOCC E7, which amplified a product 200 bp larger (data not shown). Thus, all microcystin-producing stains have maintained a consistent linkage of *uma*1 with the microcystin genes.

To assess the related genome structure of the non-toxic *Microcystis* strains, a modified version of the suppression PCR gene walking method of Siebert *et al.* (1995) was used to obtain the DNA sequence downstream of the *uma1* stop codon in the non-microcystin producing strain *M. flos-aquae* UWOCC N. Using this approach, 255 bp of non-coding sequence flanking *uma1* was obtained. Blast analysis of this strain N-derived sequence, termed *N1*, revealed no significant homology to any sequences contained within the GenBank database. Two oligonucleotide primers, N1F and N1R, were designed to amplify the *N1* region. PCR amplifications were performed using these primers on the 37 *Microcystis* strains to assess the distribution of this region within *Microcystis* strains. Only six cultures, all non-toxic, provided *N1*-specific PCR amplicons: UWOCC AubB1, N, P3, MSU28-1, MSU28-2, and K. Interestingly, the *N1* region appears not to be present within the other two *M. flos-aquae* strains, UWOCC C2 and C3.

The conservation of genome structure was then assessed within the six strains containing the *N1* sequence by performing PCR amplifications using the N1R and UMF primers. Amplicons ranging in size from 1.1 to 1.4 kb were observed with strains N, P3, MSU28-1, MSU28-2 and K (data not shown). However, a N1R/UMF derived PCR amplicon was not obtained from strain AubB1.

The ability to produce microcystin is not universal within the *Microcystis* genus. In this study we found only 20 out of 37 strains examined appear to contain the microcystin synthetase operon. This limited distribution suggests two alternative hypothesis for the current distribution of microcystin production within the genus: (a) that all *Microcystis* were originally able to produce microcystin, with the currently non-toxic strains arising through loss of the microcystin synthetase operon, possibly as a consequence of strains adapting to new ecological niches; or (b) that all *Microcystis* were originally non-toxic, with the microcystin synthetase being acquired after the evolution of the genus. This hypothesis implies that such an acquisition event provided a selective advantage to those strains able to producing microcystin in some, but obviously not all, ecological niches. Thus a survey of the genetic diversity within the *Microcystis* should allow either hypothesis (a) or (b) to be excluded. While the 16S rDNA data did not allow either hypothesis to be excluded, examination of the genome region surrounding the *mcy* operon suggests hypothesis (b) is the more likely. Firstly, all microcystin-producing strains have the same gene arrangement in this region, that is, *uma1* and *mcyC* are proximately adjacent. Secondly, considerable genome variation is apparent in the region adjacent to *uma1* in the non-toxic strains as compared with the toxic strains. Among the 19 non-toxic strains, only six had the *N1* region to the left and in relatively close proximity to *uma1*. An additional non-toxic culture (AubB1) contained the *N1* region in another part of the genome and not adjacent to *uma1*. Thus, while 6 of the non-toxic strains have *N1* in that position, the 11 other non-toxic strains apparently have other DNA of unknown sequence.

3.6 Environmental Regulation of Cellular Contents and Toxin Gene Expression

3.6.1 Effect of light intensity

Batch cultures of *M. aeruginosa* PCC7806, set up with a starting OD_{750} of 0.15, were grown under various light intensities for eight days. Within the first three days, cells under high light grew to an OD_{750} of 0.7, compared to an OD_{750} of 0.6 under medium light and 0.45 at low light. Increased growth under high light intensities continued until day 6 when the cells had reached an OD_{750} of between 1.1-1.3 (data not shown). Cell counts and analysis of cellular protein and chl-*a*, were performed at early, mid and late growth phase. Sample set one (sample 1) and sample set 3 (sample 3) were taken on day three and day eight, respectively. Sample set two (sample 2) was taken when cells had reached an OD_{750} of between 0.84-0.87, which corresponded to day 5 for low light cultures and day 4 for mid and high light cultures. At all three sampling times, a downward trend was observed for protein and chl-*a* concentrations, from low light to high light cultures. Cell numbers were also slightly reduced from low to high light intensities, when at similar (sample 2), or increased optical densities (sample 1). This trend was not observed during the late growth phase (sample 3).

Transcript levels of *mcyB* from cells grown under different light intensities, were higher at high light. For each light intensity, total RNA was extracted from samples taken during early (sample 1), mid (sample 2) and late (sample 3) growth. Equal amounts of RNA, for each sample, were subjected to RPAs using the antisense probe for *mcyB*. Levels of *mcyB* transcripts increased when cells were grown under high light intensities, from samples taken at early and mid growth phase. This transcript response at high light was not seen in the late growth phase. To standardise these results, the same RNA was hybridised with a 16S rDNA probe. No differences were seen in 16S rRNA transcripts, relative to total RNA, under all light intensities and growth phases investigated. Comparison of *mcyB* transcripts, from cells grown at low and medium light intensities and, over growth showed no obvious differences.

Transcript levels of *mcyB* and *mcyD* increased when cells were transferred from low to high light intensities and decreased when moved into the dark. *M. aeruginosa* cells were grown to mid exponential phase (OD_{750} = 0.66) under low light (16 μmol photons/m^2/s) and then exposed to high (68 μmol photons/m^2/s) and very high light (400 μmol photons/m^2/s) for periods of 0.5 to 24 hours. Transcripts of *mcyB* and *mcyD* increased within the first 0.5 hours under high light, with no further increase thereafter. While a difference in *mcyB* transcript amounts was seen from cells at 16 μmol photons/m^2/s (control) compared to 68 μmol photons/m2/s, no obvious differences were observed when cells were exposed to 68 or 400 μmol photons/m^2/s. Cells maintained in the dark for two hours, resulted in the lowest *mcyB* and *mcyD* transcript levels recorded in this study.

Light quality affected *mcyB* and *mcyD* transcripts. Responses to light intensities seen via transcript increases/decreases, posed questions regarding the nature of the signal for gene transcription. When

cells were moved from low white light (16 μmol photons/m2/s) to red light (16 μmol photons/m^2/s) for two hours, the amounts of *mcyB* and *mycD* transcripts increased to a level comparable to that seen for high light. Cells moved to the same intensity of blue light, exhibited no change in transcript amounts compared to the control at low white light.

There were no significant differences in cellular toxin content from cells grown under different light intensities. Toxin content with respect to cell numbers, was measured using the PP2A assay based on the inhibition of protein phosphatase activity. All samples exhibited toxin contents ranging from 0.95 ± 0.31 to $3.43 \pm 1.04\ 10^{-5}$ pmol/cell. The sensitivity of the assay led to high standard deviations between measurements of the same sample in repetitive assays. As a result, with the exception of cells at early growth under high light which showed a lower toxin content (1.73 ± 0.38) compared to medium (2.82 ± 0.19) and high (3.43 ± 1.04) intensities, there were no significant differences between any of the other samples.

These results show, for the first time, that light quality (in the red light spectrum) was responsible for this effect on genes involved in microcystin biosynthesis. Furthermore, transcript levels were reduced under certain artificial stress conditions.

Increased levels of *mcyB* and *mcyD* transcript seen in cells exposed to 68 and 400 μmol photons/m^2/s were comparable to those seen for red light at 16 μmol photons/m^2/s. This intensity of red light is equivalent to the content of red light found during high light exposure (68 μmol photons/m^2/s) and may thus lead to the observed 'high light effect'. The same intensity of blue light had no effect on the amount of transcripts.

At high light intensities the saturation of the light harvesting complex leads to the production of free radicals and susceptibility to photoinhibition (Walsh 1997). Thus, enhanced transcription under red and high light conditions may be due to a direct effect of light via a light receptor, via an effect on PS, or due to oxidative stress. Electron scavengers, such as MV have commonly been used to create a situation of oxidative stress via the inhibition of PS (Haertel 1992, Winterbourn 1981). Cells exposed to MV for only 10 min showed reduced *mcyB* and *mcyD* transcripts. If the "high light effect" was due to the inhibition of PS or oxidative stress, transcripts should have increased under these conditions. The addition of a second stress factor, NaCl at concentrations comparable to that in seawater, also resulted in reduced transcript amounts. As NaCl does not inhibit PS, this result together with the response to MV, may point to a more general negative effect of stress on the transcription of microcystin synthetase genes.

Comparison of transcript amounts in cells exposed to different light quantities revealed two approximate threshold intensities at which transcription may be initiated. The first increase in transcript amounts, identified in cells grown at low light (16 μmol photons/m^2/s) compared to cells exposed to the dark for two hours, was similar to the only effect of light reported for another *Microcystis* strain (Nishizawa 1999). The second quantum increase occurred between cells grown at 31 and 68 μmol photons/m^2/s, with no further transcript increases when cells were exposed to 400 μmol photons/m^2/s. Although not directly comparable, several ecological studies have observed a similar threshold when measuring toxin content in cells grown under different light conditions. Increasing microcystin content was observed when light intensity was raised from approximately 2 to 40 μmol photons/m^2/s, depending on the study, with no further increases at higher light intensities (Rapala 1997, Utkilen and Gjolme 1992, van der Westhuizen and Eloff 1985, Watanabe and Oishi 1985). This led to the conclusion that light intensities influencing the toxicity of *M. aeruginosa* are less than about 40 μmol photons/m^2/s (Utkilen and Gjolme 1992). Such light intensity is found at a depth of about 1 m during bloom conditions with intensities of 400 μmol photons/m^2/s measured at the surface (Walsh 1997). This correlates to highest toxicity measured in the surface waters of a *Microcystis* bloom during periods of calm weather (Annadotter 1991). Unfortunately, direct comparison of light intensities described by various studies is not possible due to different measuring techniques used. In this study a spherical globe measuring light from all angles was used, as opposed to a flat light meter which is likely to result in slightly lower measurements under diffused light (Li-Cor manual, WALZ). Nevertheless, the notion of a light threshold for microcystin production is of interest in the investigation of putative roles for this substance.

Due to the numerous cellular processes affected by light, we can not conclude that light is the single factor leading to these thresholds. Cell division is influenced by high light and may have a pronounced effect on transcription and/or toxin production. Highest cell division rates may be expected at light saturation intensity, which for *M. aeruginosa* PCC7806 is about 32 μmol photons/m^2/s (K. Hesse, E.

Dittmann, W. Bleiß, T. Börner and J.-G. Kohl, manuscript in preparation), falling within the second threshold suggested. It has been reported as a summary of several previous studies, that toxin production is limited by cell division rates regardless of the environmental factor under investigation (Orr and Jones 1998). Short term exposures to high light intensities, in the present study, revealed transcript increases within two hours of moving cells from low to high light, and decreases when moved from light into the dark. Cell division rates are also likely to increase and decrease under these conditions, respectively, and thus be coupled to transcription. However, cells of *M. aeruginosa* divide less than once a day which suggests that the changes in transcript amount seen after just two hours are due to a direct response to light and not entirely due to cell division (Orr and Jones 1998).

Constant transcript amounts were observed from early to late growth phase, in samples from low and medium light intensities. Reduced transcripts visualised under high light during the late growth phase, were probably due to increased culture density reducing the amount of effective light able to penetrate the culture, thus producing a 'low light effect' on *mcyB* transcription (Schaefer and Golden 1989). Alternatively, the high light response of transcripts may be repressed by a cell density response similar to the quorum sensing regulation of the biosynthesis of several other nonribosomal peptides (Dunny and Leonard 1997, Whiteley 1999). Preliminary data indicates that *mcyD* transcripts, in an *mcyB* mutant of *M. aeruginosa* PCC7806 unable to produce microcystin (Dittmann 1997), do not show the upregulated response to high light as seen in the wild-type (E. Dittmann, unpublished data). This may imply that microcystin plays some role in regulating its own biosynthesis and, taken with the putative effects of cell density, may also have a more general role as a signalling or quorum sensing molecule.

Unlike the studies mentioned above, and with the exception of cells from sample one under high light, cellular toxin content between cells grown at low, medium and high light for up to eight days, did not change significantly. Toxin content was correlated on a per cell basis, as this parameter was less affected by light intensities, than protein or chl-*a* contents. Total protein content is reduced with increasing light intensities, possibly due to the breakdown of PS components such as phycobilisomes (Raps 1985, Walsh 1997). Similarly, as *Microcystis* adapts to high light, carotenoid levels, providing protection against photooxidative damage, are increased while chl-*a* concentrations are reduced (K. Hesse, E. Dittmann, W. Bleiß, T. Börner and J-G. Kohl, manuscript in preparation).

The negative trend of total proteins with increasing light, may also have influenced microcystin content reported by previous studies. Increasing microcystin concentrations identified under high light intensities, calculated with respect to total protein, may actually reflect decreasing protein concentrations at constant toxin levels and provide an explanation of varied results from this study and others (Utkilen and Gjolme 1992). Similarly, decreasing toxin concentrations identified under high light conditions calculated with respect to dry weight (Rapala 1997, Sivonen 1990), may be a result of increasing cell mass, due to the production of high molecular weight fatty acids and carbohydrate accumulation, rather than toxin content.

Constant or decreasing (for sample one) toxin content results of our study do not correlate with our transcript analyses, over increasing light intensities. However, toxin measurements detect the amount of microcystin in the cell at the time of sampling only, and cannot distinguish whether the peptide is stored at some threshold amount, or continuously synthesised or degraded as a result of some environmental factor. Further, increases in toxicity as a result of changing light intensity from 12-37 μmol photons/m^2/s, were shown to take longer than five days (Utkilen and Gjolme 1992), and thus may not be observed in our toxin data. Hence, the identification of short-term regulatory factors upon toxin production are not possible via toxin analyses only. mRNA analysis showing increased transcription of *mcyB* and *mcyD* under high light, may indicate higher toxin production under this condition. As this is not reflected by increased cellular PP2A inhibition, we speculate that the peptide is altered to a derivative form not detected by the PP2A assay for microcystin toxicity, photo-degraded, or released by the cell. While intracellular microcystin degradation mechanisms have not been identified, both sunlight irradiation and photolysis with UV light has been implicated in microcystin decomposition and isomerisation to non-toxic forms (Tsuji 1995).

Active release of microcystins from the cell has not been shown. Increased extracellular microcystin concentrations, after exposure to high light intensities (Rapala 1997) have usually been attributed to cell lysis and subsequent leakage of the peptide (Berg 1987, Lehtimaeki 1994, Sivonen 1990). However, the recent identification of a putative ABC transporter gene *(mcyH)* located upstream of *mcyE*, may suggest the existence of a cytoplasmic or transmembrane microcystin transport system. Thus it could be suggested, from the data presented here, that the toxin is constitutively produced

under a low and medium light intensity, but exported only when a higher threshold intensity is reached. Low cellular toxin concentrations under high light at early growth may be a result of microcystin release before normal intracellular levels have been established. To offset these losses the cell may engage in increased microcystin production, as seen by higher transcript levels of the biosynthesis genes. Toxin release under different light intensities requires further investigation, as does the possibility of post-transcriptional and post-translational regulation of microcystin synthetase activity.

3.6.2 Effect of stress

Stress had a negative effect on *mcyB* transcription. To find out if the increased transcript level under red light was due to oxidative stress, cultures were exposed to various stress inducing factors. MV (1 mM), an agent causing oxidative stress by inhibiting the electron transfer between photosystem II (PSII) and PSI, was added to low and high light cultures. Ten minutes after the addition of MV to low light cultures, the level of *mcyB* transcripts was reduced to those observed in cells kept in the dark. Similar reductions in *mcyB* transcripts were seen in cells exposed to high light prior to addition of MV (data not shown). Cells under low light and exposed to 250 mM NaCl for one hour, showed a decreased level in *mcyB* transcript similar to those seen in cells exposed to MV. Reduced transcripts seen for both stress factors do not correlate to the increased transcripts seen under red light.

4. CONCLUSIONS

The microcystins are a family of polyketide/peptide derived environmental toxins produced by diverse genera of cyanobacteria. Their complex biosynthetic pathway is one of many multienzyme-linked modular systems involving domains of PKS and NRPS. Cyanobacteria are a prominent source of such compounds, and will provide information on the functional organization of complex systems, their plasticity and evolution, and strategies for manipulation to generate new bio-products by combinatorial biology. At the same time possible clues are expected to emerge on the functional role of these metabolites in the ecosystem, and factors controlling their expression and thus promoting their environmental hazard.

We have analyzed the microcystin biosynthetic gene cluster of *Microcystin aeruginosa* PCC7806, a producer of microcystin-LR, which is composed of the ß-amino-polyketide moiety Adda, linked into the cyclic heptapeptide D-Glu-N-Me-dehydro-Ala-D-Ala-Leu-D-methyl-iso-Asp-Arg. This region spans 55 kb consisting of two oppositely transcribed gene clusters, encoding 10 proteins, 6 being multifunctional enzymes composed of PKS and NRPS domains. Two defined knock-out mutants were generated proving the involvement of this cluster in microcystin biosynthesis. Comparative sequence analysis assigns almost all of the 48 catalytic functions required for microcystin synthesis to this cluster and allows the identification of the primary precursors as phenylacetate, malonylCoA, S-adenosyl-methionine (SAM), glutamate, serine, alanine, leucine, D-methyl-iso-aspartate, and arginine. Among the reactions involved are peptide bonds between ß- and □-carboxyl groups, SAM-dependent C-, N- and O-methyl transfers, and dehydration of a seryl side chain. The modular architecture of microcystin synthetase provides new insights on the organization of these complex systems.

The NMT-specific primers proved to be reliable in identifying toxigenic *Microcystis* cultures. Of the 18 laboratory strains that gave a positive reaction in the phosphatase inhibition assays for microcystin, none lacked the NMT region of *mcyA*. Therefore, primers MSF and MSR may prove useful as genetic probes enabling the identification and management of toxigenic *Microcystis* blooms. The ability to produce microcystin is not universal within the *Microcystis*. In this study we found only 20 out of 37 strains examined appear to contain the microcystin synthetase operon. It is tempting to speculate that this function was acquired by horizontal gene transfer, possible from one of the distantly related cyanobacterial genera capable of producing microcystin (Luukkainen 1993, Rouhiainen 1995). Additionally, data supporting a recent acquisition of microcystin production does not provide any clues as to what the true ecological function of microcystin is, other than the selective force for toxicity is geographically widespread but limited to particular niches. Further work exploring the ecological function(s) of microcystin biosynthesis is certainly warranted.

From our results we conclude that light has a positive effect on *mcyB* and *mcyD* transcription and that this is not due to oxidative stress. Certain stress factors have a negative influence on transcription. We propose that the microcystin synthetase gene cluster is regulated by light quality, either directly or via another regulatory factor, and that transcription requires two thresholds of light intensity for initiation and upregulation. Cell division, density and growth do not appear to influence transcription directly, but may still be involved in a post-transcriptional regulation of microcystin synthetase gene expression. Alternatively, the lack of correlation between increasing *mcyB* and *mcyD* transcription and cellular toxin content may suggest microcystin release from the cell for a putative, yet unknown, role of this peptide under high light conditions.

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