This thesis is dedicated to Maralize,
who is the reason for my being.

Si quid facebamus scimus, is non scientiae nominaverat.

(If we knew what we were doing, it wouldn't be called science.)

-Albert Einstein

MOLECULAR CHARACTERISATION OF TOXIN-PRODUCING AND NON TOXIN-PRODUCING STRAINS OF *MICROCYSTIS AERUGINOSA*

by

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Submitted in fulfillment of the requirements for the degree

MAGISTER SCIENTIAE

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Bloemfontein

May 2001

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Acknowledgements

I would like to thank the following people and institutions:

Prof. J.U. Grobbelaar for financial support and expertise during this study and preparation of the manuscript.

Prof. A.-M. Botha Oberholster for suggestions, guidance and bearing with me during the course of the study.

The University of the Free State, Department of Botany and Genetics for their facilities and materials provided.

The Water Research Commission for funding this project.

The National Research Foundation for the bursary provided.

Mr. Tim Downing of UPE for providing the microcystin-LR standard and helpful suggestions.

The Department of Microbiology and Biochemistry for the use of their facilities and support.

Prof. Derek Litthauer for help, encouragement and faith in me.

Special thanks to Dr. Esta van Heerden for many hours of patience, encouragement and being a friend.

Dr. Koos Albertyn for insightful suggestions during the course of this study and preparation of the manuscript.

Dr. Jeanette Lötter for teaching me the intricacies of the HPLC.

My sincerest thanks to Cornelia Casaleggio for countless helpful ideas, infinite kindness, never-ending patience, and unselfish willingness to help.

Barbara Mashope for dragging me through the final sections of this manuscript.

Thanks to my colleagues in the Molecular Biology Lab for lively, interesting discussions, comments and general indulgence.

Much appreciation to my mother and brother, family and friends for their love, interest and support.

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List of Abbreviations

aa	Amino acid
Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
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_2O	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
GC	Gas chromatography
HPLC	High performance liquid chromatography
IPTG	Isopropyl-β-D-galactoside
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
PCC	Pasteur Culture Collection
PCR	Polymerase Chain Reaction
PP	Protein phosphatase
PPi	Inorganic pyrophosphate
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
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
tRNA	Transfer ribonucleic acid
UV	Ultraviolet
UV	University of the Free State
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.

LD_{50}

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

Klenow

One unit is the enzyme activity which 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 1μg λDNA 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 catalyzes the exchange of 1 nmole of ^{32}P from pyrophosphate into [γ, β-^{32}P]ATP in 20 minutes at 37 °C.

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1 Introduction

Microcystis aeruginosa is a blue-green alga with worldwide occurrence that can form seasonal cyanobacterial blooms (Hauman, 1982). This organism produces a vast number of peptides, some of which are highly toxic (Carmichael, 1986). These toxins have been implicated in many fatalities, both in livestock and humans (Falconer, 1991). The most commonly occurring toxin is microcystin-LR, a cyclic heptapeptide hepatotoxin (Carmichael, 1992). Effects of exposure to microcystins include skin irritation (Falconer *et al.*, 1983), possible liver cancer as a result of prolonged periods of exposure (Nishiwaki-Matsushima *et al.* 1992), and death in severe cases (Falconer *et al.*, 1981). The mechanism of toxicity is exerted by the general inhibition of dephosphorylation of protein phosphatases 1 and 2A, leading to hyperphosphorylation in the cytosol (Yoshiziwa *et al.*, 1990).

The molecular basis of toxin-production in *M. aeruginosa* was partially elucidated by Meiβner *et al.*, (1996) and Dittmann *et al.*, (1997). Meiβner, *et al.*, (1996) found that both toxin-producing and non toxin-producing strains of *M. aeruginosa* contained sequences that revealed a high degree of homology with several well-characterised peptide synthetases. In blotting experiments, a PCR fragment based on a portion of one of these peptide synthetases hybridised exclusively to restricted genomic DNA from toxin-producing strains indicating that this peptide synthetase was involved in toxin production.

Towards the end of 1997 Dittmann and co-workers performed homologous recombination in a toxin-producing strain that inactivated this peptide synthetase (*mcyB*) and arrested microcystin production in the toxin-producing strain PCC 7806 (Dittmann *et al.*, 1997). These results lead to the conclusion that the basic difference between toxin-producing and non toxin-producing *M. aeruginosa* strains is the presence or absence of the peptide synthetase, *mcyB*, in toxin producing and non toxin-producing strains respectively (Dittmann *et al.*, 1997).

The aim of this study was to ultimately provide a fast, accurate, robust and relatively easy way of screening *M. aeruginosa* blooms on a genetic/molecular level for potential toxin production. This would be accomplished by firstly, examining toxin-producing and non toxin-producing strains on a molecular level, secondly, assessing the genetic differences between toxin-producing and non toxin-producing strains and thirdly, to use this information to develop a molecular screening tool that could potentially be used to screen naturally occurring blooms for the presence of *mcyB*.

For the purposes of this particular study eight geographically unrelated strains were obtained from various sources. Seven of the strains investigated were reported to be toxin-producing, while the last strain was reportedly non toxin-producing. The strains were maintained under standard conditions and genomic DNA was extracted. Four specific primer pairs were designed, based on the sequence of *mcyB*, and polymerase chain reactions were performed. The fragments generated by PCR were cloned into a plasmid vector and sequenced using PCR primers, various internal primers and universal primers. Two PCR fragments were randomly labelled and used to screen other *M. aeruginosa* strains for the presence of *mcyB*.

Based on the conclusions from the paper by Dittmann *et al.* (1997), expected results in this study would include, firstly, that the oligonucleotide primer pairs would yield PCR fragments with only genomic DNA from toxin-producing strains, secondly, that if the PCR fragments obtained were sequenced, they would show a high degree of homology with *mcyB*, and thirdly, that if PCR fragments were labeled and used as probes to screen other strains of *M. aeruginosa*, the probes would exclusively hybridise to restricted genomic DNA from toxin-producing strains.

2 Literature Review

2.1 Cyanobacteria and their Toxins

The cyanobacteria are an extremely diverse and widely distributed group of organisms. They are prokaryotes possessing cell walls composed of peptidoglycan and lipopolysaccharide layers instead of the cellulose of green algae. They form one of the two systematic groups of the oxyphotobacteria, the other group being the prochlorophytes. The cyanobacteria consist of multi- and unicellular organisms, all of which possess chlorophyll *a*. Traditionally the cyanobacteria are classified into five orders: the *Chroococcales*, *Pleurocapsales*, *Oscillatoriales*, *Nostocales* and *Stigonematales* (Skulberg *et al.*, *1993)*. *Microcystis* spp. are classified as members of the *Microcystis* cluster of the genus *Synechocystis*, order *Chroococcales*.

It is generally believed that the increased occurrence of cyanobacterial blooms is, among other factors, a result of agricultural eutrophication of surface waters. Other factors resulting from the impounding of waterways for irrigation and domestic consumption also play an important role in the stimulation of bloom formation (Jones, 1990). Toxic blooms of *Microcystis* spp. usually take place in eutrophic stagnant waters during warm months of the year (Carmichael, 1986). The occurrence of toxic blooms is likely to escalate with an increase in the use of fertilisers, irrigation, animal-based agriculture and construction of water holding facilities such as ponds, lakes and reservoirs (Stotts *et al.*, 1993).

Cyanobacteria are capable of producing two kinds of toxins, the cyclic peptide hepatotoxins and the alkaloid neurotoxins. Serious illness such as hepatoenteritis, asymptomatic pneumonia and dermatitis may result from consumption of, or contact with water contaminated with toxin-producing cyanobacteria (Falconer *et al.*, 1983; Hawkins *et al.*, 1985; Turner *et al.*, 1990).

The neurotoxins include anatoxin-a, a depolarising neuromuscular blocking agent, anatoxin-a(s), an anti-cholinesterase, and saxitoxin and neosaxitoxin that inhibit nerve conduction by blocking sodium channels. The hepatotoxins include the pentapeptide nodularin, cylindrospermopsin, an alkaloid and most relevant to this study, the cyclic heptapeptide microcystins (Carmichael, 1994).

2.2 Microcystin-LR

Microcystis (order *Chroococcales*), *Anabaena* (order *Nostocales*), *Nostoc* (order *Nostocales*) and *Oscillatoria* (order *Oscillatoriales*), are able to produce microcystins. More than 50 variants of microcystin have been identified and characterised (Bourne *et al.*, 1996). Bishop and co-workers isolated the most common microcystin, microcystin-LR, in 1959 from a Canadian strain of *Microcystis aeruginosa* (Bishop *et al.*, 1959). Microcystins are monocyclic heptapeptides that have two unusual amino acids, N-methyl-dehydroalanine (Mdha) and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) (Nishiwaki-Matsushima *et al.*, 1992). The total structure of microcystin-LR was established as cyclo-D-alanine-L-leucine-erythro-β-methyl-D-isoaspartic acid-L-arginine-Adda-D-isoglutamic acid-N-methyl-dehydroalanine (Mdha) (Rinehart *et al.*, 1988).

Variations in structures of the microcystins were first observed in amino acids 2 and 4, L-leucine and L-arginine. Other microcystins are characterised 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.

A few esters of glutamic acid have been observed for amino acid 6, replacing γ-linked glutamic acid itself, and

N-methylserine or serine is sometimes found as amino acid 7. Variations in the Adda subunit (amino acid 5) include O-acetyl-O-demethyl-Adda and (6Z)-Adda (Rinehart *et al.*, 1988).

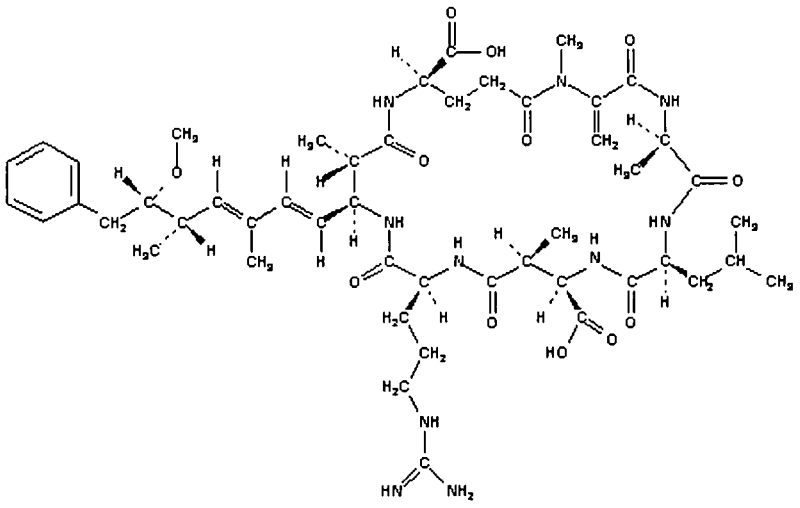

Figure 2.1 Chemical structure of microcystin-LR.

The Adda and D-glutamic acid portions of the microcystin-LR molecule play highly important roles in the hepatoxicity of microcystins. Esterification of the free carboxyl group of glutamic acid leads to essentially inactive compounds. Some variations in the Adda subunit exert little effect, specifically the O-demethyl and the O-demethyl-O-acetyl analogs. However, the overall shape of the Adda molecule seems to be critical since the [(6*Z*)-Adda](*cis*) isomer is inactive (Rinehart *et al.*, 1988).

The reason for the importance of these subunits is unknown, but there are two possible explanations. They may provide a necessary steric configuration that is directly involved in a carrier protein conveying hepato-specificity to the molecule, and/or may be important at an active site involving intracellular inhibition of protein phosphatases (Stotts *et al.*, 1993). The three dimensional structure of the hydrophobic core also seems to be an essential factor in the recognition and/or maintaining the proper orientation for the Adda residue, thereby determining toxicity (Rudolph-Bohner *et al.*, 1994).

Toxicity, thus appears to require that:

- the peptide be cyclic (linear peptides are inactive),
- the glutamic acid carboxyl group be free,
- a non-polar amino acid be attached to the γ-carboxyl of glutamic acid, and
- the Adda residue have a 6*E* double bond rather than the 6*Z* analogue (Rinehart *et al.*, 1988).

Interestingly, Quinn *et al.*, (1993) have carried out molecular modeling studies that indicate a similarity in the shape of okadaic acid and microcystin in which the carboxyl group of okadaic acid occupies a position similar to that of the carboxyl group of the glutamic acid portion of microcystin. Also, the methyl ester of okadaic acid is, like the methyl ester of microcystin-LR, inactive (Nishiwaki-Matsushima *et al.*, 1992).

2.3 Synthesis of Microcystins

For a complex peptide such as microcystin to be synthesised, there has to be genetic material present in the concerned organism. Several possible origins of this genetic material have been investigated:

2.3.1 Chloroplast DNA

Shi *et al.* (1995) used a polyclonal antibody against microcystins in conjunction with immuno-gold labelling to localise microcystins in a toxin-producing strain (PCC 7820) and non toxin-producing strain (UTEX 2063) of *M. aeruginosa*. No specific labelling was found in the non toxin-producing strain. Most of the specific labelling in the toxin-producing strain occurred in the thylakoid and nucleoid regions. The cell wall and sheath area also displayed specific labelling, but to a lesser extent. No microcystins were found in cellular inclusions with storage products such as lipid bodies, polyhedral bodies, cyanophycin granules and membrane-limited inclusions. These results suggest that microcystins are not compounds that the

cell stores, but that they may be involved in specific cell activities such as regulation of protein phosphorylation.

2.3.2 Plasmids

Genetic control of toxin production by plasmids commonly found in some strains of *M. aeruginosa* has also been investigated. Vakeria *et al.* (1985) applied plasmid-curing agents to toxin-producing strains of *M. aeruginosa* and did not find any significant decrease in toxicity. Also, to support this argument, Schwabe and co-workers (1988) found toxin-producing strains that contained no plasmids. On the other hand, evidence has been presented of a South African strain (WR 70) that exhibited decreased toxicity after treatment with plasmid-curing agents (Hauman, 1982).

2.3.3. Thiotemplate Mechanism

Synthesis of most proteins can be described in terms of the genetic code where DNA serves as a template for mRNA and proteins are then assembled on ribosomes using aminoacylated tRNA's. As early as 1954, Fritz Lipmann predicted a poly- or multienzymatic pathway of peptide synthesis (Lipmann, 1954) and this mechanism has been verified for various types of peptides (Laland & Zimmer, 1973). Laland and Zimmer (1973) were the first authors to propose the term 'thiotemplate mechanism' to distinguish this mechanism from other mechanisms of non-ribosomal peptide synthesis. The term was formulated as a result of studies into the synthesis mechanism of *Bacillus brevis* peptide antibiotics. In this mechanism the peptide bond is made possible by the unique structural feature of the thioester moiety (Laland & Zimmer, 1973).

On comparing ribosome-mediated protein synthesis with the thiotemplate mechanism, many similarities are apparent, most notably: (i) in both systems the amino acids are activated through the formation of an amino acid adenylate, (ii) the activated amino acyl residue is transferred to a receptor molecule and the peptide chain grows from the *N*-terminal end by insertion of the next amino acid at the activated

C-terminal and (iii) during the synthesis the growing chain is covalently linked to a macromolecule. The most important difference between the two systems is that the sequence of the final peptide product in the thiotemplate mechanism is determined by a protein template compared to DNA in the ribosomal system (Laland & Zimmer, 1973).

Peptides synthesised by the thiotemplate mechanism share a few notable chemical characteristics. Table 2.1 describes these characteristics as well as how these apply to microcystins where applicable.

Table 2.1 *General characteristics of peptide synthetase products and applicability to microcystins (Adapted from Ljones* et al., *1968; Sand* et al., *1967).*

Characteristic of Peptide Synthetase Products	Microcystins
Often cyclic	MC-LR
Contain both D- and L-amino acid residues	MC-LR: D-alanine, L-leucine, etc.
Contain N-methylated amino acids	N-methyl-dehydroalanine
Contain unusual amino acids and other non-amino acid moieties	Adda, Mdha
Molecular weights of between 300 – 3000 kDa	MC-LR: 995.2 kDa
Micro-organisms generally synthesise a group of closely related peptides rather than a single entity	MC-LR, MC-YR, MC-RR, etc.
Members of such a group usually differ from each other by one or a small number of amino acids	Usually amino acids 2 and 4

Peptide synthetases form part of a superfamily of adenylate-forming enzymes (Conti *et al.*, 1996). The domains from different peptide synthetases share homologous regions with other adenylate-forming enzymes such as 4-coumarate:CoA ligase (EC 6.2.1.12) (Lozoya *et al.*, 1988), acetyl CoA synthetase (EC 6.2.1.1) (Connerton *et al.*, 1990) and firefly luciferase (EC 1.13.12.7) (De Wet *et al.*, 1987).

In 1992, Turgay and co-workers demonstrated that there are certain repetitive, highly conserved domains present in the peptide synthetases depending on the number of amino acids to be activated. Sequence comparisons of these domains reveal highly conserved motifs. These sequence similarities

can provide a general approach for identifying the genes encoding peptide synthetases (Borchert *et al.*, 1992).

Activation of each amino acid takes place at the AMP-binding site by cleavage of the α, β-phosphate bond of ATP, forming AMP and inorganic pyrophosphate (PP_i) (Ljones *et al.*, 1968; Rapaport *et al.*, 1987). This site also determines the specificity of the amino acid to be bound (Conti *et al.*, 1997). The activated amino acid is then transferred to the phosphopantetheine attachment site. This 4'-phosphopantetheine prosthetic group is attached through a serine residue and acts as a 'swinging arm' for the transport of the activated amino acids to the condensation site. The condensation site catalyses a condensation reaction to form peptide bonds between amino acid adenylates (Fig. 2.2) (Stachelhaus *et al.*, 1998).

Elongation of the peptide chain is not a repeated cycle of reactions as in polyketide formation, rather it is a single cycle of sequential and similar reactions. The intermediates remain in an active state as thioesters and transfer of the growing peptide chain is mediated by the successive transthiolation of the cofactor 4'-phosphopantetheine (Gilhuus-Moe *et al.*, 1970; Kleinkauf *et al.*, 1970; 1971). Termination of the chain is achieved by cyclisation with terminal or internal peptide ester bond formation or by modification or hydrolysis of the activated C-terminus (Marahiel, 1992).

Arment and Carmichael (1995) were the first to speculate on the possibility of the thiotemplate mechanism being involved in the synthesis of microcystins. Several genes encoding peptide synthetases have been isolated from different strains of *M. aeruginosa* such as HUB 5-2-4 (Meißner *et al.*, 1996), PCC 7820, PCC 7806, EAWAG 120a and EAWAG 167 (Niederberger & Neilan, 1998). Towards the end of 1997 a peptide synthetase, termed *mcyB* was positively identified as a role-player in microcystin-LR production in PCC 7806 (Dittmann *et al.*, 1997).

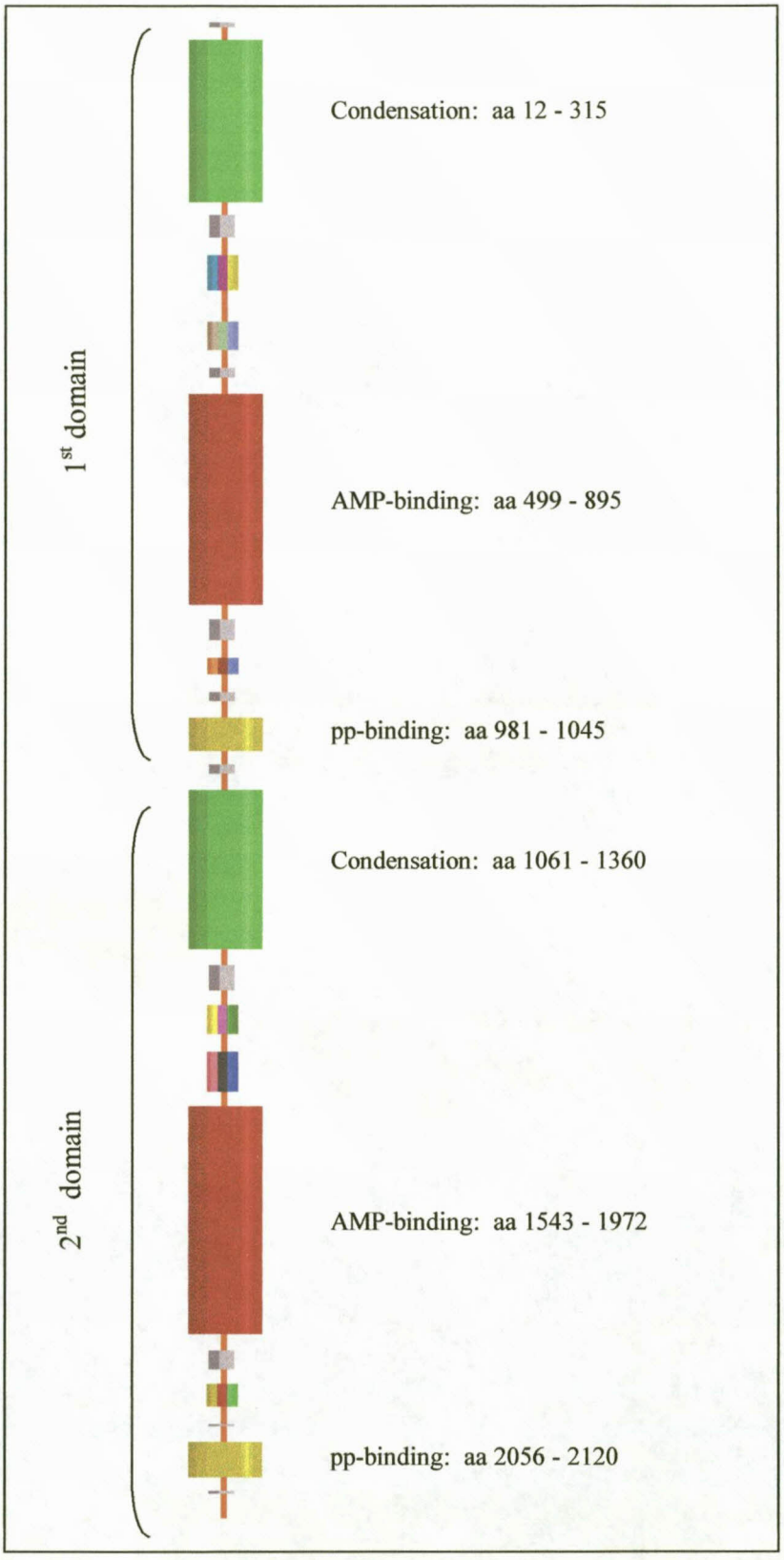

Figure 2.2 *Schematic representation of mcyB.* (Niederberger & Neilan, 1998).

2.4 External Factors Affecting Synthesis

Toxin production and growth of toxin-producing strains of *M. aeruginosa* depend to a certain extent on various physical, chemical and biological factors such as light, temperature, pH, nutrients and some other miscellaneous factors:

- A variety of "optimal" light intensities for toxin production have been described by numerous authors (Rapala *et al.*, 1997; Sivonen, 1990; Utkilen & Gjolme, 1995; Van der Westhuizen & Eloff, 1985; Watanabe & Oishi, 1985). These discrepancies are mainly due to differing measuring techniques employed in the various studies. In a recent publication the quality of light, i.e. 16 µmol photons $m^{-1}s^{-1}$ in the red light spectrum, increased toxin production a *M. aeruginosa* strain (Kaebernick *et al.* 2000).
- Some strains express different toxicities and synthesise different microcystins at different temperatures. The optimum temperature for maximum growth is 32°C, while the highest toxicity is observed at 20°C (Krüger & Eloff, 1977; Van der Westhuizen & Eloff, 1985).
- The highest growth rate occurs at approximately pH 9.0, but toxicity is greater at higher or lower pH values (Van der Westhuizen & Eloff, 1983).
- Omission of nitrogen or inorganic carbon causes an approximately tenfold decrease in toxicity (Carmichael, 1986).
- Toxin-producing strains that are repeatedly subcultured in media enriched by nutrients undergo a decrease or loss of toxin production over time (Carmichael, 1994).
- 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 clear how Fe^{2+} deficiency modulates microcystin production, but it has been noted that as cyanobacteria experience iron stress, they appear to compensate for some of the effects of iron loss by synthesising new polypeptides (Lukač & Aegerter, 1993).

- The most important biological factor influencing toxicity appears to be natural algaecides produced by other phytoplankton species that mainly inhibit photosynthesis by affecting thylakoid integrity (Marwah *et al.*, 1995).

From these results it would seem as if higher levels of microcystin production are induced by stressful growth conditions. Considering the complex structure of microcystins, it appears unlikely that they are waste products or that their presence is fortuitous or accidental. On the contrary, the energy cost involved in the production of microcystins is likely to be high and can only be justified if they meet particular needs and requirements. As with other secondary metabolites, microcystins are produced from monomeric substrates, products and intermediates of primary metabolism (Rinehart *et al.*, 1988).

The persistence in cyanobacteria of the genetic information for the regulation and catalysis of such complex products, despite the frequency of deleterious mutations would seem to indicate specific biological functions. Also, besides being widely produced, microcystins can be present at up to 0.2 µg microcystin per µg chlorophyll *a* (Lawton *et al.*, 1994).

It is unlikely that the production of such abundant products would have been retained throughout cyanobacterial evolution unless they have biological functions.

2.5 Detection of Microcystins

Accurate detection and quantitation of toxins in cyanobacterial blooms are extremely important due to the serious health risks involved. There are several biological and physiochemical screening methods available for the detection of microcystins.

The majority of routine testing of blue-green algal toxicity is done using male Swiss Albino mice, of an approximate weight of 25 g to 30 g. Toxicity is assayed by intraperitoneal injection of 0.1 - 1.0 mL of material into mice followed by 24 h of observation. At the end of 24 h all animals, still alive, are sacrificed for post-mortem examination of tissue injury. After injection, the animals become progressively pale due to blood loss and die within 15 min to 4 h after injection from circulatory failure.

Autopsy shows extensive haemorrhage and swelling of the liver, with minor signs of damage to other tissues (Falconer *et al.*, 1981). Animals subjected to a non-lethal dose show a dose-dependent congestion of the liver that demonstrates sinusoidal breakdown and infiltration of erythrocytes into areas of disorganised hepatocytes (Naseem *et al.*, 1991). This method is, for obvious reasons, extremely inhumane and fortunately other methods are available.

Another widely used method for the detection of microcystins has been described by Lawton *et al.* (1994). This method involves breaking *M. aeruginosa* cells mechanically and removing the cell debris by centrifugation. The supernatant is then dissolved in a mobile phase which is separated on a high performance liquid chromatography (HPLC) column.

Toxins can then be detected based on characteristic retention times.

A physiochemical method has been reported, based on the detection of 3-methoxy-2-methyl-4-phenylbutric acid (MMPB) by gas chromatography (GC). MMPB is produced as an oxidation product of microcystins using a flame ionisation detector or HPLC with a fluorescence monitor (Sano *et al.*, 1992; Tanaka *et al.*, 1993). This method requires tedious procedures such as extraction, cleanup, oxidation, post-treatment in order to eliminate reagents used, and derivatisation for GC and HPLC analysis. This physiochemical method is less sensitive than the biological method described, but screening is more accurate because it measures MMPB derived directly from the Adda moiety (Tanaka *et al.*, 1993).

Harada *et al.* (1996) described a chemical screening method for microcystins in cyanobacteria, which consists of the formation of MMPB by ozonolysis, and the detection of MMPB by thermospray-liquid chromatography/mass spectrometry or electron ionisation-gas chromatography/mass spectrometry using selected ion monitoring. This method is applicable as a simple, selective screening method for microcystins and their accurate quantitation, and can be performed within 30 minutes. The most remarkable feature of this method is that it is directly applicable to samples in the solid state without any complicated operations such as extraction and cleanup procedures. This also means that other solid samples such as shellfish, fish, animal tissues and sediment could be directly analysed. The only negative aspect of this method is that it cannot distinguish between individual microcystins.

Biological methods include enzyme-linked immunosorbent assay (ELISA) (Chu *et al.*, 1989) and protein phosphatase (PP) assay (Holmes, 1991). These methods are very sensitive and convenient for treating a large number of samples. Problems encountered with these methods include the following: ELISA

the inhibited enzymes (Falconer & Yeung, 1992; Eriksson *et al.*, 1990; Yoshiziwa *et al.*, 1990). Evidence of increased phosphorylation of cytokeratins after exposure of hepatocytes to microcystin, and a relocation from the insoluble cytoskeletal fraction to the cytosol fraction, supports the idea that the major toxic action is exerted by increasing phosphorylation of intermediate filament cytokeratins (Eriksson *et al.*, 1990).

A study conducted by Delaney and Wilkins in 1995 demonstrated that microcystin-LR is a potent insecticide, comparable in efficacy to various other insecticides such as rotenone, malathion and carbofuran. The mechanism of toxicity in insects however, is unknown. There is speculation that the acute hepatoxicity of microcystin-LR in mammals mask other, more chronic effects such as initiation of the inflammatory response (Naseem *et al.*, 1991) and/or disruption of the immune system (Adams *et al.*, 1989).

In addition to microcystins Henning *et al.* (1992) found as yet undescribed substances in crude extracts from *M. aeruginosa*. These substances result in disruption of cell membranes and liberation of lactate dehydrogenase (LDH) in primary and permanent Chang liver cell lines.

2.6.2 Tumours

Microcystins have also been implicated in causing liver cancer in humans exposed to low levels over a period of time (Falconer, 1991; Nishiwaki-Matsushima *et al.*, 1992; Yoshiziwa *et al.*, 1990). Nishiwaki-Matsushima *et al.*, (1992) found that to date, microcystin was the most potent liver tumour promoter which they had analyzed. The mechanism of tumour promotion by microcystin-LR is likely to be as a result of the inhibition of dephosphorylation by protein phosphatases 1 and 2A. This results in hyperphosphorylation of particular proteins concerned with a range of actions in the cell cycle (Falconer, 1991).

An area of importance with respect to microcystin toxicity is the influence of hyperphosphorylation of the cell cytoskeleton, which results in a transition to an apparently mitotic state. This change relates to tumour promotion, since increased mitosis is an essential part of accelerated tissue growth. The loss of cell-cell contact resulting from microcystin toxicity could be expected to reduce the normal contact inhibition of cell replication in organs, which is also related to tumour growth (Falconer & Yeung, 1992).

2.7 Control and Degradation

2.7.1 Chemical

Lam and co-workers (1995) found that most of the microcystin-LR present in cells remains inside the cell until the cell is lysed. To control cyanobacterial blooms, cells are usually lysed in the presence of chemicals (e.g. Reglone A, NaOCl, $KMnO_4$, Simazine and $CuSO_4$) that inhibit new cell wall synthesis, enzymatic reactions or photosynthesis (Kenefick *et al.*, 1993; Lam *et al.*, 1995). Any sudden release of microcystins into the surrounding water can present a significant hazard to livestock and humans using the water (Lam *et al.*, 1995).

Treatments with lime and alum, on the other hand have been found to control blooms mainly by cell-coagulation and sedimentation without any significant increase in microcystin concentration in the surrounding water (Kenefick *et al.*, 1993; Lam *et al.*, 1995). It would seem, then, that these treatments would be favorable for the chemical control of *Microcystis* spp. blooms if the sedimented cells are removed.

It has been demonstrated, however, that microcystins persist in dried crusts of lakes formed as water levels recede during dry seasons. Large quantities of microcystins leach from the dry material upon re-wetting within 48 hours (Jones *et al.*, 1995). This could present a significant problem

with coagulation and sedimentation treatments, as the water would not be suitable for consumption for up to three weeks before biodegradation commences (Jones, 1990).

2.7.2 Biological

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

Newman and Barrett (1993) demonstrated that decomposing barley straw effectively inhibits growth of *M. aeruginosa* to 6 % 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 mixture of chemicals, are so far unidentified, but there are several probabilities: firstly, antibiotics may be produced by fungal flora active in decomposition of the straw; secondly, modified cell wall components released during decomposition may have an effect on cyanobacterial growth; and thirdly phenolic compounds and other aromatic compounds produced during biodegradation of cell walls may also contribute to the effect. The inhibitory effect seems to be algistatic rather than algicidal, therefore, the presence of decomposing barley straw can help prevent the development of blue-green algal blooms by inhibiting a rapid population increase if applied when population numbers are low (Newman and Barrett, 1993).

Marwah and co-workers (1995) found that *Oscillatoria late-virens* produces an algicidal by-product that interacts with toxin-producing species of *M. aeruginosa*. This natural algaecide abruptly inactivates photosystem II-mediated electron flow, reduces pigments and protein content by

affecting thylakoid integrity and lowers toxicity of the microcystins. The limitation of this treatment method is the uncontrolled growth of protozoa and bacteria as a result of decaying substrates released into the water. Application at the onset of bloom formation should be advantageous because the release of decaying substrates would be limited.

3 Materials and Methods

3.1 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 PCC 7806 and PCC 7813 were obtained from the Pasteur Institute Culture Collection, France; UV 027 from the University of the Free State Culture Collection, South Africa; CCAP 1450/1 obtained from the Culture Collection of Algae and Protozoa, Institute of Freshwater Ecology, UK; NIES 88, NIES 89, NIES 91, NIES 99 from the National Institute for Environmental Studies, Japan. All these strains were received as axenic, maintained as such and microscopically verified prior to further experiments.

Table 3.1 Table of M. aeruginosa *strains used in the study describing the sources strains were obtained from as well as toxicity of the various strains.*

Strain	Source	Toxicity
PCC 7806	Pasteur Culture Collection, France	Toxin-producing
PCC 7813	Pasteur Culture Collection, France	Toxin-producing
UV 027	University of the Free State Culture Collection	Toxin-producing
NIES 88	National Institute for Environmental Studies, Japan	Toxin-producing
NIES 89	National Institute for Environmental Studies, Japan	Toxin-producing
NIES 91	National Institute for Environmental Studies, Japan	Toxin-producing
NIES 99	National Institute for Environmental Studies, Japan	Toxin-producing
CCAP 1450/1	Institute of Freshwater Ecology, UK	Non toxin-producing

Strains were maintained at a temperature of approximately 24 °C in liquid BG-11 nutrient medium containing 17.65 mM $NaNO_3$, 0.18 mM $K_2HPO_4.3H_2O$, 030 mM $MgSO_4.7H_2O$, 0.25 mM $CaCl_2.2H_2O$, 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 $MnCl_2.4H_2O$, 0.77 mM $ZnSO_4.7H_2O$, 1.61 mM $Na_2MoO_4.2H_2O$, 0.37 mM $CuSO_4.5H_2O$ and 0.17 mM $Co(NO_3)_2.6H_2O$. Cultures were grown under constant light of approximately 60 µmol quanta/m^2/s at pH 8.0.

3.2 DNA Analysis

3.2.1 DNA Isolation

Approximately 15 mL culture of strains PCC 7813, UV 027 and CCAP 1450/1 was aliquoted into JA20 centrifuge tubes and centrifuged for 10 minutes at 4 000 rpm in a Beckman Model J2-21 centrifuge to separate the cells from the growth medium. The supernatant was removed and the cell pellet resuspended in 500 µL 1X TE buffer (pH 8)(10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and transferred to sterile 1.5 mL Eppendorf tubes. Cells were lysed by the addition of 5 mg lysozyme and then incubated at 50 °C for 30 minutes. Subsequently, 100 µg proteinase-K and 10% SDS (sodium dodecyl sulfate) were added and the cells were incubated at 50 °C for an additional 10 minutes. An equal volume phenol-chloroform-isoamylalcohol (25:24:1 v/v) was added, thoroughly vortexed and centrifuged at 14 000 rpm in a Sigma 2MK centrifuge for 5 minutes. The supernatant was transferred to a fresh 1.5 mL Eppendorf tube. This step was repeated at least two times until the interphase appeared relatively clean. To quantitatively remove phenol from the reaction, an equal volume chloroform was added. The tubes were vortexed and centrifuged at 14 000 rpm for 5 minutes. This step was repeated as previously until the interphase appeared relatively clean. The supernatant was transferred to a clean 1.5 mL Eppendorf tube and an equal volume cold 100 % ethanol and 0.2 M NaCl was added.

To facilitate precipitation of the genomic DNA, tubes were stored at 4 °C for at least 2 h. This mixture was centrifuged at 14 000 rpm for 20 minutes at 4 °C and the

supernatant removed by aspiration. The pellet was washed with 800 µL cold 70 % ethanol and then centrifuged at 14 000 rpm for 5 minutes at 4 °C. The supernatant was removed and the pellet vacuum-dried in a SpeedVac Concentrator SVC 100H (Savant). The pellet was resuspended in 50 - 500 µL ddH_2O (double distilled water), aliquoted into 15 µL volumes and stored at -20 °C until further analysis.

DNA concentrations were determined by visualisation on 1 % TAE (40 mM Tris-acetate, 1mM EDTA, pH 8.0) agarose gels (Techcomp Ltd.) as well as spectrophotometrically (Beckman DU650 Spectrophotometer).

3.2.2 Polymerase Chain Reaction (PCR)

The PCR reaction was optimized based on the Taguchi method 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 % Triton®X-100, 0.2 mM of each dATP, dTTP, dGTP and dCTP, 2 mM $MgCl_2$, 2.5 U Taq DNA Polymerase, all from Promega, and 0.8 pmol of any of two of the appropriate primers (Roche) (Table 3.2).

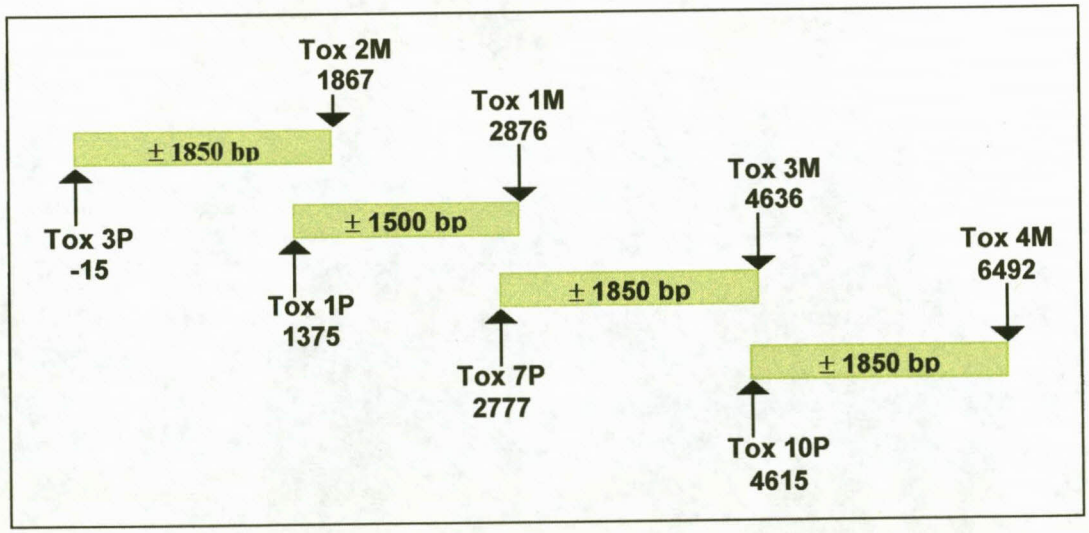

Figure 3.1 *Schematic representation of relative binding positions of primers in* mcyB *as well as approximate sizes of expected products.*

Table 3.2 *Description of primers used in the study describing expected sizes of products, relative binding positions, orientation and melting temperatures.*

Group	Primer	Sequence (5' – 3')	Orientation	Tm
1 ± 1850 bp	Tox 3P	GGAGAATCTTTCATGGCAGAC	Forward	62.4 °C
	Tox 4P	GCGTTGCTTGATGATTCAAC	Forward	57.9 °C
	Tox 5P	GCGATTCTTCTCAGTCGC	Forward	55.6 °C
	Tox 1P	CGATTGTTACTGATACTCGCC	Forward	57.9 °C
	Tox 2P	GGAACAAGTTGCACAGAATCCGC	Forward	62.4 °C
	Tox 2M	CCAATCCCTATCTAACACAGTACCT CGG	Reverse	65.1 °C
2 ± 1500 bp	Tox 1P	CGATTGTTACTGATACTCGCC	Forward	57.9 °C
	Tox 2P	GGAACAAGTTGCACAGAATCCGC	Forward	62.4 °C
	Tox 2M	CCAATCCCTATCTAACACAGTACCT CGG	Reverse	65.1 °C
	Tox 6P	GGGATCAAGACGCTTTTG	Forward	53.7 °C
	Tox 13P[§]	CATCAGGTTCAACGGGAAAC	Forward	57.9°C
	Tox 1M	TAAGCGGGCAGTTCCTGC	Reverse	58.2 °C
3 ± 1850 bp	Tox 7P	CCTCAGACAATCAACGGTTAG	Forward	53.7 °C
	Tox 8P	CTCTGACGGTAGCCACTATTC	Forward	59.8 °C
	Tox 9P	GCCTAATATAGAGCCATTGCC	Forward	57.9 °C
	Tox 3M	CGTGGATAATAGTACGGGTTTC	Reverse	58.4 °C
4 ± 1850 bp	Tox 10P	GCCTAATATAGAGCCATTGCC	Forward	59.8 °C
	Tox 11P	CCTTCTAGCTATGCCGGATG	Forward	59.4 °C
	Tox 12P	GAACTGGCTGAATGGCATC	Forward	56.7 °C
	Tox 4M	CCAGTGGGTTAATTGAGTCAG	Reverse	57.9 °C

The PCR-reaction was 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 (Fig. 3.2).

Primer used in strain PCC 7813
[§] Primer used in strain UV 027

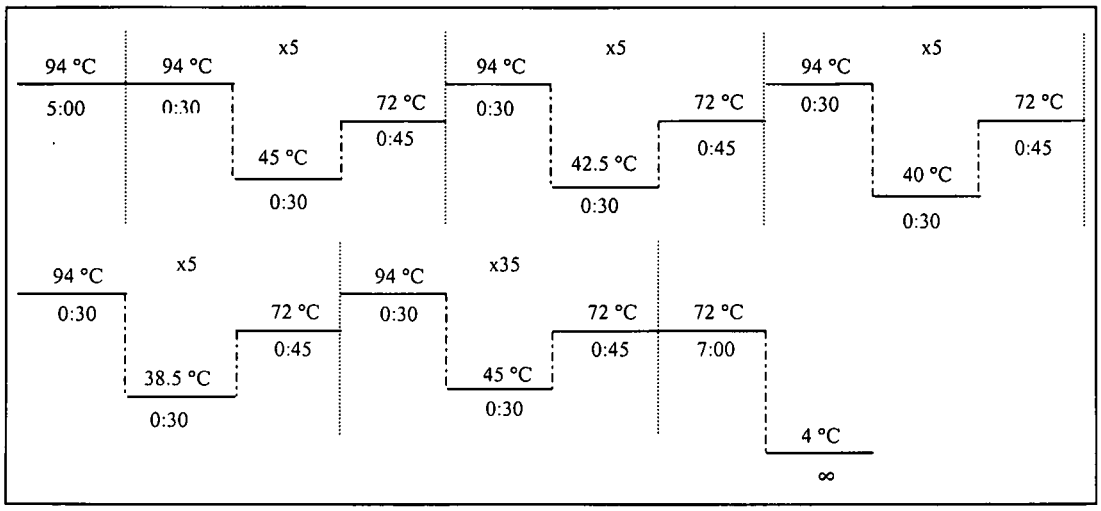

Figure 3.2 *Schematic diagram depicting the PCR cycle used to amplify the respective fragments from* mcyB *in strains PCC 7813, UV 027 and CCAP 1450/1.*

The products were analyzed by agarose gel electrophoresis through horizontal slab gels of 1 % agarose (Techcomp Ltd.) dissolved in 1X TAE buffer containing 0.15 µg/mL ethidium bromide (Sigma). The generated fragments were separated at 85 mV for 1 h, visualized under UV-light (Herolab UVT-28 M) and photographed.

3.2.3 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 guaninidine-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 in a table top centrifuge (Denver Instruments) 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 flow-through discarded. The tube was centrifuged for an additional 1 minutes 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.

3.2.4 Preparation of Competent E.coli Top 10 Cells

E.coli Top 10 cells were inoculated into 100 mL LB-media (10 g Bacto®-tryptone, 5 g Bacto®-yeast extract, 5 g NaCl) overnight at 37°C with shaking. Subsequently, a 200 µL aliquot of this preculture was inoculated into 5 mL LB-media and incubated at 37°C with shaking. Optical densities at 600 nm, were monitored until and OD of 0.9 - 0.95 was reached, after which the cells were centrifuged at 5 000 rpm for 5 minutes at 4°C. The pellets were resuspended in a 10 mL ice-cold solution of 80 mM $CaCl_2$ and 50 mM $MgCl_2$ and incubated for 10 minutes on ice. This step was repeated twice. The final pellet was resuspended in 2 mL ice-cold 0.1 mM $CaCl_2$ and mixed with an equal volume of 50 % glycerol. The cells were aliquoted into 80µL volumes, snap-frozen in liquid nitrogen and stored at -70°C (Tang *et al.* 1994).

3.2.5 Cloning into pGem®T-Easy (Promega)

Fragments generated with primer pairs Tox 3P/2M, Tox 1P/1M, Tox 7P/3M and Tox 10P/4M from PCC 7813 and UV 027 were subsequently cloned into the pGem®T-Easy vector (Promega). Approximately 10 % of the PCR product, 50 ng pGem®T-easy (Promega) vector and 3 Weiss units/µL T4 DNA Ligase (Promega) were added to 5 µL 2X Ligation Buffer (60 mM Tris-HCl, pH7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10 % polyethylene glycol). These reactions were made up to a final reaction volume of 10 µL with ddH_2O and incubated overnight at 4 °C. The ligation reactions were centrifuged briefly and half of each reaction was added to 500 µL competent *E.coli* cells, mixed and placed on ice for 30 minutes. This mixture was then heat shocked at 42 °C for 60 seconds and placed on ice for 2 minutes.

To this, 870 µL LB-medium and 40 mM glucose was added and then incubated at 37 °C for 1 h. The cells were centrifuged

at 4 000 rpm in a table top centrifuge (Denver Instruments) for 1 minute and the supernatant discarded. The cells were resuspended in 100 µL LB-media, plated out on LB/IPTG (isopropylthio-β-D-galactoside)/X-gal (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 in a Sigma 2MK centrifuge 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 % Triton®X-100). Lysozyme, 0.15, mg was added and the cells incubated at room temperature for 5 minutes. To facilitate lysis the cells were then incubated at 95 °C for 1 minute and centrifuged at 14 000 rpm in a Sigma 2MK centrifuge for 15 minutes at 4 °C. The pellet was removed, 5 % CTAB (N-cetyl-N-N-N-trimethylammonium bromide) was added to the supernatant and centrifuged at 14 000 rpm for 5 minutes in a Sigma 2MK centrifuge.

The supernatant was discarded, the pellet resuspended in 300 µL 1.2 M NaCl and 750 µL cold 100 % 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 µL ddH_2O.

The inserts were verified by restriction analysis with approximately 1 µg plasmid DNA, 5 U *Eco*RI, 50 mM Tris-HCl, 10 mM $MgAc_2$, 10 mM $MgCl_2$, 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 (Herolab UVT-28 M).

3.3 Sequencing

Sequencing of the fragments were performed using the DYEnamic™ ET Terminator Cycle Sequencing Premix Kit (Amersham Pharmacia Biotech, Inc.) and the ABI Prism® Big Dye® Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). Sequencing reactions were performed according to the various 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 was 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 was analyzed 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/).

3.4 Southern Blot

3.4.1 Labelling of Tox 7P/3M/PCC 7813 Fragment

A PCR fragment generated from toxin producing strain PCC 7813 with primer pair Tox 7P/3M, spanning the phosphopantetheine attachment site from the first domain from *mcyB* and part of the condensation site of the second domain (Fig. 2.2), was randomly labelled with digoxigenin

with the DIG DNA Labelling and Detection Kit (Roche). PCR fragments were diluted to 0.5 µg - 3 µg and denatured at 94 °C for 10 minutes. On ice 2 µL Hexanucleotide Mix (62.5 A_{260}), 40 µM dATP, 40 µM dCTP, 40 µM dGTP, 26 µM dTTP, 14 µM DIG-dUTP; pH 7.5 and 2 U Klenow enzyme was added and then incubated at 37 °C for approximately 24 hours.

To stop the reaction 20 mM EDTA, pH 8.0 was added. Labelled DNA was precipitated by the addition of 0.1 M LiCl and 75 µL cold 100 % ethanol and then incubated at -70 °C for 30 minutes. The reactions were centrifuged at 14 000 rpm for 15 minutes at 4 °C in a Sigma 2MK centrifuge and the supernatant removed. The pellet was washed by the addition of 50 µL cold 70 % ethanol and centrifuged at 14 000 rpm for 5 minutes at 4 °C. The supernatant was removed and the pellet vacuum-dried in a SpeedVac Concentrator SVC 100H (Savant). The pellet was resuspended in 50 µL TE-buffer.

3.4.2 Quantification of Tox 7P/3M/PCC 7813 Probe

A dilution series of Control DNA (Roche) and the Tox 7P/3M/PCC 7813 probe ranging from approximately 10 pg to 0.01 pg was spotted onto a positively charged nylon membrane (Roche). Labelled fragments were UV-crosslinked to the membrane in a GS Gene Linker™ UV Chamber (Bio-Rad). The membrane was equilibrated in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5 and 0.3 % (v/v) Tween® 20 for 2 minutes and then blocked for 30 minutes in 1 % (w/v) Blocking Reagent (Roche) dissolved in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5.

Polyclonal sheep anti-digoxigenin (75 mU) conjugated to alkaline phosphatase was added to 1 % (w/v) Blocking Reagent (Roche) dissolved in 0.1 M maleic acid and 0.15 M NaCl, pH 7.5 and incubated for 30 minutes. The membrane was washed twice for 15 minutes in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5 and 0.3 % (v/v) Tween® 20 and equilibrated in

0.1 M Tris-HCl, 50 mM $MgCl_2$ and 0.1 M NaCl, pH 9.5 for 2 minutes.

The color reaction was performed in 10 mL 0.1 M Tris-HCl, 0.05 M $MgCl_2$ and 0.1 M NaCl, pH 9.5 with 9.2 mg/mL NBT (nitroblue tetrazolium salt) and 4.5 mg/mL BCIP (5-bromo-4-chloro-3-indolyl phosphate)/X-phosphate (toluidinium salt) in DMF (dimethylformamide) for approximately 16 hours in the dark. Spot intensities between the labelled fragments and Control DNA were compared to estimate the concentrations of the respective DIG-labelled probes.

3.4.3 DNA Extraction

DNA was extracted from strains PCC 7806, PCC 7813, UV 027, NIES 88, NIES 89, NIES 91, NIES 99 and CCAP 1450/1 as described in Section 3.2.1.

3.4.4 Genomic DNA Restriction Analysis

Approximately 1 ng of DNA from all strains, i.e. PCC 7806, PCC 7813, UV 027, NIES 88, NIES 89, NIES 91, NIES 99 and CCAP 1450/1 was incubated at 37 °C with 25 U *Pvu*II, 10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM DTE (dithioerythritol) at pH 7.5 (Roche). Approximately 50 % of each reaction was loaded onto a 0.8 % TAE agarose gel (Techcomp Ltd.) containing 0.15 mg ethidium bromide (Sigma), separated at 85 mV for 90 minutes, visualized under UV-light (Herolab UVT-28 M) and photographed.

3.4.5 Transfer to Nylon Membrane

Digested genomic DNA was blotted onto a nylon membrane by vacuum transfer. The gel was depurinated with 0.25 N HCl and 0.5 M NaOH, 1.5 M NaCl for approximately 7 minutes each. The agarose gel was placed onto a positively charged nylon membrane (Roche) and a vacuum of approximately 50 mBar applied. Transfer of the DNA fragments was accomplished using 20X SSC buffer (0.3 M NaCitrate, 3 M NaCl, pH 7.0). The membrane was then washed in 20X SSC buffer briefly and

the DNA UV-crosslinked to the membrane in a GS Gene Linker ™ UV Chamber (Bio-Rad).

3.4.6 Hybridisation with Tox 7P/3M/PCC 7813 Probe

The membrane was incubated at 50 °C for at least 30 minutes in Dig Easy Hyb (Roche). Approximately 250 ng of the DIG-labelled probe, generated with primer pair Tox7P/3M from strain PCC 7813, was denatured at 100 °C for 10 minutes and added to 5 mL pre-heated (50 °C) Dig Easy Hyb (Roche). The membrane was probed overnight at 50 °C in a roller tube.

3.4.7 Detection with NBT/BCIP

The membrane was washed twice in 2X SSC buffer (30 mM NaCitrate, 0.3 M NaCl, pH 7.0) and 0.1 % SDS for 10 minutes and twice in 0.5X SSC buffer (7.5 mM NaCitrate, 75 mM NaCl, pH 7.0) and 0.1 % SDS for 15 minutes. The membrane was then equilibrated for 2 minutes in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5 and 0.3 % (v/v) Tween® 20 for 2 minutes and blocked for 30 minutes in 1 % w/v Blocking Reagent (Roche) dissolved in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5.
Polyclonal sheep anti-digoxigenin (75 mU) conjugated to alkaline phosphatase was added to 2.5 mL Dig Easy Hyb (Roche) and incubated for 30 minutes. The membrane was then washed twice for 15 minutes in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5 and 0.3 % (v/v) Tween® 20 and equilibrated in 0.1 M Tris-HCl, 50 mM $MgCl_2$, 0.1 M NaCl (pH 9.5) for 2 minutes.

The color reaction was performed in 0.1 M Tris-HCl, 50 mM $MgCl_2$, 0.1 M NaCl (pH 9.5) with 9.2 mg/mL NBT (nitroblue tetrazolium salt) and 4.5 mg/mL BCIP (5-bromo-4-chloro-3-indolyl phosphate)/X-phosphate (toluidinium salt) in DMF (dimethylformamide) for 4 - 16 hours in the dark.

3.4.8 Stripping of Membrane

The membrane was incubated in DMF (dimethylformamide), heated to 50 °C until the blue color had been removed and

then thoroughly rinsed in ddH_2O. The DIG-labelled probe was removed by washing the membrane twice in 0.2 M NaOH, 0.1 % SDS at 37 °C. The membrane was then rinsed twice in 2X SSC.

3.4.9 Labelling of Tox 1P/1M/PCC 7813 Fragment

A second fragment from strain PCC 7813, generated with primer pair Tox 1P/1M, spanning a part of the AMP-binding site from the first domain from *mcyB* was labelled as described in Section 3.4.1.

3.4.10 Quantification of Tox 1P/1M/PCC 7813 Probe

The DIG-labelled Tox 1P/1M/PCC 7813 probe was quantified as described in Section 3.4.2.

3.4.11 Hybridisation with Tox 1P/1M/PCC 7813 Probe

Hybridisation with the Tox 1P/1M/PCC 7813 probe was performed as described in Section 3.4.6.

3.4.12 Detection with NBT/BCIP

Detection was performed as described in Section 3.4.7.

3.5 Toxin Analysis

3.5.1 Toxin Extraction and HPLC Analysis

Approximately 15 mL *M. aeruginosa* cells from each strain were harvested and centrifuged at 4 000 rpm in a Beckman Model J2-21 Centrifuge for 15 minutes. The supernatant was discarded and the cells resuspended in 3 mL ddH_2O. This suspension was then subjected to ultrasonication continiously for 15 minutes at 4 °C, and centrifuged at 14 000 rpm for 20 minutes in a Sigma 2MK centrifuge. Breaking of the cells was confirmed visually with a Zeiss Axioscop MC 80 microscope. The supernatant was filtered

through 45 µm Whatman nitrocellulose filters and 10 - 20 µL of the filtrate was used in toxin assays.

The mobile phase consisted of acetonitrile, 10 mM ammonium acetate, (26:74 v/v) at pH 6.0 at approximately 23 °C with a flow-rate of 0.8 mL/min. Microcystin-LR standard (generously provided by Mr. Downing*) was analysed to identify characteristic retention times for microcystin-LR. Samples were manually injected into a Phenomenex 250 x 4.6 mm, 5µ, Jupiter, 300 A C_{18} column and separated for 25 minutes. Absorbance values at 238 nm were detected with a JPD-10A VP Liquid Chromatograph and integrated with a C-R6A Chromatopac (Shimadzu).

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4 Results

4.1 Polymerase Chain Reaction

Four PCR primer pairs, based on the sequence of *mcyB* in *M. aeruginosa* strain PCC 7806, were used to amplify in total approximately 6.5 kb from a target sequence in strains PCC 7813 and UV 027, respectively. Genomic DNA was extracted from toxin-producing strains PCC 7813, UV 027 and non toxin-producing strain CCAP 1450/1. Four pairs of oligonucleotide primers were synthesised based on the sequence of *mcyB* from strain PCC 7806. These primer pairs were used in PCR reactions with genomic DNA from strains PCC 7813, UV 027 and CCAP 1450/1. A wide variety of parameters were tested, including reaction components and cycling parameters before it was possible to successfully generate PCR products with any of the strains tested.

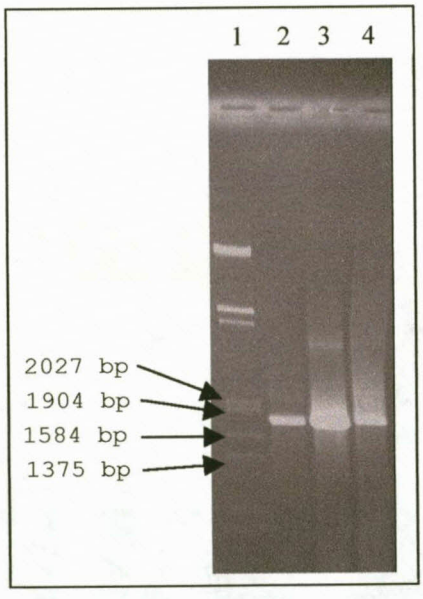

Figure 4.1
PCR amplification of a ± 1850 bp product in M. aeruginosa *strains with primer pair Tox 3P/2M.*

Lane1: Eco*RI digested λDNA;*
Lane2: *± 1850 bp fragment from PCC 7813;*
Lane3: *± 1850 bp fragment from UV 027;*
Lane4: *± 1850 bp fragment from CCAP 1450/1.*

Fig. 4.1 showed the PCR amplification of the expected size fragments with primer pair Tox 3P/2M from strains PCC 7813, UV 027 and CCAP 1450/1. The size of the products amplified correlated well with the expected size of 1866 bp from the known sequence of *mcyB*.

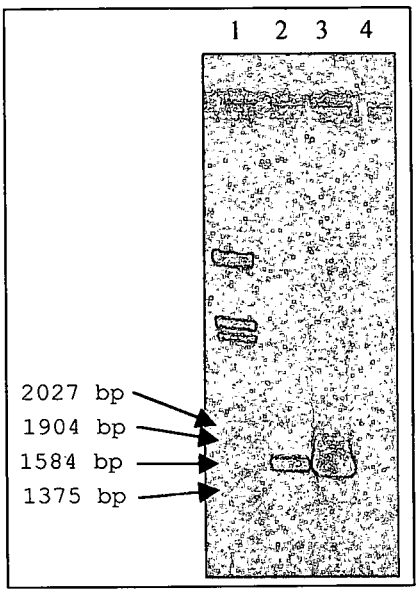

Figure 4.2
PCR amplification of a ± 1500 bp product in M. aeruginosa *strains with primer pair Tox 1P/1M.*

Lane1: Eco*RI digested λDNA;*
Lane2: *± 1500 bp fragment from PCC 7813;*
Lane3: *± 1500 bp fragment from UV 027;*
Lane4: *No amplification with CCAP 1450/1.*

In Fig. 4.2 the results from the PCR amplification with primer pair Tox 1P/1M is shown. A PCR product of ± 1500 bp was amplified from strains PCC 7813 and UV 027. The size of the fragments successfully amplified with this primer pair corresponded to the expected size, i.e. 1401 bp, based on the sequence of *mcyB*. Primer pair 1P/1M failed to amplify PCR product from strain CCAP 1450/1.

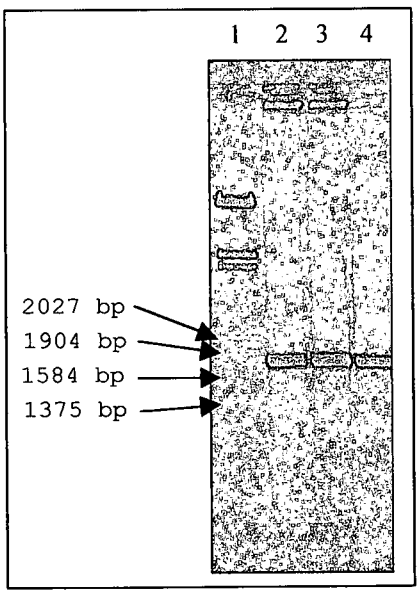

Figure 4.3
PCR amplification of a ± 1850 bp product in M. aeruginosa *strains with primer pair Tox 7P/3M.*

Lane1: Eco*RI digested λDNA;*
Lane2: *± 1850 bp fragment from PCC 7813;*
Lane3: *± 1850 bp fragment from UV 027;*
Lane4: *± 1850 bp fragment from CCAP 1450/1.*

Amplification with primer pair Tox 7P/3M is shown in Fig. 4.3. A ± 1850 bp product was amplified from strains PCC 7813, UV 027 and CCAP 1450/1 respectively. The expected size of the fragments, based on the sequence of *mcyB* from strain PCC 7806, was 1859 bp, which corresponded well with

the size of fragments successfully amplified using these primers.

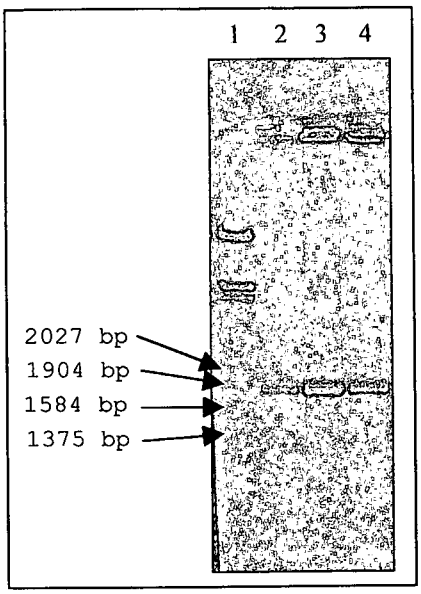

Figure 4.4
PCR amplification of a ± 1850 bp product of M. aeruginosa *strains with primer pair Tox 10P/4M.*

Lane1: Eco*RI digested λDNA;*
Lane2: *± 1850 bp fragment from PCC 7813;*
Lane3: *± 1850 bp fragment from UV 027;*
Lane4: *± 1850 bp fragment for CCAP 1450/1.*

Fig. 4.4 showed the amplification of an approximately 1850 bp product with primer pair Tox 10P/4M for strains PCC 7813, UV 027 and CCAP 1450/1 respectively. The sizes of the fragments amplified correlated well with the expected size of 1877 bp based on the known sequence of *mcyB*.

4.2 Sequencing

All the fragments generated with primers targeted to amplify *mcyB* from toxin-producing strains PCC 7813 and UV 027 were cloned into pGem®T-easy (Promega). The fragments were subsequently sequenced with primers used to generate the fragments, various internal primers as well as T7 and Sp6 that bind on the pGem®T-easy vector (Promega). Sequencing data was subsequently assembled according to basepair similarities. The assembled sequences were deposited in the Genbank database as Accession number AY034601 for PCC 7813 and Accession number AY034602 for UV 027. The sequences generated were analysed and certain regions identified based on similar sequences present in other peptide synthetases (Appendix A).

The DNA sequences were translated to amino acids and compared to the translated sequence of *mcyB* as well as to another peptide synthetase, *grsA*, encoding gramicidin S, in the Genbank database. The amino acid sequences also share similarities and homologous regions (Appendix B).

No structural information on the 3D conformation of microcystin synthetase is available at present, but based on the degree of homology between the amino acid sequence of *mcyB* and *grsA* (Appendix B) it is highly possible that these two molecules will have a similar 3D structure. Fig. 4.5 is a graphic representation of the phenylalanine-activating subunit of gramicidin S synthetase (PheA). During the synthesis of gramicidin S, this subunit catalyses the activation of L-phenylalanine to the corresponding acyl-adenylate as well as inversion of the configuration of the amino acid with the concurrent conversion of ATP to AMP (Conti *et al.*, 1997).

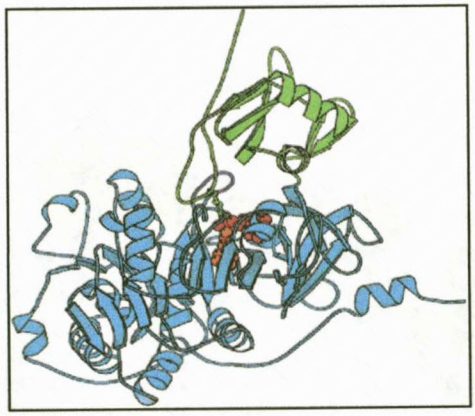

Figure 4.5
Structure of the phenylalanine-activating subunit of gramicidin S synthetase (PheA) (Conti et al., *1997).*

Fig. 4.5 shows the binding of phenylalanine (orange) and AMP (red) to the phenylalanine activating site (PheA) (Conti *et al.*, 1997). The AMP-binding site of the microcystin synthetase would fold in a similar way because of the amino acid sequence similarity between these two domains in microcystin synthetase and gramicidin S synthetase.

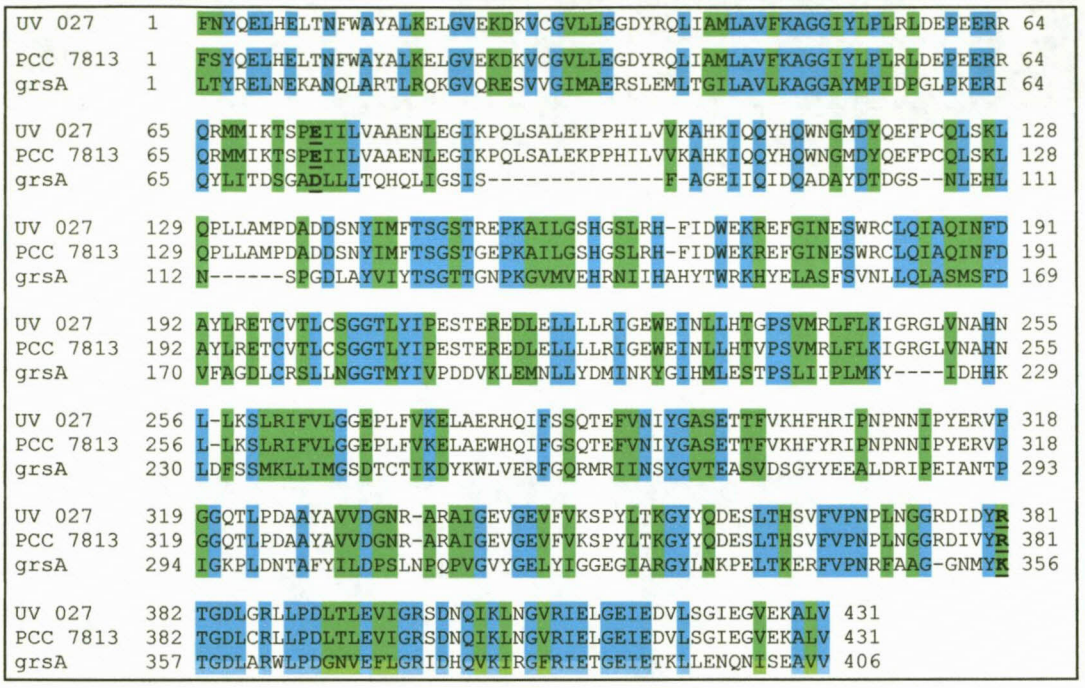

Figure 4.6 *Alignment of amino acid sequences from toxin-producing strains PCC 7813 and UV 027 with the corresponding portion of PheA.*

Fig. 4.6 shows the alignment of AMP-binding site from the second domain from microcystin synthetase from the toxin-producing strains PCC 7813, UV 027 and the amino acid sequence of PheA.

The AMP-binding site has been identified as the specificity determining site in PheA where binding of the amino acid to be activated takes place (Conti *et al.*, 1997). Based on this statement it is realistic to expect that the AMP-binding sites from microcystin synthetase and PheA should differ with respect to the amino acids in the specificity determining pocket of the enzyme as the two enzyme modules are specific for the activation of different amino acids. It is also realistic to expect that these amino acids would not be completely different, as the type of interaction between the amino acid residues involved in binding the substrate amino acid is likely to be similar.

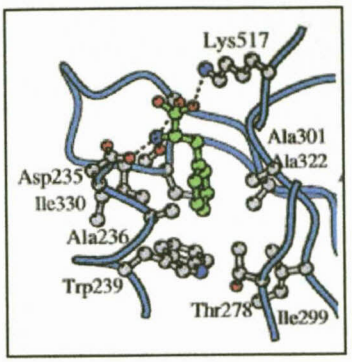

Figure 4.7
Diagram showing the side chains of PheA that line the specificity pocket for the phenylalanine substrate (indicated in green) (Conti et al., *1997).*

Fig. 4.7 shows the side chains of PheA that line the specificity determining pocket for the phenylalanine substrate. The two amino acid residues involved in the binding of the substrate are indicated as Lys 517 and Asp 235. As explained earlier, the conformational structure of microcystin synthetase and mechanism will probably be similar to that of PheA, but the amino acids involved in forming the specificity pocket will probably differ. In Fig. 4.6 the two amino acid residues involved in the binding with the phenylalanine substrate, Lys 517 (K) and Asp 235 (D), are underlined and printed in bold. As indicated in green in Fig. 4.6, the amino acid substitutions in microcystin syntetase are conserved substitutions with respect to PheA.

4.3. Southern Blot

4.3.1 Genomic DNA Restriction Analysis with PvuII

Genomic DNA of each of the strains investigated was digested with *Pvu*II and separated on an agarose gel confirm proper digestion (Fig. 4.8).

As can be seen in Fig. 4.8, proper digestion was possible for strains PCC 7806, PCC 7813, UV 027, CCAP 1450/1, NIES 88, and NIES 91. Genomic DNA from strains NIES 89, and NIES 91, was not completely digested by *Pvu*II.

Band and DNA-smear intensities differed because of the variation in the quantity of DNA extracted from the various strains. This was also reflected in the Southern Blots represented in Fig. 4.10 and 4.12.

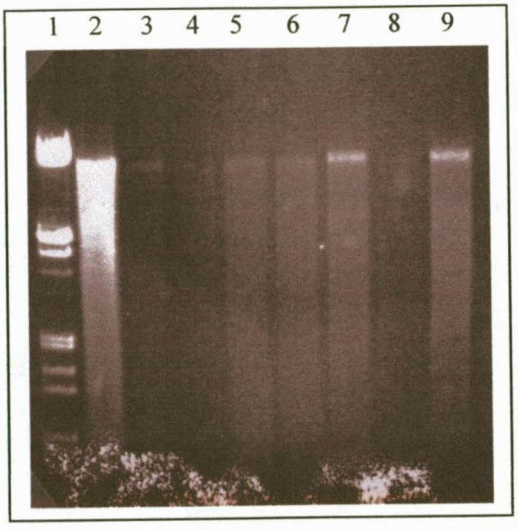

Figure 4.8
PvuII *digested genomic DNA.*

Lane 1: EcoRI *digested λDNA;*
Lane 2: *Genomic DNA from PCC 7806*
Lane 3: *Genomic DNA from PCC 7813*
Lane 4: *Genomic DNA from UV 027*
Lane 5 *Genomic DNA from CCAP 1450/1*
Lane 6: *Genomic DNA from NIES 88*
Lane 7: *Genomic DNA from NIES 89*
Lane 8: *Genomic DNA from NIES 91*
Lane 9: *Genomic DNA from NIES 99.*

4.3.2 DIG-Labelling of Tox 7P/3M/PCC 7813 Fragment

A PCR-fragment of ± 1850 bp, generated with primers Tox 7P/3M from the toxin-producing strain PCC 7813 (Fig. 4.3, lane 2) was randomly labelled with digoxigenin to synthesize a probe which could be used in screening *M. aeruginosa* strains for the presence of *mcyB*. This fragment represented the phosphopantetheine attachment site from the first domain from *mcyB* and part of the condensation site of the second domain, as has been verified by sequencing.

4.3.3 Probe Quantification

The labelled Tox 7P/3M/PCC 7813 fragment was quantified by a dilution series to assess the labelling efficiency and concentration of synthesised probe (Fig. 4.9).

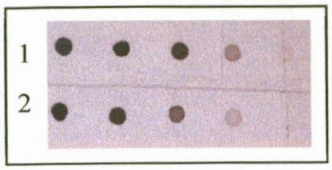

Figure 4.9
Dilution series ranging from approximately 10 pg to 0.01 pg to quantify fragments generated with primers Tox 7P/3M from PCC 7813 randomly labelled with digoxigenin.

Row 1 *Randomly labelled Tox 7P/3M/PCC 7813 probe*
Row 2 *Labelled Control DNA.*

4.3.4 Probing with Tox 7P/3M/PCC 7813 Probe

The profiles generated by restriction of total genomic DNA with *Pvu*II was transferred to a positively charged nylon membrane and probed with the randomly labelled dioxigenin Tox 7P/3M/PCC 7813 fragments. This probe hybridised to an approximately 13 kb fragment in all strains which corresponded well to the expected size of 12.973 kb.

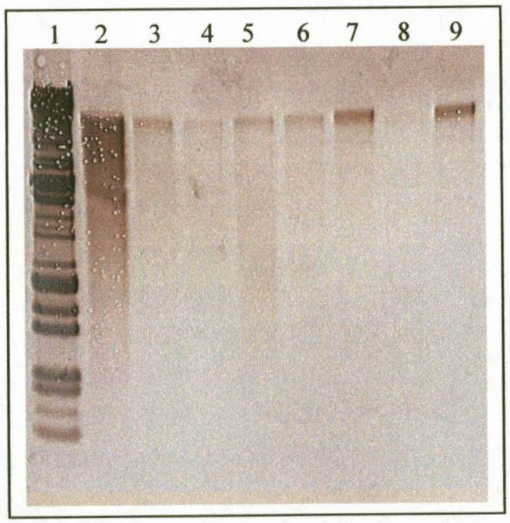

Figure 4.10
Hybridisation of the Tox 7P/3M/PCC 7813 probe randomly labelled with digoxigenin to a ± 13 kb fragment generated with Pvu*II.*

Lane1: Eco*RI digested λDNA;*
Lane2: *± 13 kb fragment from PCC 7806*
Lane3: *± 13 kb fragment from PCC 7813*
Lane4: *± 13 kb fragment from UV 027*
Lane5: *± 13 kb fragment from CCAP 1450/1*
Lane6: *± 13 kb fragment from NIES 88*
Lane7: *± 13 kb fragment from NIES 89*
Lane8: *± 13 kb fragment from NIES 91*
Lane9: *± 13 kb fragment from NIES 99.*

Fig. 4.10 showed the hybridization of the Tox 7P/3M/PCC 7813 probe to an approximately 13 kb fragment with all toxin-producing strains as well as the non toxin-producing strain CCAP 1450/1.

4.3.5 DIG-Labeling of Tox 1P/1M/PCC 7813 Fragment

A second PCR-fragment of ± 1500 bp, obtained from PCC 7813 with primer pair Tox 1P/1M (Fig. 4.2, lane 2) was labelled

with dioxigenin. This particular fragment was chosen because no PCR product was visibly amplified with strain CCAP 1450/1 with this primer pair. This fragment represented part of the AMP-binding site from the first domain from *mcyB*.

4.3.6 Probe Quantification

The labelled Tox 1P/1M/PCC 7813 fragments were quantified by a dilution series to assess the labelling efficiency (Fig. 4.11).

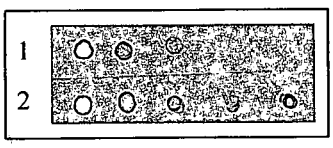

Figure 4.11
Dilution series ranging from approximately 10 pg to 0.01 pg to quantify fragments generated with Tox 1P/1M from PCC 7813 randomly labelled with digoxigenin.

Row 1 *Labelled Control DNA*
Row 2 *Randomly labelled Tox 1P/1M probe.*

4.3.7 Probing with Tox 1P/1M/PCC 7813 Probe

The membrane was stripped, re-probed and detected as described. The profiles generated by restriction of total genomic DNA with *Pvu*II were probed with the randomly labelled dioxigenin Tox 1P/1M/PCC 7813 PCR fragments. This probe hybridised to an approximately 9 kb fragment in all strains screened. This fragment size corresponded well to the expected size of 9.367 kb.

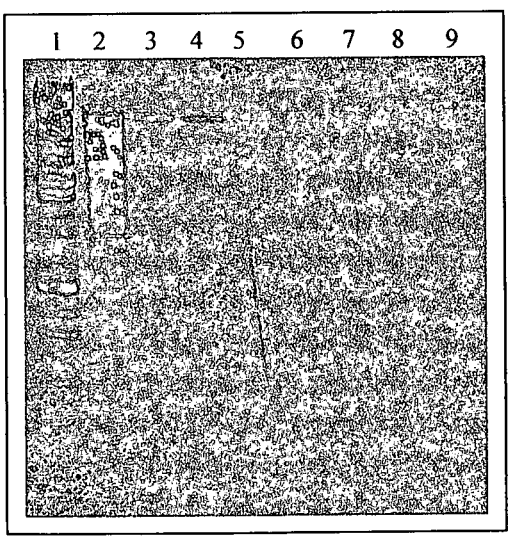

Figure 4.12
Hybridisation of the Tox 1P/1M/PCC 7813 probe randomly labelled with digoxigenin to a ± 9 kb fragment generated with Pvu*II. Lane1:* Eco*RI digested λDNA;*
Lane2: *± 9 kb fragment from PCC 7806*
Lane3: *± 9 kb fragment from PCC 7813*
Lane4: *± 9 kb fragment from UV 027*
Lane5: *± 9 kb fragment from CCAP 1450/1*
Lane6: *± 9 kb fragment from NIES 88*
Lane7: *± 9 kb fragment from NIES 89*
Lane8: *± 9 kb fragment from NIES 91*
Lane9: *± 9 kb fragment from NIES 99.*

In Fig. 4.12 hybridization of the Tox 1P/1M/PCC 7813 probe is shown. An approximately 9 kb hybridization product was obtained for all toxin-producing strains as well as the non toxin-producing strain CCAP 1450/1.

4.4 Toxin analysis

4.4.1 HPLC-analysis

Microcystins were extracted from each strain reported to be toxin-producing, i.e. PCC 7806, PCC 7813, UV 027, NIES 88, NIES 89, NIES 91 and NIES 99. The same procedure was carried out on the non toxin-producing strain CCAP 1450/1. Extracts were analyzed by HPLC on a C_{18} column at 238 nm. A characteristic peak, with a retention time of approximately 17.9 minutes was identified from the microcystin-LR standard.

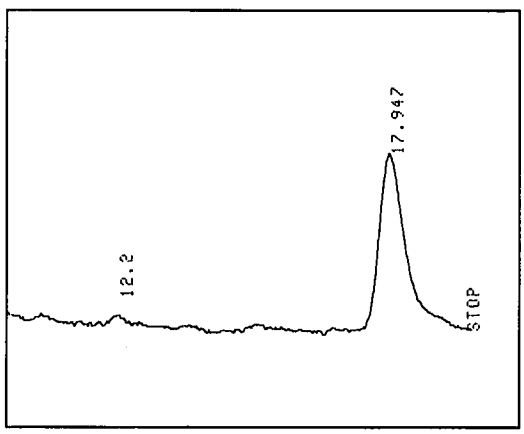

Figure 4.13
Characteristic HPLC profile of microcystin-LR standard at 238 nm.

A HPLC profile of the microcystin-LR standard is shown in Fig. 4.13. The major peak was located at a retention time of 17.9 minutes.

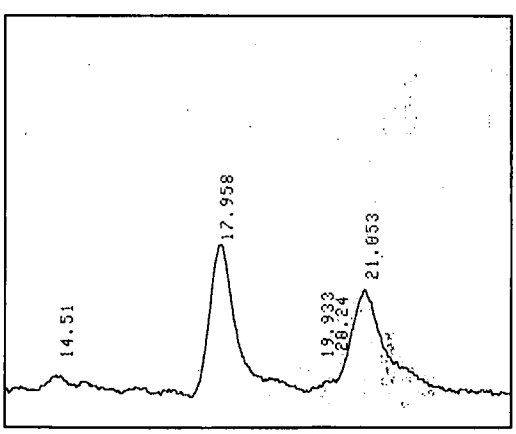

Figure 4.14
Typical HPLC profile of a toxin-producing M. aeruginosa *strain at 238 nm.*

A typical HPLC profile from toxin-producing strains, in this particular case, PCC 7806 is represented in Fig. 4.14. A significant peak was located at a retention time of approximately 17.9 minutes. Similar peaks were identified for all other toxin-producing strains. This retention time corresponded to that found for microcystin-LR standard.

Microcystin-LR was also detected for strain CCAP 1450/1, which was obtained from the Institute of Freshwater Ecology, UK as a non toxin-producing strain (Fig. 4.15).

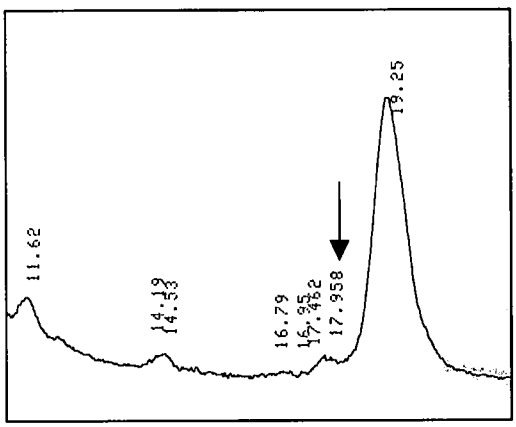

Figure 4.15
HPLC profile of a CCAP 1450/1 extract at 238 nm.

The HPLC profile from extracts of strain CCAP 1450/1 is shown in Fig. 4.15. A small peak was observed at a retention time of 17.9 minutes, similar to microcystin-LR standard.

5 Discussion

5.1 Introduction

The term 'thiotemplate mechanism' was first proposed by Laland & Zimmer in 1973 to describe a non-ribosomal pathway of protein synthesis observed in *Bacillus brevis*. In this pathway large, multifunctional enzymes, termed peptide synthetases, catalyse the activation and peptide bond formation of amino acids to form protein products (Laland & Zimmer, 1973).

In 1996 Meiβner and co-workers showed that both toxin-producing as well as non toxin-producing strains of *M. aeruginosa* contain sequences that showed extensive homology with other peptide synthetases. They isolated a PCR fragment from a toxin-producing strain of *M. aeruginosa* that hybridised only to DNA from other toxin-producing strains (Meiβner *et al.*, 1996). This fragment was targeted in a recombination experiment performed by Dittmann and co-workers (1997), that confirmed the involvement of this gene, termed *mcyB*, in microcystin production in strain PCC 7806. The conclusion was therefore made that the basic difference between toxin-producing and non toxin-producing strains of *M. aeruginosa* is the presence of *mcyB* (Dittmann *et al.*, 1997).

5.2 Polymerase Chain Reaction

To ascertain if the results presented by the previously cited publications could be expanded to include *M. aeruginosa* populations in general, it was attempted to identify *mcyB* in other toxin-producing strains, namely strain PCC 7813 and the South African strain UV 027, while using a non toxin-producing strain (CCAP 1450/1) as a negative control.

Based on the conclusions by Dittmann *et al.* in 1997, the expectation was that if the basic difference between toxin-producing and non toxin-producing strains was indeed

the presence or absence of *mcyB* that it would be possible to amplify PCR products with all four primer pairs in toxin-producing strains, while no products would be amplified in a non toxin-producing strain.

After a wide range of variables were optimized, it was possible to amplify PCR products for both toxin-producing strains using all four primer pairs (Fig. 4.1 - 4.4). The sizes of the fragments amplified from both strains, i.e. approximately 1850 bp with primer pair Tox 3P/2M, 1500 bp with primer pair Tox 1P/1M, 1850 bp with primer pair Tox7P/3M and 1850 bp with primers Tox 10P/4M, correspond well to the expected sizes calculated from the sequence of *mcyB* in the Genbank database (Niederberger & Neilan 1998, http://www.ncbi.nlm.nih/gov). Collectively, these fragments span approximately 7 kb which correlate well with the known size of approximately 6.5 kb of *mcyB*. These results could indicate that a complete copy of *mcyB* is present in the genomes of both strain PCC 7813 and UV 027.

If identical PCR conditions were employed for the non toxin-producing strain, it was possible to successfully amplify PCR products with three of the four primer pairs, i.e. Tox 3P/2M, Tox 7P/3M and Tox 10P/4M (Fig. 4.1, 4.3, 4.4). The sizes of the fragments amplified, again correlated well with the expected fragment sizes calculated. If the three PCR fragments amplified in strain CCAP 1450/1 did indeed represent portions of *mcyB*, and corresponded to similar fragments amplified in toxin-producing strains, these three fragments would hypothetically include the start codon, the condensation site, part of the AMP-binding site and the phosphopantetheine attachment site from the first domain as well as the condensation site, the AMP-binding site and the phosphopantetheine attachment site from the second domain and the stop codon, respectively.

No PCR product was amplified with primer pair Tox 1P/1M in strain CCAP 1450/1 (Fig. 4.2). The most likely reason for non-amplification in this strain was that one or both of the

primers did not bind to CCAP 1450/1 template DNA. Sequence comparison of *mcyB* in strains PCC 7813, UV 027 and two sequences in the Genbank database showed that the region directly upstream of primer Tox 1P was much more variable than any other part of the gene. It might therefore have been possible that primer Tox 1P could not bind to CCAP 1450/1 template DNA. Sequencing results showed that the target region of primer Tox 1M was conserved and it was therefore more likely that the inability to amplify fragments with these two primers was due to non-binding of primer Tox 1P. This remains to be verified, but the only fact that can be stated with any measure of certainty is that strain CCAP 1450/1 contained an anomalous feature in this particular region of its genome.

The successful amplification of PCR fragments in strain CCAP 1450/1 seemed to contradict the publication of Dittmann *et al.* (1997) which asserts that the basic difference between toxin-producing and non toxin-producing strains of *M. aeruginosa* is the presence or absence of *mcyB*. In the study undertaken by Dittmann *et al.*, (1997) the primers employed amplified a part of the AMP-binding site of the first domain from *mcyB*, which correlates to the fragment not amplified in this study in strain CCAP 1450/1 with primer pair Tox 1P/1M.

Also, it is known that both toxin-producing and non toxin-producing strains of *M. aeruginosa* contain sequences homologous to peptide synthetases (Meiβner *et al.*, 1996). Combined with the low stringency conditions employed in the PCR cycle, the PCR fragments generated with primer pairs Tox 3P/2M, Tox 7P/3M and Tox 10P/4M could therefore simply confirm this for strain CCAP 1450/1. This, however, seems unlikely as the sizes of the various fragments amplified are highly similar to fragments amplified from toxin-producing strains and to the fragment sizes expected from the known sequence of *mcyB*. In addition, as set out in Table 3.2 and confirmed by sequencing, all primers employed were highly

specific, and non-specific binding therefore, seems improbable.

5.3 Sequencing

PCR products from toxin-producing strains PCC 7813 and UV 027 were cloned into pGem®T-Easy and sequenced. Results confirmed that PCR fragments from both strains represent portions of *mcyB* and that these fragments show > 99 % homology with the known sequence of *mcyB*. Fragments amplified with primer pair Tox 3P/2M in strain PCC 7813 and UV 027 (Fig. 4.1) represent the first portion of the first domain from *mcyB*, and include the start-codon, ATG, bases 1 - 3 (Appendix A). The ± 1500 bp products amplified in both toxin-producing strains with primer pair Tox 1P/1M (Fig. 4.2) represent the second portion of the first domain of *mcyB* and span a portion of the first AMP-binding site. Primer pair Tox 7P/3M generated fragments for both strains (Fig. 4.3) that represent the first portion of the second domain from *mcyB* and include the phosphopantetheine attachment and the condensation site. PCR fragments generated with primer pair Tox 10P/4M in these strains (Fig. 4.4) represent the second portion of the second domain of *mcyB* and include a portion of the AMP-binding site and the phosphopantetheine attachment site as well as the stop codon, TGA, bases 6379 - 6381, see Appendix A. These results indicate that a complete, and theoretically functional copy of *mcyB* is present in both toxin-producing strains tested.

5.4 Southern Blot with Tox 7P/3M/PCC 7813 Probe

If the presence or absence of *mcyB* is indicative of toxicity, one would expect that a probe based on a conserved region of this gene would exclusively hybridise to restricted genomic DNA from toxin-producing strains.

A probe synthesised from PCR products from the toxin-producing strain PCC 7813 with primer pair Tox 7P/3M, spanning the first phosphopantetheine attachment site and the second condensation site, hybridised to an approximately 13 kb fragment in all strains investigated (Fig. 4.10). The size of this fragment correlates well to the expected calculated size. Hybridisation of this probe to *Pvu*II digested genomic DNA from toxin-producing strains and non toxin-producing strain CCAP 1450/1 confirms that toxin-producing strains and the non toxin-producing strain CCAP 1450/1 do possess this particular portion of a copy of *mcyB* (Fig. 4.10).

Referring to the PCR results presented earlier, it was possible to successfully amplify fragments for strain CCAP 1450/1 using primer pair Tox 7P/3M (Fig. 4.3). The successful amplification of this fragment indicates that target sequences for a probe based on this fragment, would indeed be present in the CCAP 1450/1 genome. Hybridisation of this particular probe to restricted genomic DNA of strain CCAP 1450/1 is, therefore, not surprising.

5.5 Southern Blot with Tox 1P/1M/PCC 7813 Probe

To resolve the results obtained from the hybridisation of the Tox 7P/3M/PCC 7813 probe to *Pvu*II digested genomic DNA from the non toxin-producing strain CCAP 1450/1, a second probe was synthesised. PCR fragments from strain PCC 7813 generated with primer pair Tox 1P/1M was labelled with DIG. Referring to PCR results discussed earlier, it was not possible to successfully amplify PCR products with this particular primer pair in strain CCAP 1450/1 (Fig. 4.2). The expectation was that if no PCR products were amplified for this particular region, there would be no target sequences present in the genome for the probe to hybridise to.

Analysis of the sequence of *dnaN* predicted a ± 9 kb fragment, which according to literature and PCR results discussed earlier, would exclusively hybridise to target sequences in digested genomic DNA from toxin-producing strains. Hybridisation of this probe yielded an approximately 9 kb fragment for all strains investigated including CCAP 1450/1 (Fig. 4.12).

Results from this particular hybridisation experiment would suggest that target DNA for the Tox 1P/1M/PCC 7813 probe is indeed present in the CCAP 1450/1 genome. This, however, does not necessarily mean that CCAP 1450/1 possesses a complete and functional copy of *mcyB*, only that target sequences for at least a part of the Tox 1P/1M/PCC 7813 probe is present. As discussed previously, the inability to successfully amplify a PCR product from strain CCAP 1450/1 with primer pair Tox 1P/1M only indicates that target sequences for one of the primers, probably Tox 1P are absent. It is entirely possible that there is enough target DNA present for the Tox 1P/1M/PCC 7813 probe to hybridise to.

Hybridisation of the Tox 1P/1M/PCC 7813 probe to restricted genomic DNA from CCAP 1450/1 corroborate PCR and hybridisation results obtained earlier, confirming that CCAP 1450/1 possesses at least partial elements of *mcyB*.

5.6 Toxin Analysis with HPLC

To ascertain if the results obtained by molecular techniques were an accurate representation of toxin production in the various *M. aeruginosa* strains, all strains were subjected to HPLC-analyses.

Microcystin-LR was found to be present in all toxin-producing strains investigated (Fig. 4.14) indicating that these strains possess a complete, functional and expressed copy of *mcyB*. Microcystin-LR was also detected in

strain CCAP 1450/1 (Fig. 4.15). This shows firstly, that this particular strain does possess a copy of *mcyB*, corroborating the PCR and hybridisation results, and secondly, according to HPLC results, that the gene is functional and expressed.

The Institute of Freshwater Ecology, UK was contacted and enquiries made as to the precise nature of strain CCAP 1450/1: Is this strain toxin-producing or not? Does non-toxic mean that the strain produces no toxin at all, or that it produces neglible amounts of toxin? In an e-mail received from the Institute, it was stated that they "have no record of it being tested for toxicity (Personal communication)[‡]". In a later communication it was stated that strains sold to research facilities are not necessarily monocultures and that different 'strains', i.e. toxin-producing and non toxin-producing, may dominate at different times in the culture. It is, therefore, entirely possible that a culture may not produce toxins at first, while at a later stage another toxin-producing type may dominate in the culture (Personal communication)[‡]. This may to some extent explain the loss of toxin production observed in cultured strains (Carmichael, 1994).

Results obtained during this study may thus simply be a reflection of the change in dominance in a *M. aeruginosa* culture and conclusions and assumptions made based on the work of Dittmann *et al.* (1997), could be true. PCR results obtained with primer pair Tox 1P/1M for the non toxin-producing strain could reflect dominance of a non toxin-producing strain, i.e. absence of *mcyB* in the culture. PCR results with the remaining three primer pairs and Southern Blot results could reflect dominance of a toxin-producing strain, i.e. presence of *mcyB* in the CCAP 1450/1 culture. This is possible as a time lapse of approximately 2 years occurred between onset and conclusion of results.

[‡] Jean Tompkins: ccap@ife.ac.uk

6 Conclusion

PCR and Southern Blot results indicated that toxin-producing strains of *M. aeruginosa* investigated in this study contained a copy of *mcyB*. Basepair sequence alignment and amino acid alignment of strains PCC 7813 and UV 027 with known sequences revealed a high degree of homology. Sequencing revealed that the portion of DNA analysed in both strains contained start and stop codons and that this gene was, therefore, complete and potentially functional. HPLC analysis verified that the gene was functional and expressed in toxin-producing strains investigated. Based on these results, it was not possible to say with any measure of certainty that *mcyB* was involved in toxin production in *M. aeruginosa* strains investigated in this study, as no proper negative control was available.

It was almost impossible to draw any certain conclusions from the results regarding strain CCAP 1450/1. It is important to keep in mind that the inconsistent results obtained with this 'non toxin-producing' strain were only a reflection of the observations made from strain CCAP 1450/1 during the course of this particular study. These results should not necessarily be extrapolated to include non toxin-producing strains in general.

Firstly, it should be determined if the results presented here regarding this strain are merely indicative of a change in dominance in the culture over time, or if the results are indeed an accurate reflection of the genetic content of non toxin-producing strains. It seems unlikely that the latter is the case as an immense region of genetic material codes for microcystin synthetase. It is possible that the results presented here are an accurate representation of CCAP 1450/1, but a myriad of factors could render *mcyB* ineffectual in other non toxin-producing strains.

Secondly, more non toxin-producing strains should be obtained and very importantly, these strains should be monitored for toxin production on a regular basis, before and during a molecular investigation. PCR fragments

obtained with primer pairs Tox 3P/2M, Tox 7P/3M and Tox 10P/4M from CCAP 1450/1 should be cloned and sequenced and new primers designed to elucidate the exact nature of the anomaly present in strain CCAP 1450/1 in the region represented by primer pair Tox 1P/1M.

Research into the mechanism of toxin production in *M. aeruginosa* is ongoing worldwide and it has since been discovered that *mcyB* forms part of a large gene cluster (Niederberger & Neilan, 1998). Inactivation of *mcyB* by Dittmann *et al.*, (1997) has proven that *mcyB* is essential for microcystin synthesis, but the mere presence of this single gene could in no way be indicative of toxin production in *M. aeruginosa*. The fact that the *mcyB* gene is contained in a gene cluster and that each gene in the cluster has to be intact, functional and expressed for toxin to be synthesised, renders it imprudent to attempt to develop a molecular screening tool based only on the presence of this one gene.

The best possible way to develop a molecular screening tool is to investigate the possible presence of a molecular activation/inactivation mechanism involved in transcription of the microcystin synthetase gene cluster. In a recent publication, transcriptional levels of the microcystin synthetase gene cluster has been linked to light quality (Kaebernick *et al.*, 2000). This phenomenon should be further investigated to verify conclusively that an increase in transcriptional levels of the gene cluster correlates to higher levels of toxin production. If this can be verified, a molecular screening tool based on the presence, expression and functionality of the gene/genes involved in regulation, in conjunction with the same parameters for all the genes in the gene cluster could be developed.

However, taking into account the immense region of DNA spanned by the microcystin synthetase gene cluster, approximately 55 kb (Tillett *et al.*, 2000), as well as an unknown region of DNA coding for the hypothetical regulatory

protein/proteins, and considering that a single basepair mutation, i.e. insertion or deletion, could render any of the genes "non-sense", the author regards the development of a single molecular screening tool for toxin production in *M. aeruginosa* as unwise. Further underscoring this argument, is the fact that changing toxicities in naturally occurring *Microcystis* spp. blooms are probably due to altering strain compositions as well as the regulation of toxin synthesis by certain environmental conditions (Kaebernick *et al.* 2000).

The environmental factors influencing toxin production, either directly or indirectly via regulating mechanisms, should be identified and studied extensively so that this knowledge can be applied to preventing, controlling or at least understanding naturally occurring *Microcystis aeruginosa* blooms.

In retrospect, the aims of this study, namely 'to provide a fast, accurate, robust and relatively easy way of screening *M. aeruginosa* blooms on a genetic/molecular level for potential toxin production', was highly optimistic and would require a lot of further research.

Summary

The main aim of this study was to attempt to develop a molecular screening tool for naturally occurring blooms of *M. aeruginosa* based on the presence or absence of the gene *mcyB*. This peptide synthetase has previously been implicated in toxin production in *M. aeruginosa* (Dittmann *et al.*, 1997).

Geographically unrelated strains of *M. aeruginosa* were obtained from the Pasteur Institute, France; the National Institute for Environmental Studies, Japan; the Institute of Freshwater Ecology, UK; and the University of the Free State culture collections. Based on conserved regions present in known sequences of *mcyB* four primer pairs were designed. The strains were maintained under standard conditions and total genomic DNA was extracted from toxin-producing strains PCC 7813, UV 027 and non toxin-producing strain CCAP 1450/1.

PCR reactions were performed and the fragments generated with the various primer pairs were compared with expected fragment sizes. PCR products of the expected size were amplified in both toxin-producing strains with all four primer pairs, signifying that these toxin-producing strains possess a copy of *mcyB*. It was also possible to generate PCR fragments with three primer pairs from the non toxin-producing strain CCAP 1450/1. These results indicated that this strain contained at least partial elements of *mcyB*.

Fragments amplified by PCR from toxin-producing strains were cloned into pGemT®-Easy (Promega) and sequenced. Basepair and translated amino acid alignment of the assembled fragments showed a high degree of homology with previously deposited sequences of *mcyB* in the Genbank database.

A fragment amplified by PCR from strain PCC 7813 with primer pair Tox 7P/3M was randomly labelled and used as a probe to screen unrelated strains of *M. aeruginosa* for the presence of *mcyB*. This probe hybridised to a fragment of the

expected size in all toxin-producing strains as well as the non toxin-producing strain confirming PCR results that all strains contain this particular portion of *mcyB*.

A second probe generated from strain PCC 7813 with primer pair Tox 1P/1M representing the fragment of *mcyB* not amplified by PCR in strain CCAP 1450/1 was synthesised. This probe hybridised to a fragment of the expected size in all toxin-producing strains and the non toxin-producing strain. Hybridisation of this probe to *Pvu*II digested DNA from CCAP 1450/1 indicated that there was enough target DNA in the CCAP 1450/1 genome for the Tox 1P/1M/PCC 7813 probe to hybridise to, hinting at the possibility that this strain also possess a complete copy of the gene.

Crude cell extracts were made from all strains investigated and analysed by HPLC for the presence of microcystin-LR. Microcystin-LR was detected in all toxin-producing strains as well as the 'non toxin-producing' strain CCAP 1450/1.

The Institute of Freshwater Ecology where this strain was obtained from was contacted and enquiries made. From replies received it became known that firstly, the Institute has never tested the strain for microcystin-LR production and that secondly, the strains are not monocultures. The most probable explanation for the anomalous results gathered from strain CCAP 1450/1 is that a toxin-producing *M. aeruginosa* type dominated in the culture for the duration of this study.

Opsomming

Daar is gepoog om 'n molekulêre siftingsmetode te ontwikkel vir natuurlike opbloeie van *M. aeruginosa*, gebasseer op die aan- of afwesigheid van die geen *mcyB*. Hierdie peptied-sintetase is voorheen geïmpliseer in toksienproduksie in *M. aeruginosa* (Dittmann *et al.*, 1997).

Geografies onverwante stamme van *M. aeruginosa* is bekom vanaf die Pasteur Instituut, Frankryk; die National Institute for Environmental Studies, Japan; die Insitiute for Freshwater Ecology, Verenigde Koninkryk; en die Universiteit van die Vrystaat kultuurversamelings. Vier inleierpare is ontwerp gebasseer op gekonserveerde gedeeltes aanwesig in bekende basispaarvolgordes van *mcyB*. Die stamme is gegroei by standaard toestande en totale genomiese DNS is geëkstraheer vanuit toksienproduserende stamme PCC 7813 en UV 027 en die nie-toksienproduserende stam CCAP 1450/1.

PKR reaksies is gedoen en die fragmente wat gegenereer is met die onderskeie priemstukke was vergelyk met die verwagte fragmentgroottes. PKR-produkte van die verwagte grootte is geamplifiseer in beide toksienproduserende stamme met al vier inleierpare, wat aangedui het dat hierdie stamme 'n kopie van *mcyB* bevat het. Dit was ook moontlik om PKR-fragmente te genereer met drie inleierpare vanuit die nie-toksienproduserende stam CCAP 1450/1. Hierdie resultate het aangedui dat hierdie stam ten minste gedeeltelike elemente van 'n kopie van *mcyB* bevat het.

Fragmente wat deur PKR geamplifiseer is vanuit toksienproduserende stamme is in pGemT®-Easy (Promega) gekloneer en die basispaaropeenvolging is bepaal. Basispaarvolgorde en getranslerde aminosuurinlynstelling van die saamgevoegde fragmente het 'n hoë mate van homologie getoon met voorheen-gedeponeerde basispaarvolgordes in die Genbank databasis.

'n Fragment geamplifiseer deur PKR vanuit stam PCC 7813 met inleierpaar Tox 7P/3M is lukraak gemerk en gebruik as 'n peiler om onverwante stamme van *M. aeruginosa* vir die

teenwoordigheid van *mcyB* te sif. Hierdie peilstuk het aan 'n fragment van die verwagte grootte in al die toksienproduserende stamme gehibridiseer asook aan die genomiese DNS van die nie-toksienproduserende stam. Dit bevestig PKR resultate dat alle stamme hierdie spesifieke gedeelte van *mcyB* bevat. 'n Tweede peilstuk is gemaak van 'n PKR-produk van stam PCC 7813 met inleierpaar Tox 1P/1M. Hierdie fragment verteenwoordig die gedeelte van *mcyB* in stam CCAP 1450/1 wat nie met PKR geamplifiseer is nie. Hierdie peilstuk het aan 'n fragment van die verwagte grootte gehibridiseer in alle toksienproduserende stamme asook die nie-toksien-produserende stam CCAP 1450/1. Hibridisering van hierdie peilstuk aan *Pvu*II-gesnyde genomiese DNS van CCAP 1450/1 het aangedui dat daar genoeg teikenvolgorde-DNS aanwesig was in die CCAP 1450/1-genoom vir die Tox 1P/1M/PCC 7813 peilstuk om te kon hibridiseer, wat ook kon aangedui het dat hierdie stam selfs 'n volledige kopie van die *mcyB* geen kon bevat het.

Kru sel-ekstrakte is gemaak van alle stamme wat ondersoek is en dit is geanaliseer deur HDVC vir die aanwesigheid van mikrosistien-LR. Mikrosistien-LR is gevind in alle toksienproduserende stamme asook die 'nie-toksien-produserende' stam CCAP 1450/1.

Die Institute of Freshwater Ecology, waarvandaan die stam verkry is, is gekontak en navraag is gedoen. Met terugvoer wat verkry is, het die aan die lig gekom dat eerstens, die Instituut nog nooit die stam getoets het vir mikrosistien-LR produksie nie, en tweedens dat die stamme nie reinkulture is nie. Die waarskynlikste verklaring vir die onreëlmatige resultate waargeneem van stam CCAP 1450/1 is dat 'n toksienproduserende *M. aeruginosa* tipe dominant was vir die duur van hierdie studie.

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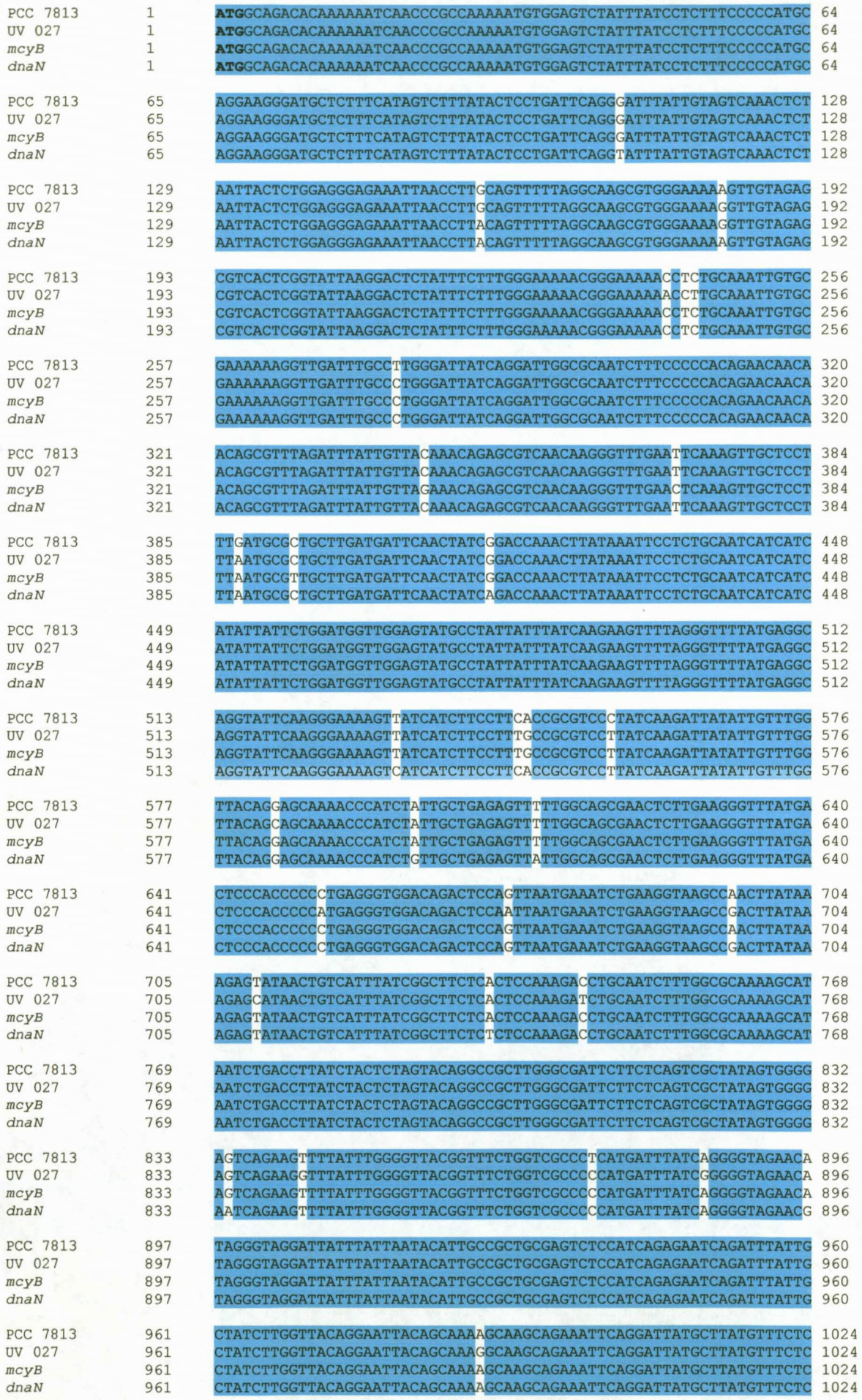
Appendix A

(Sequence Alignment)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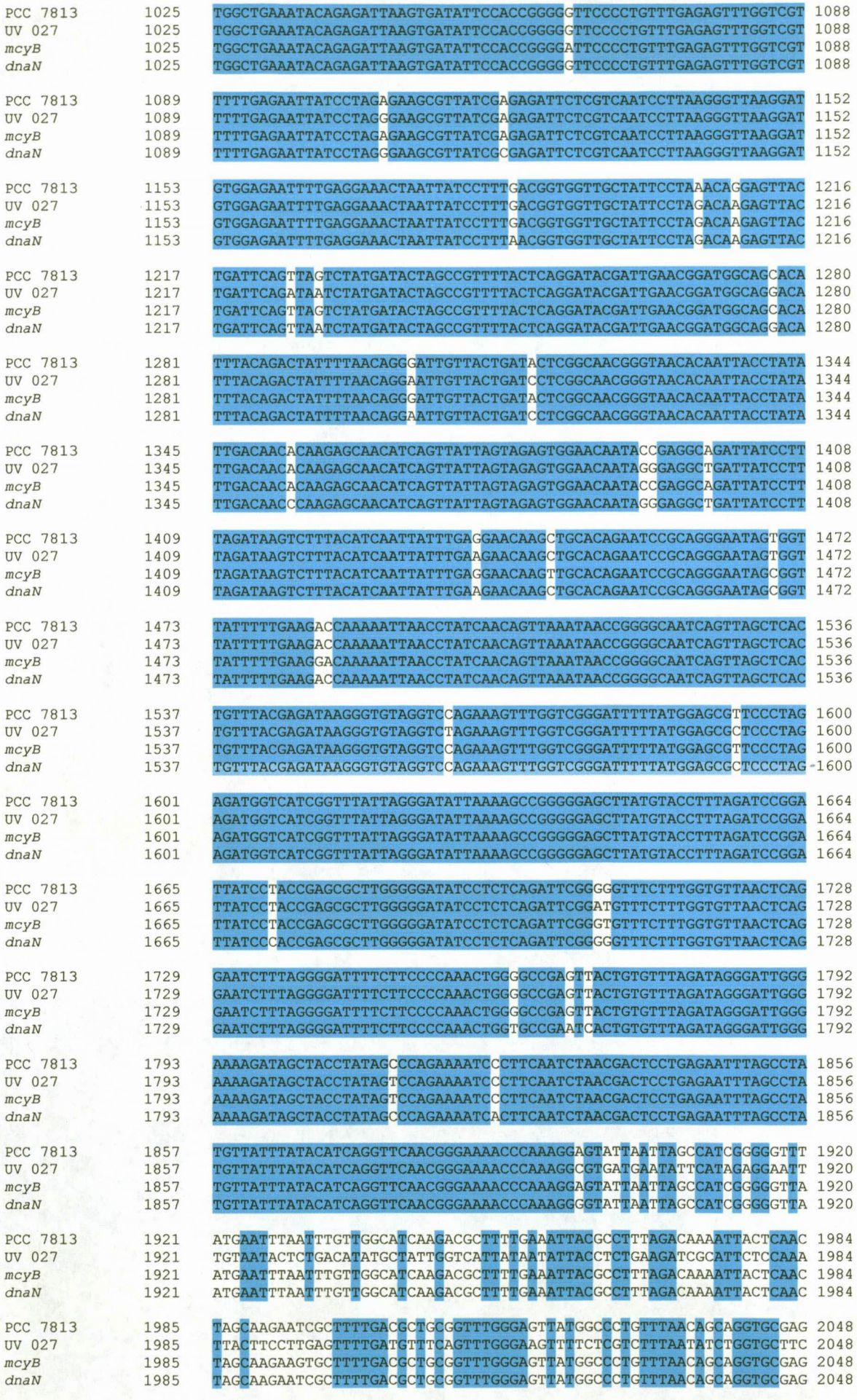
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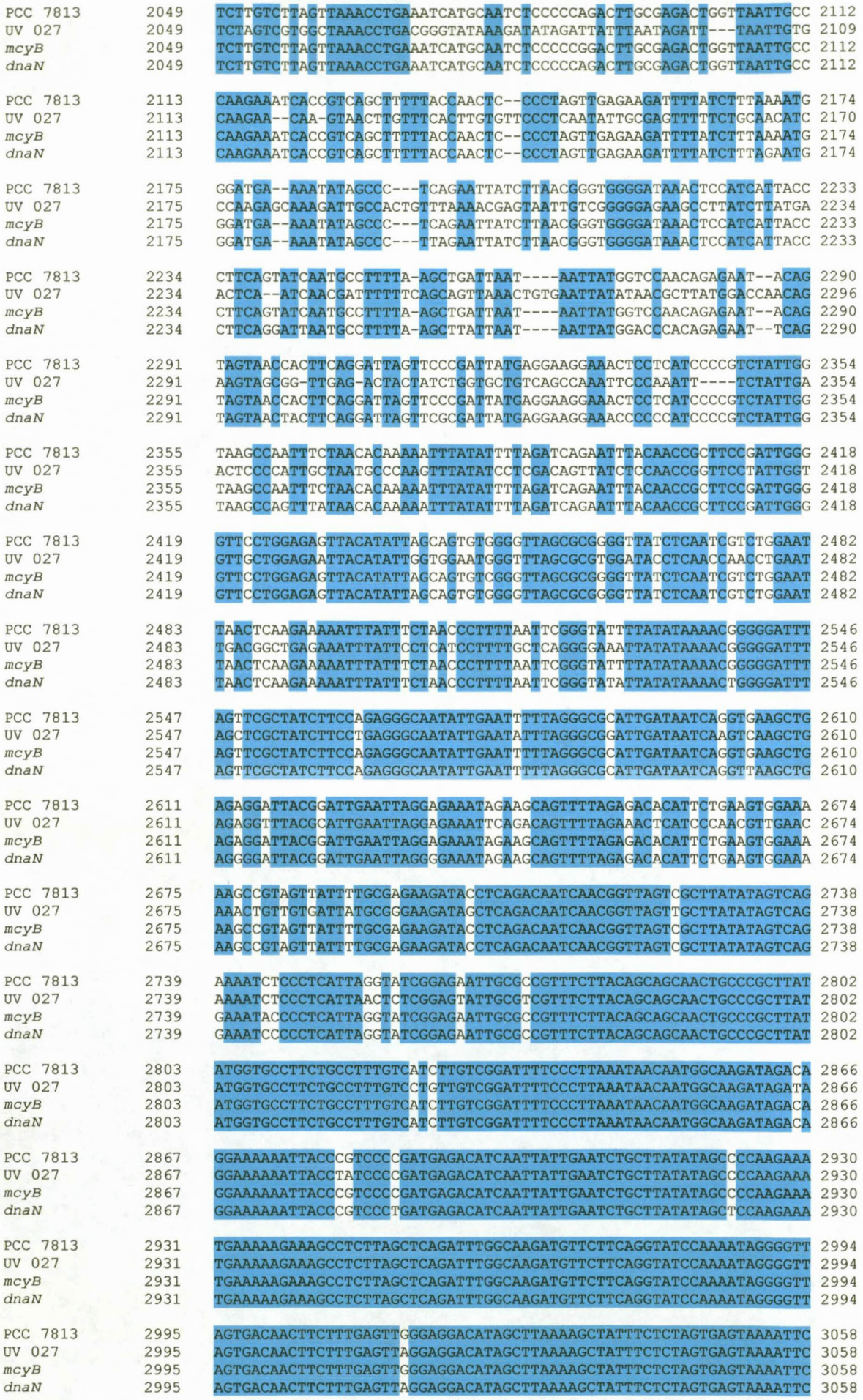
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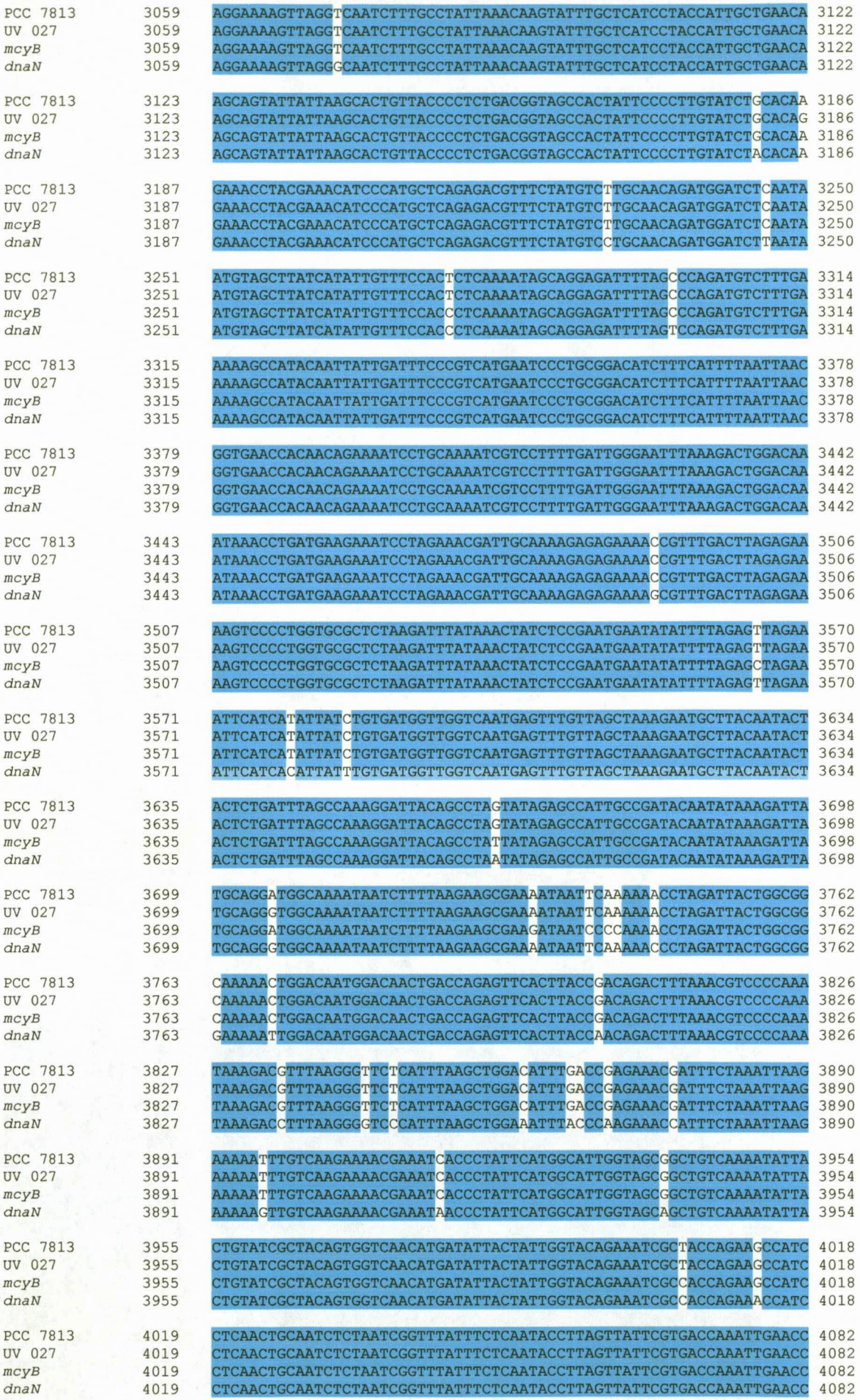
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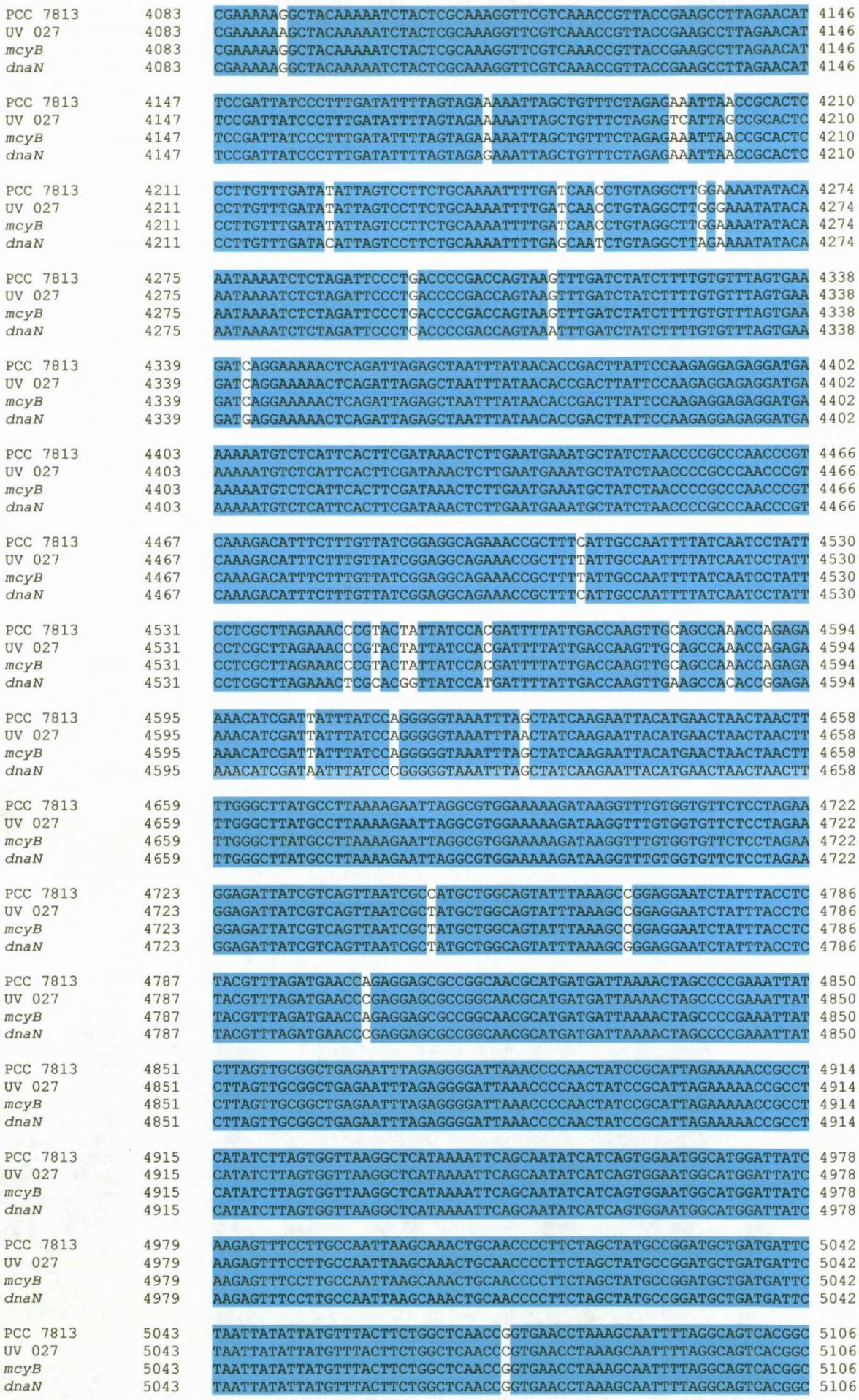
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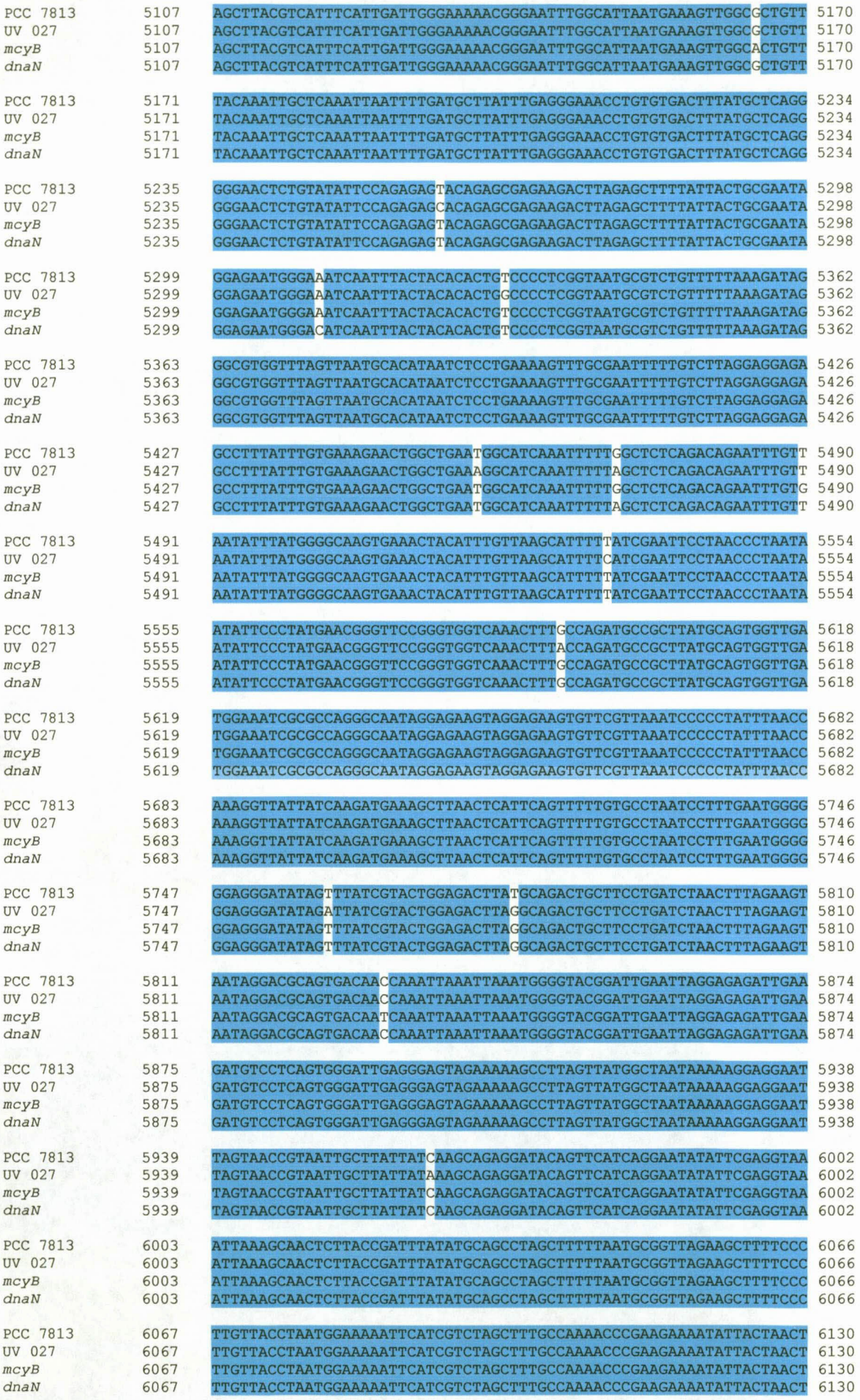
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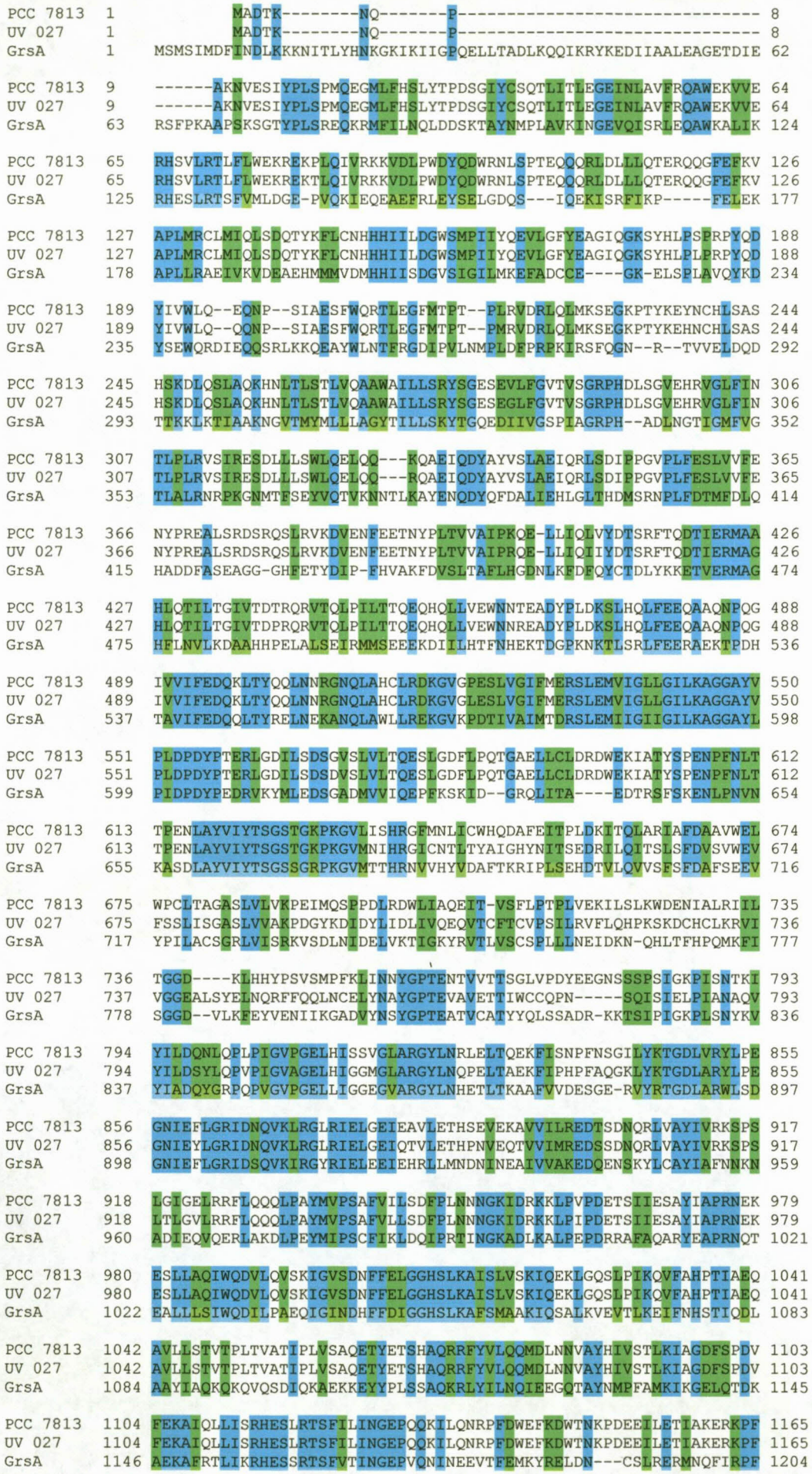
Appendix B

(Amino Acid Alignment)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GrsA 178 APLLRAEIVKVDEAEHMMMVDMHHIISDGVSIGILMKEFADCCE----GK-ELSPLAVQYKD 234
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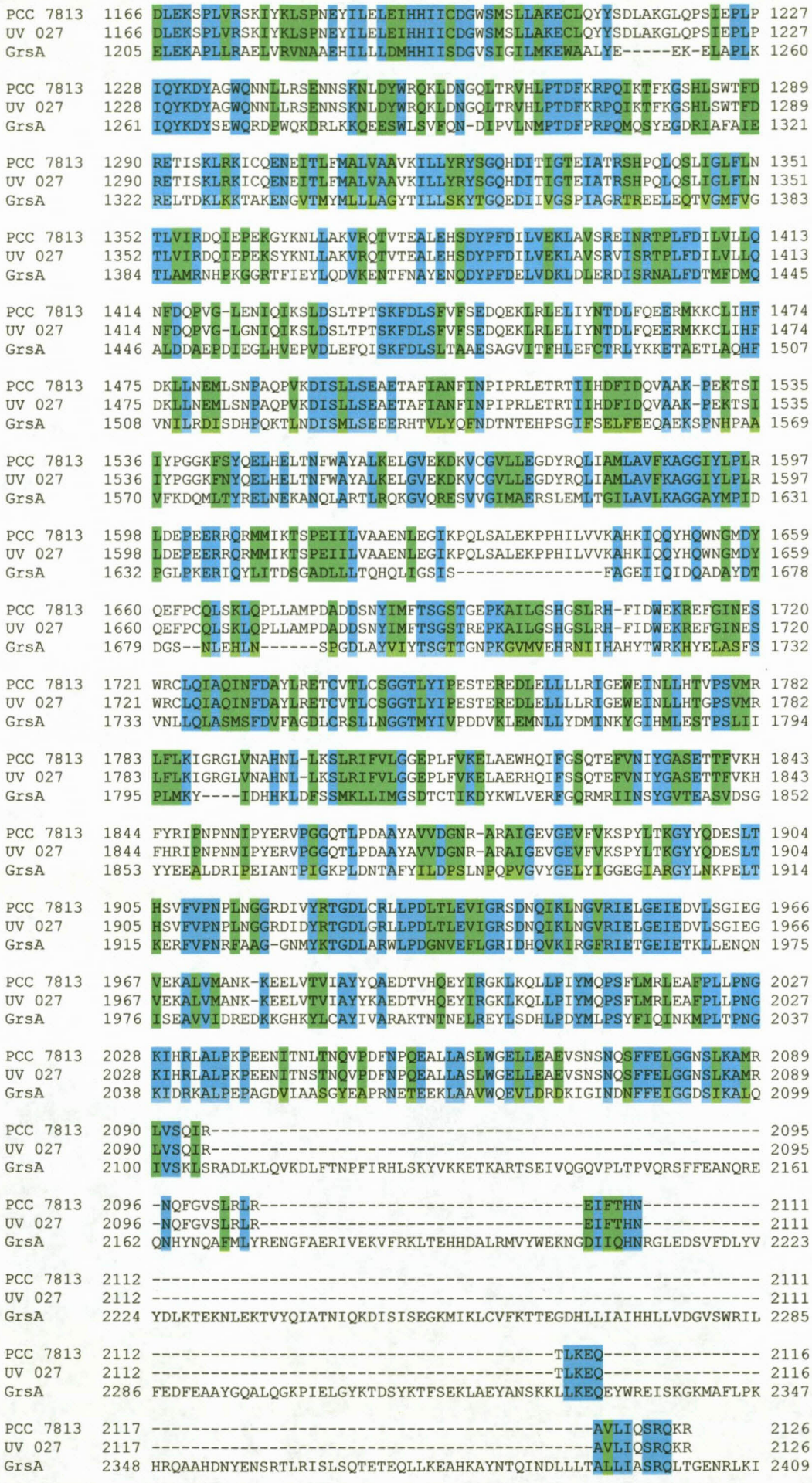
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