Chapter 17: The genetics and genomics of cyanobacterial toxicity

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Introduction

The past ten years has witnessed major advances in our understanding of natural product biosynthesis, including the genetic basis for toxin production by a number of groups of cyanobacteria. Cyanobacteria produce an unparalleled array of bioactive secondary metabolites; including alkaloids, polyketides and non–ribosomal peptides, some of which are potent toxins. This paper addresses the molecular genetics underlying cyanotoxin production in fresh and brackish water environments. The major toxins that have been investigated include microcystin, cylindrospermopsin, nodularin, the paralytic shellfish poisons (PSP), including saxitoxin, and the anatoxins.

Non–ribosomal peptide synthesis is achieved in prokaryotes and lower eukaryotes via the thiotemplate function of large, modular enzyme complexes, known collectively as peptide synthetases. Most non–ribosomal peptides from microorganisms are classified as secondary metabolites, that is, they rarely have a role in primary metabolism, growth, or reproduction but have evolved to somehow benefit the producing organism. Most cyanobacterial genera have either been shown to produce non–ribosomal peptides or have them encoded within their genomes. Early work on the genetics of cyanobacterial toxicity led to the discovery of one of the first examples of hybrid peptide–polyketide synthetases. This enzyme complex directed the production of the cyclic heptapeptide microcystin and, as one

of the largest known bacterial gene clusters, is encoded by more than 55 kb. Orthologs of microcystin synthetase have been found in several strains of *Microcystis* and other genera of toxic cyanobacteria, including *Planktothrix*, *Nostoc*, *Anabaena*, *Nodularia*, *Phormidium*, and *Chroococcus*. The homologous gene cluster in *Nodularia* is predicted to be responsible for the synthesis of the pentapeptide nodularin, providing evidence of genetic recombination and possibly transfer during the evolution of these compounds. Genomic information related to microcystin and nodularin synthesis has also indicated their environmental and cellular regulators, as well as associated transport mechanisms.

More recently, hybrid peptide and polyketide synthetic pathways have been implicated in the production of the alkaloid cylindrospermopsin. Other predicted biosynthetic pathways are also under scrutiny and are being used in the search for candidate gene loci involved in PSP and anatoxin production. The correlation between toxicity and salt tolerance may raise future concerns as these cyanobacteria could compromise the safety of recycled and desalinated drinking water supplies.

The pattern of acquisition of genes responsible for cyanobacterial toxicity is not, on the whole, related to the evolution of potentially toxic species and the global distribution of toxic strains has been the topic of several phylogeographical studies. The exceptions to this include the species *Nodularia spumigena* and Australian strains of *Anabaena circinalis* that produce saxitoxin. Toxin biosynthesis gene cluster–associated transposition and the natural transformability of certain species allude to a broader distribution of toxic taxa. The information gained from the discovery of these toxin biosynthetic pathways has enabled the genetic screening of various environments for drinking water quality management. Understanding the role of these toxins in the producing microorganisms and the environmental regulation of their biosynthesis genes may also suggest the means for controlling toxic bloom events.

Genes involved in the biosynthesis of cyanotoxins

This section of the paper describes the characterization of the biosynthetic gene clusters that have either, by mutation, or by functional prediction been shown to be required for toxin production. Where possible the chemical structures of the toxins will not be revised here unless recent related genetic or enzymology data is available. Other genes encoding cofactors and regulators, for example, are also intimately linked to cyanotoxin production and, where appropriate, these will also be reviewed. Due to the

number of toxins to be considered and their distribution across various cyanobacterial genera, each toxin biosynthetic pathway will be discussed separately, in the chronological order of their elucidation.

Microcystin synthetases

The structurally related hepatotoxins microcystin and nodularin are synthesized nonribosomally by the thiotemplate functions of large multifunctional enzyme complexes containing both nonribosomal peptide synthetase (NRPS) and polyketide synthetase (PKS) domains. The gene clusters encoding these biosynthetic enzymes, *mcyS* (microcystin) and *ndaS* (nodularin), have recently been sequenced and partially characterized in several cyanobacterial genera including *Microcystis*, *Anabaena*, *Planktothrix*, and *Nodularia* (Dittmann et al. 1997; Tillett et al. 2000; Christiansen et al. 2003; Moffitt et al. 2004; Rouhiainen et al. 2004). These fundamental studies have afforded insight into the evolution of cyanotoxin biosynthesis, and have provided the groundwork for current PCR–based toxic cyanobacteria detection methods.

The microcystin biosynthesis gene cluster, *mcyS*, was the first complex metabolite gene cluster to be fully sequenced from a cyanobacterium. In *M. aeruginosa* PCC7806, the *mcyS* genomic locus spans 55 kb and comprises 10 genes arranged in two divergently transcribed operons (*mcyA–C* and *mcyD–J)*. The larger of the two operons, *mcyD–J*, encodes a modular PKS (McyD), two hybrid enzymes comprising NRPS and PKS modules (McyE and McyG), and enzymes putatively involved in the tailoring (McyJ, F, and I) and transport (McyH) of the toxin. The smaller operon, *mcyA–C* encodes three NRPS (McyA–C). Interestingly, the arrangement of ORFs in the *mcyS* cluster was found to be different in the organisms *Microcystis*, *Anabaena* and *Planktothrix* (Fig. 1). The *Anabaena mcyS* cluster adheres to the 'co–linearity' rule of NRPS pathways that predicts the order of catalytic processes involved in the biosynthesis of a non–ribosomal metabolite is generally the same as the order of the genes which encode their catalytic enzymes (Kleinkauf and von Dohren 1996), however the *Microcystis* and *Plankothrix* clusters do not adhere to this rule.

The formation of Adda (3–amino–9–methoxy–2,6,8,–trimethyl– 10–phenyl–4,6–decandienoic acid) putatively involves enzymes encoded by *mcyD–G* and *mcyJ*, based on bioinformatic analyses and homology to related enzymes. The hybrid NRPS/PKS enzyme, McyG, constitutes the first step in Adda biosynthesis–the putative activation of phenylacetate, catalyzed by the NRPS adenylation domain. The activated phenylacetate is transferred to the 4–phosphopantetheine cofactor of the first carrier do-

main. Phenylacetate is then extended by several malonyl–CoA elongation steps and subsequently modified by C–methylations, reduction and dehydration, all catalyzed by the PKS modules of McyD, E, and G. The aminotransferase domain of McyE converts the polyketide to a β–amino acid in the final step of Adda biosynthesis. The NRPS module of the second hybrid PKS/NRPS enzyme, McyE, is thought to be involved in the activation and condensation of D–Glu with Adda. No phospopantetheine transferase, that catalyzes the post–translational modification of the holo–NRPS, is associated with the *mcyS* gene cluster.

The *mcyF* ORF was originally predicted to encode a glutamate racemase, responsible for the epimerisation of the L–Glu residue of microcystin (Tillett et al. 2000; Nishizawa et al. 2001). A subsequent study by Sielaf et al. (2003), contested this theory and provided evidence that McyF acts exclusively as an Asp racemase to form the D–*erythro* β–methylaspartate (D–MeAsp) residue. It was proposed that the D–Glu residue is provided by a L–Glu racemase resident external to the *mcyS* gene cluster. However, earlier feeding experiments with isotopic substrates identified that the D–MeAsp residue is formed via a novel pathway (Moore et al. 1991) that would be consistent with McyF acting as a Glu racemase. Mutagenesis experiments in *P. agardhii* have shown that the production of Adda also involved an O–methylation step catalyzed by the putative monofunctional tailoring enzyme, McyJ (Christiansen et al. 2003).

The remaining biosynthetic enzymes in the microcystin biosynthesis pathway (NRPSs) are putatively involved in the specific activation, modification, and condensation of substrate amino acids onto the linear peptide chain that is then cyclized to produce microcystin. In *M. aeruginosa* PCC7806, initially, McyA adds L–Ser to the growing chain, followed by the addition of D–Ala. This step is followed by the addition of L–Leu and D–MeAsp residues (McyB) followed by the addition of L–Arg (McyC), and subsequent thioesterase–dependent cyclization and release of the final peptide product (Tillett et al. 2000).

An additional monofunctional tailoring enzyme, McyI is hypothesized to play a role in the modification of microcystin. McyI shows greatest homology to a group of D–3–phosphoglycerate dehydrogenase (D–3–PGDH) enzymes from various archaeal species. In *E. coli*, D–3–PGDH enzymes are responsible for the first step in the pathway for serine biosynthesis (Sugimoto and Pizer 1968). The role of this enzyme in microcystin synthesis is unknown, however, the MeDha residue in microcystin is produced from L–Ser, therefore McyI may play a role in the production of L–Ser or conversion of L–Ser to MeDha.

An ABC transporter–like gene *mcyH*, is believed to be involved in the transport of microcystin (Pearson et al. 2004). This transporter may be responsible for the thylakoid localization of the toxin (Shi et al. 1995; Young et al. 2005) or for the extrusion of the toxin under certain growth conditions, including exposure to high and red light (Kaebernick et al. 2000).

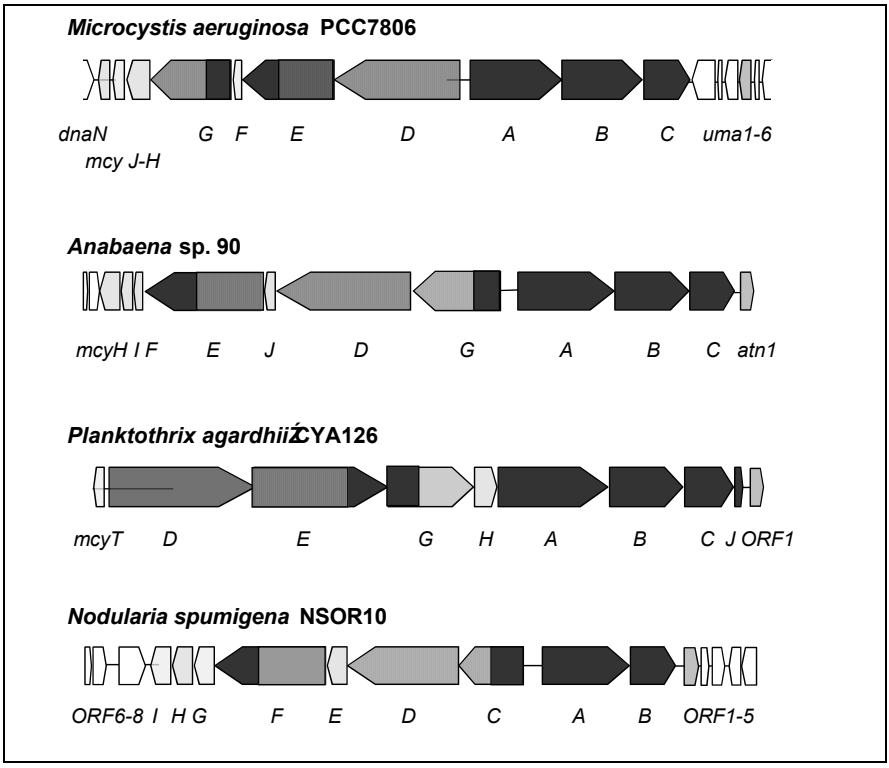

Fig. 1. The microcystin biosynthesis gene clusters and flanking regions found in *M. aeruginosa*, *Anabaena*, and *P. agardhii*, as well as the nodularin biosynthetic genes from *N. spumigena*. The key describes the scheme for polyketide synthase, peptide synthetase, and tailoring enzymes.

Nodularin synthetase

The nodularin biosynthesis gene cluster *ndaS*, from *Nodularia spumigena* NSOR10, has also been recently sequenced and characterized (Moffitt and Neilan 2004). The 48 kb region of the genome consists of nine ORFs (*ndaA–I*) transcribed from a bidirectional regulatory promoter region (Fig. 1). While most of the *ndaS* encoded genes have homologs in the *mcyS* cluster, their arrangement adheres more closely to the 'co–linearity' rule of NRPS pathways that predicts the order of catalytic processes involved in the biosynthesis of a non–ribosomal metabolite is generally the same as the order of the genes which encode their catalytic enzymes (Kleinkauf and von Dohren 1996).

The proposed pathway for nodularin biosynthesis is similar to that proposed for microcystin. Functional assignment of the enzymes was based on bioinformatic analysis and homology to the microcystin synthetase enzymes. The Adda side–chain is produced via a mixed NRP/PKS pathway from a phenylacetate starter unit and several malonyl–CoA extensions (NdaC, D and F). The NRPS module of the hybrid NRP/PKS, NdaF, subsequently adds D–Glu to the growing chain. Two NRPS enzymes, NdaA and B, complete the cyclic pentapeptide by adding the final amino acid residues, L–Thr, D–MeAsp and L–Arg. The NRPS modules responsible for the activation of D–Ala and D–Leu in *mcyS* (McyA and B) are absent from *ndaS* as nodularin lacks these moieties.

The *ndaS* cluster also encodes several putative monofunctional enzymes that may play a role in the modification and transport of nodularin. *ndaE* encodes an O–methyltransferrase, *ndaG* encodes a putative L–Asp/L–Glu racemase, and *ndaI* encodes an ABC transporter. Also encoded within the *ndaS* cluster is a D–3–PGDH homolog, NdaH, that shares 71% identity with McyI. It has been hypothesized that NdaH may therefore catalyse the conversion of MeThr to MeDhb in the final peptide structure (Moffitt and Neilan 2004).

Cylindrospermopsin synthetase

Cylindrospermopsin (Fig. 2) consists of a tricyclic sulfated and methylated guanidinium backbone, which is linked to hydroxymethyluracil (Ohtani et al. 1992). Its biosynthesis has been partially elucidated by labeled precursor feeding studies (Burgoyne et al. 2000). Glycine, five intact acetates, and an S–adenosylmethionine methyl group are confirmed cylindrospermopsin precursors. Biosynthesis of cylindrospermopsin is initiated by the production of guanidinoacetate from glycine and an, as yet unidentified,

guanidino donor. The molecular backbone of cylindrospermopsin is then assembled by successive condensations of five intact acetates to the guanidinoacetate starter unit, followed by tailoring reactions, such as C-methylation, ketoreduction, sulfation and cyclizations to complete biosynthesis. Not all parts of the cylindrospermopsin molecule are accounted for, however. For instance, the origin of the guanidino and uracil moieties remains to be investigated (Burgoyne et al. 2000).

The molecular structure and feeding experiments of cylindrospermopsin suggested that it is produced by a mixed non–ribosomal peptide and polyketide pathway. Using a reverse genetic approach, gene fragments of two putative cylindrospermopsin biosynthesis genes were detected in cylindrospermopsin–producing strains of *Cylindrospermopsis raciborskii* and *Anabaena bergii* (Schembri et al. 2001). Phylogenetic screening in this study revealed that the presence of these two genes was directly linked to cylindrospermopsin production. The partial cylindrospermopsin biosynthesis gene cluster from *Aphanizomenon ovalisporum* was sequenced in a later study (Shalev–Alon et al. 2002), and revealed the presence of an amidinotransferase (*aoaA*) adjacent to a hybrid NRPS/PKS (*aoaB*) and a type I PKS (*aoaC*) (Fig. 2b). Enzymes encoded by these three genes are believed to initiate cylindrospermopsin biosynthesis (Shalev–Alon et al. 2002). AoaA is believed to synthesise the guanidinoacetate starter unit from glycine and an unidentified guanidino donor. It provided the highest homology to vertebrate arginine:glycine amidinotransferase. Functional modelling of AoaA revealed that residues corresponding to those involved in arginine and glycine substrate binding in the human amidinotransferase were conserved with regard to the amino acid and the topology in AoaA (Kellmann et al. 2005). Contrary to the study by Burgoyne et al (2000), which could not detect any incorporation of labelled arginine into guanidinoacetate, the data presented strongly suggested that arginine is the natural guanidino donor in cylindrospermopsin biosynthesis. However, this needs to be verified experimentally. Following guanidinoacetate synthesis, the hybrid NRPS/PKS, AoaB, is believed to recruit guanidinoacetate for polyketide extension. Catalytic domains present in AoaB are adenylation domain, acyl carrier protein, β–ketoacylsynthase and acyltransferase. To date, an NRPS with an adenylation domain that activates guanidinoacetate has not been reported. Substrate–binding residues of the AoaB adenylation domain differed from those of other adenylation domains, which may reflect the structure its substrate guanidinoacetate (Kellmann et al. 2005). AoaB may thus activate guanidinoacetate and add the first acetate extender unit. While *aoaC* has only been partially sequenced, it contains a β–ketoacylsynthase, an acyltransferase and a dehydrogenase domain. AoaC may

add a further acetate extender unit and also reduce one of the carboxylate carbons (Fig. 2b).

Saxitoxin and the paralytic shellfish toxin biosynthesis

Paralytic shellfish poisoning (PSP) toxins are natural alkaloids. Like the cyclic peptide toxins, PSP toxins are considered secondary metabolites. Almost 30 years after the correct structure of saxitoxin, the parent compound of PSP toxins, was elucidated by X–ray crystallography (Schanz et al. 1975), there has been much speculation as to how this peculiar alkaloid is naturally produced. Nevertheless, very few investigations have contributed to the knowledge of saxitoxin and related compound biosynthesis. The genes encoding enzymes involved in PSP toxin biosynthesis are at present unknown.

In the early 1980s, however, Shimizu and colleagues documented fundamental studies concerning the biosynthesis of the saxitoxin carbon perhydropurine–skeleton (Shimizu et al. 1984; Shimizu 1986a, 1986b). According to feeding experiments carried out with ^{13}C, ^{15}N, and ^{2}H–labelled precursors in the dinoflagellate *Alexandrium tamarense* and the cyanobacterium *Aphanizomenon flos–aquae*, saxitoxin was shown to be derived from arginine and acetate via a Claisen–type condensation between C2 of arginine (or ornithine) and C1 of acetate. This reaction is uncommon, although it characterizes the first step of porphyrin biosynthesis with the condensation of succinate and glycine to form aminolevulinic acid (Shimizu 1996). Subsequently, the amino group of arginine is transformed into a guanidino group by transfer of an amidino moiety from another molecule of arginine. Two guanido groups originating from arginine are incorporated into tricyclic carbon backbone of saxitoxin, however the origin of the carbamate side–chain has not been reported. The side chain C13 originates from the S–adenosylmethionine methyl group. In total, to synthesize one saxitoxin molecule three arginine, one acetate, and one methionine molecule were required. This sequence of biochemical reactions was unprecedented in the literature and it remains unique.

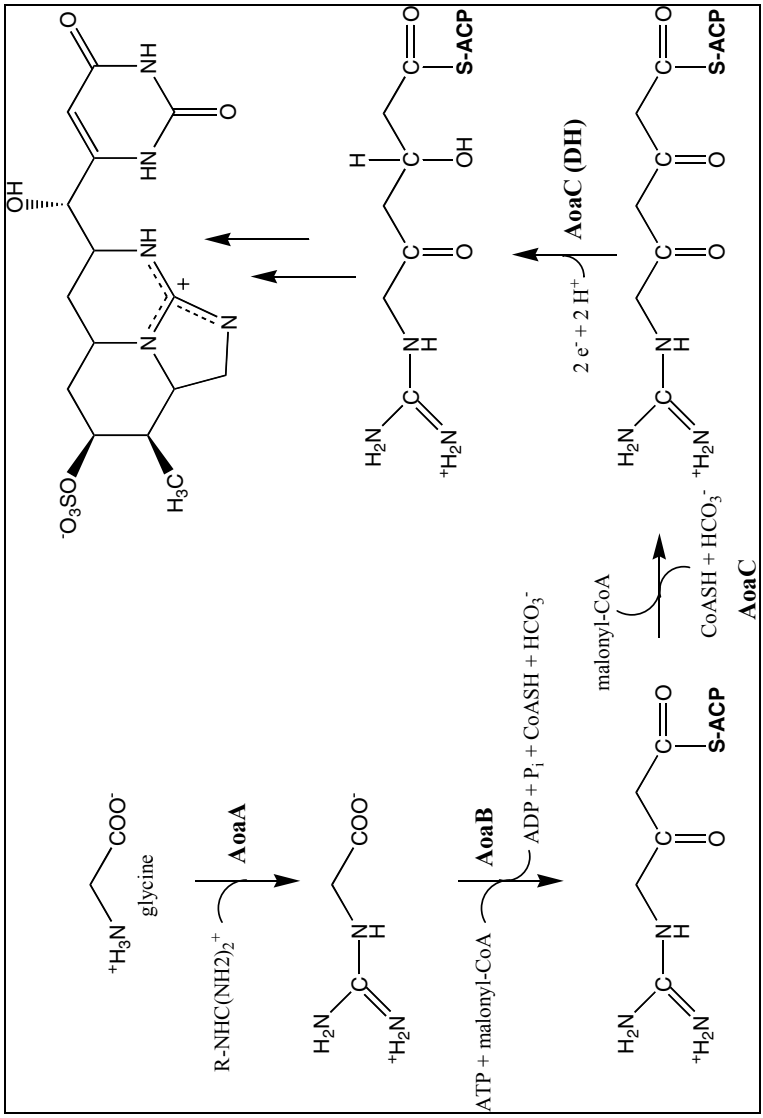

Fig. 2. (a) Biochemical reactions that three putative cylindrospermopsin biosynthesis gene products AoaA, AoaB and AoaC are thought to catalyse

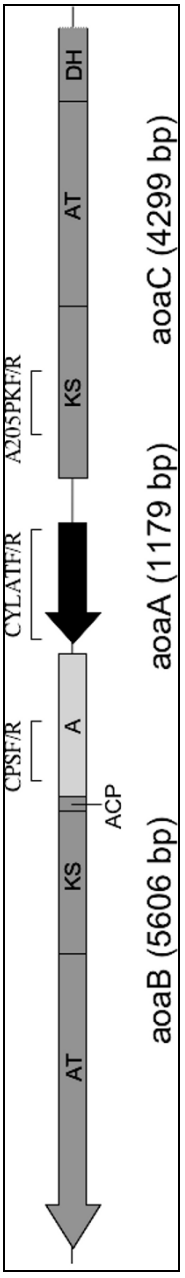

Fig. 2. (b) arrangement of aoaA, aoaB and aoaC in *A. ovalisporum* The light grey shading indicates non–ribosomal peptide synthetase, dark grey polyketide synthase modules. The brackets with primer names indicate the regions that were amplified and sequenced for phylogenetic analysis. A: aminoacyl adenylation; AT: acyl transferase; DH: dehydrogenase, KS: β–ketosynthase, ACP: acyl–carrier protein.

No biosynthetic enzymes have yet been identified and shown to be involved in saxitoxin production. Recent research, however, has identified certain candidate enzymes that may be somehow linked to saxitoxin production. Using differential display, Taroncher–Oldenburg and Anderson (2000) characterized differential gene expression in the toxic dinoflagellate *Alexandrium fundyense* during the early G_1 phase of the cell cycle, coinciding with the onset of toxin production in this organism (Taroncher–Oldemburg et al. 1997). An S–adenosylhomocysteine hydrolase, a methionine aminopeptidase, and a histone–like protein were isolated, although none of these genes show any correlation with the proposed saxitoxin biosynthetic pathway. On the other hand, Sako and co–workers (2001) purified and characterized a N–sulfotransferase from the toxic dinoflagellate *Gymnodinium catenatum* that was uniquely and specifically capable of transferring a sulphate residue from 3'–phosphoadenosine 5'–phosphosulfate (PAPS) to the N–21 carbamoyl group present in saxitoxin and gonyautoxin2+3. This enzyme, a monomeric 60 kDa protein, showed activity with only three substrates, each yielding a distinct product: saxitoxin→gonyautoxin 5, gonyautoxin 2→C–1, and gonyautoxin 3→C–2. The activity of this N–21 sulfotransferase suggested a possible pathway for saxitoxin synthesis in dinoflagellates by which saxitoxin represents the basic structure for the production of all other PSP derivatives. The hypothesized sequence of conversions, saxitoxin→gonyautoxin 2+3→C–toxins, is the reverse of a speculated pathway based on the time–dependent accumulation of various saxitoxin analogues during the synchronised growth of *A. fundyense* (Taroncher–Oldenburg et al. 1997). Another sulfotransferase, highly specific for saxitoxin analogues, has been purified from the PSP–producing dinoflagellate *G. catenatum* (Yoshida et al. 2002). This sulfotransferase converted 11–hydroxy saxitoxin, a synthetic analogue that is believed to be a natural intermediate, into gonyautoxin–2. Sequence information for these two sulfotransferases could not be obtained due to their low yield and instability. It is also possible that the enzymes do not normally play a role in saxitoxin biosynthesis since N–sulfotransferase activity has been detected in a non–toxic strain of *G. catenatum*, and was absent in toxic *A. tamarense* (Oshima 1995).

By exploring the differences between genomes of saxitoxin–producing and non–toxic *A. circinalis* strains, a carbamoyl–phosphate synthase, a S–adenosylmethionine dependent–methyltransferase, a transposase, an acetyltransferase, and several toxic–strain specific hypothetical and regulatory proteins, including the 60 kDa chaperonin GroEL, were identified (Pomati and Neilan 2004; Pomati et al. 2004a). A toxic strain specific gene coding for a putative Na^+ dependent transporter was recently recovered from a

saxitoxin–producing strain of *A. circinalis* and was suggested to be involved in Na^+–specific pH homeostasis (Pomati et al. 2004a). This gene was successfully applied as a molecular probe in the laboratory for the environmental screening of saxitoxin–producing strains of *A. circinalis*. It has not as yet been possible, however, to demonstrate the direct involvement of any of these genes mentioned above in the biosynthesis of saxitoxin.

Biosynthesis of anatoxins

Anatoxin–a is a neurotoxic alkaloid produced by a number of cyanobacterial species including *Anabaena flos–aquae, Aphanizomenon flos–aquae,* and *Planktothrix* sp. (formerly *Oscillatoria* sp.) (Carmichael 1992). It has previously been proposed (Gallon et al. 1990) that anatoxin–a is synthesized from ornithine via putrescine through the activity of ornithine decarboxylase, similar to the structurally related tropane alkaloid pathways in plants. Subsequent studies (Gallon et al. 1994) suggested the involvement of arginine and Δ^1–pyrroline in the same hypothetical pathway but results were not definitive and could be attributed to trans–amination. Later studies (Hemscheidt et al. 1995b) have shown that C1 of the glutamic acid is retained during the formation of the toxin and is not lost by decarboxylation and therefore is incompatible with the hypothesis that the carbon atoms of the pyrrolidine ring are derived in the same way as are the tropane alkaloids. It was further shown that a five–carbon unit is derived intact from an amino acid precursor of the glutamic acid family while the other carbons are derived form acetate extender units.

Recently elucidated toxin biosynthesis pathways in cyanobacteria seem to mainly involve polyketide synthases (PKS) and non–ribosomal peptide synthetases (NRPS) e.g. microcystin, nodularin, jamaicamides and cylindrospermopsin. It is therefore proposed that the anatoxin–a biosynthesis genes would encode a mixed PKS/NRPS system for activating an amino acid of the glutamic acid family followed by the incorporation of 3 acetates in a polyketide manner.

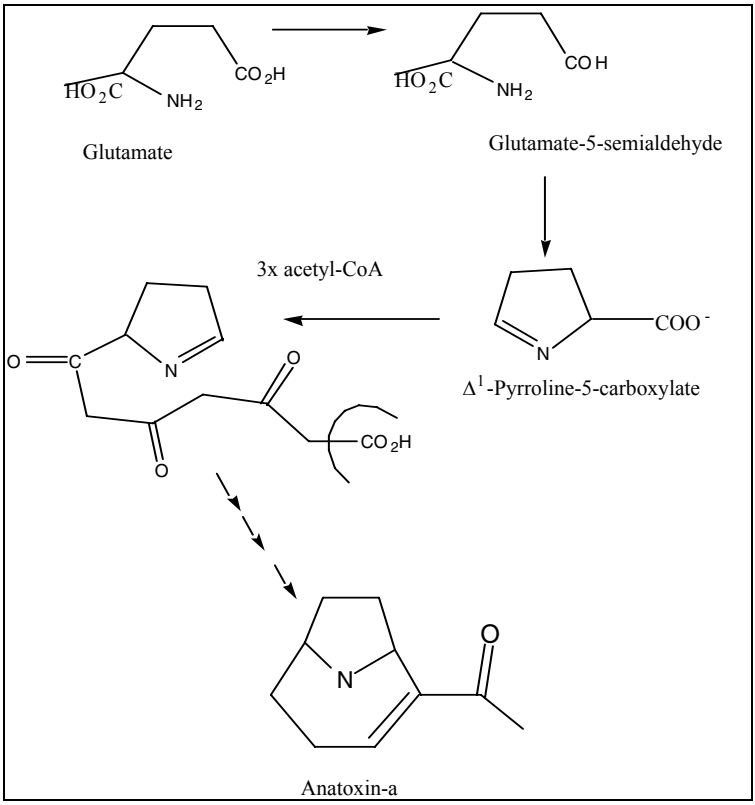

Fig. 3. Putative biosynthetic pathway of anatoxin–a. An amino acid of the glutamic acid family is activated followed by the addition of three acetate extender units in a polyketide manner, further steps may include reductions and a cyclization which are putatively encoded by the corresponding PKS tailoring domains. Note that the first two steps might also be involved in the proline biosynthesis pathway.

Due to the structural similarity of homoanatoxin–a and anatoxin–a it is believed that they are produced via the same pathway (Hemscheidt et al. 1995b), the terminal carbon of homoanatoxin–a seems to be derived from L–methionine via S–adenosyl–methionine (Namikoshi et al. 2004).

For anatoxin–a(s), feeding experiments have shown that the carbons of the triaminopropane backbone and the guanidino group are derived from L–arginine (Moore et al. 1992), whereas (2S,4S)–4–hydroxyarginine is an intermediate (Hemscheidt et al. 1995a).

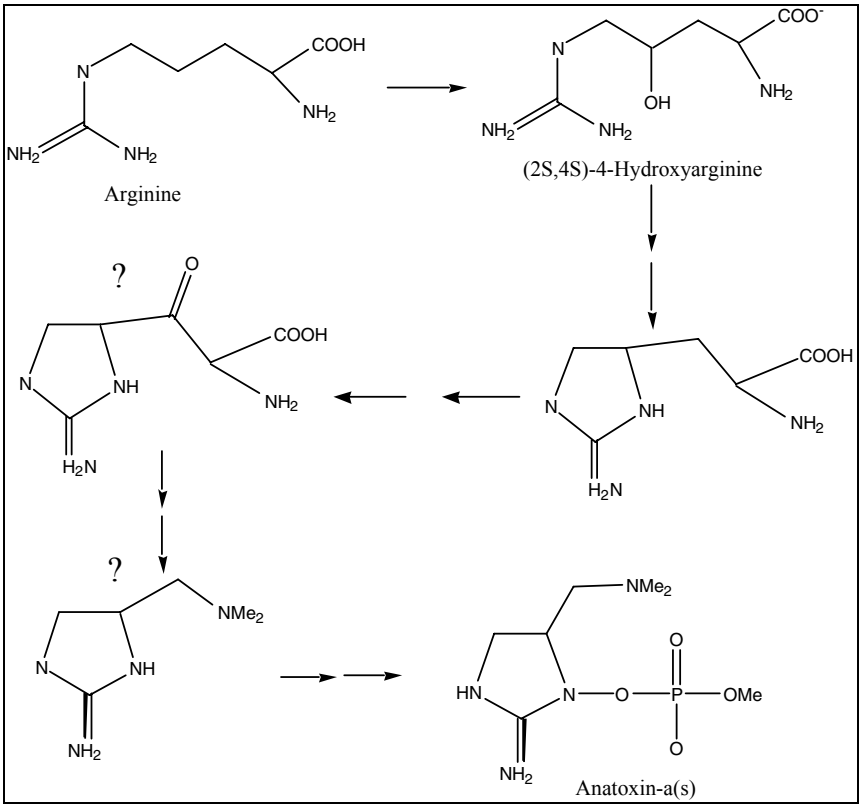

Fig. 4. Putative biosynthetic pathway of anatoxin–a(s). L–Arginine is a precursor and (2S,4S)–4–hydroxyarginine an intermediate, all other intermediates are hypothetical.

Biological and environmental factors influence the genetic expression of cyanotoxins

Most previous work in this area has investigated the end–products of gene expression. Due to the fact that most investigations have not been performed on a single set of strains, the experimental designs have not been standardized and the data not interpreted against the same specific growth controls. For the most part, production of a toxin by cyanobacteria appears to be constitutive. Evidence suggests, however, that the conditions of a bloom environment may alter the levels of toxin produced. Various studies have focused on the typically encountered environmental factors that may influence changes in the production of toxin. The variable ecological pa-

rameters that toxic cyanobacteria are exposed to include, temperature, light, nitrogen, iron, phosphate, predators, and other microorganisms.

Microcystin production and microcystin synthetase gene regulation

Hepatotoxin production in cyanobacteria is thought to be influenced by a number of different physical and environmental parameters, including nitrogen, phosphorous, trace metals, growth temperature, light, and pH (Sivonen 1990; Lukac and Aegerter 1993; van der Westhuizen and Eloff 1985; Song et al. 1998). However, due to the fact that most regulatory investigations have not been standardized, the subject of hepatotoxin regulation remains a somewhat contentious issue.

Several batch culture experiments have suggested that high microcystin production in cyanobacteria is correlated with high nitrogen and phosphorus concentrations (Sivonen in 1990; Vezie et al. 2002). Conversely, low iron concentrations have been correlated with increased toxin production (Lukac and Aegerter 1993). While these results suggest that microcystin production is influenced by nutrients and trace metals, the observed toxin fluctuations were probably due to the indirect affects of nitrogen, phosphorous and iron on cell growth rate. Long et al (2000) observed that under nitrogen–limited conditions, fast growing *M. aeruginosa* cells are smaller, of lower mass and contain higher intracellular levels of toxin than slow–growing cells. These results strongly suggest a positive linear relationship between the microcystin content of cells and their specific growth rate. This generalized model for microcystin regulation may also explain the conflicting results yielded from other batch culture investigations where variables such as temperature, light and pH have been tested (Orr and Jones 1998).

Microcystin has been shown to bind iron(III) and other cations and as such it has been considered that microcystin may be produced as a siderophore. Microcystin content was shown to be inversely proportional to the concentration of iron(III) in the media of a *Microcystis* culture (Lukac et al. 1993; Utkilen et al. 1995; Bickel et al. 2000). These studies may indicate microcystin synthesis is strain–specific, or a more complex response to light and iron (III) is associated with other external influences, including light levels and the presence of organic complexes.

Studies into the effect of different light intensities on microcystin production have revealed conflicting results. Whilst some studies indicate that production of microcystin is higher in response to low light intensities, others have indicated that the production (cellular levels) of microcystin is

highest under high light conditions (Utkilen et al. 1992; van der Westhuizen et al. 1985; Watanabe et al. 1985; Sivonen et al. 1990; Rapala et al. 1997; Kaebernick et al. 2000). Downing et al. (2005) have recently proposed that most of the differences seen in the published work regarding microcystin production is due to variations in nitrogen uptake and assimilation, possibly in response to carbon fixation. This data then proposes that biologically available nitrogen and phosphorus are the real determinants of microcystin production in the environment, rather than other influences on growth rate, including light and temperature. Interestingly, growth of *Anabaena* at different temperatures has been correlated with the production of certain microcystin isoforms, as discussed later.

While most toxin regulation studies have focused on direct measurements of cellular toxin, the description of the *mcy* gene cluster by Tillett et al. (2000) enabled a closer examination of microcystin regulation at the molecular level. Kaebernick et al. (2000) used the RNase protection assay to measure the transcription of *mcyB* and *mcyD* under a variety of different light conditions. High light intensities and red light were correlated with increased transcription, while blue light led to reduced transcript levels. Interestingly, the authors observed two light thresholds, between dark and low light (0 and16 μmol of photons m^{-2} s^{-1}), and medium and high light (31 and 68 μmol of photons m^{-2} s^{-1}), at which a significant increase in transcription occurred. The same group later discovered that transcription of *mcy* genes occurs via two polycistronic operons, *mcyABC* and *mcyDEFGHIJ,* from a central bidirectional promoter between *mcyA* and *mcyD* (Kaebernick et al. 2002). Interestingly, alternate transcriptional start sites were identified for both operons when cells were cultured under high or low light intensities (ie. 68 or 16 μmol of photons m^{-2} s^{-1} respectively). Interestingly, this central regulatory region of *mcyS* also contains sequence motifs for both Fur and NtcA DNA binding proteins.

Several studies have reported an increase in extracellular microcystin content following exposure of cultures to high light conditions (Rapala et al. 1997; Bottcher et al. 2001; Kaebernick et al. 2000; Wiedner et al. 2003). Wiedner et al. (2003), found that on average, extracellular microcystin concentrations were 20 times higher when cells were cultures at 40 μmol of photons m^{-2} s^{-1} compared to those grown at 10 μmol of photons m^{-2} s^{-1}. However, it is important to note that the extracellular microcystin concentrations at both irradiances accounted for only 2.47 and 0.22% of the total microcystin content, respectively. Kabernick et al. (2001) proposed that microcystin may be constitutively produced under low and medium light intensities, and exported when a higher threshold intensity is reached. The recently identified ABC transporter McyH, may be responsi-

ble for this apparent export, however increased cell lysis and leakage of the toxin at higher irradiances can not be ruled out at this stage. Further investigation is clearly required.

An insertional inactivation mutant was produced via the homologous recombination of a chloramphenicol resistance cassette into the *mcyB* of *M. aeruginosa* PCC7806 (Dittmann et al. 1997). Comparative analysis of the proteomes of the wild–type and the mutant cultures resulted in the identification of a protein, MrpA, that was only strongly expressed in the wild–type and not by the non–toxic mutant (Dittmann et al. 2001). This protein and another protein encoded by a gene downstream of *mrpA* were homologous to RhiA and RhiB from *Rhizobium leguminosarum*. The RhiABC proteins are thought to play a role in nodulation and are regulated via a quorum–sensing mechanism. In addition, the *mrpA* transcripts were up–regulated in response to blue light. The results of this work led to the conclusion that microcystin may act as an extracellular signalling molecule and may be associated with a light–sensing mechanism in *Microcystis*.

Regulation of nodularin gene transcription and toxin production

Like *mcyS*, the *ndaS* gene cluster is transcriptionally regulated by a bi–directional promoter region. Analysis of transcription of the *ndaS* cluster found that it is transcribed as two polycistronic mRNA, *ndaAB, ORF1,* and *ORF2,* and *ndaC* (Moffitt 2003). The two genes downstream of *ndaAB*, *ORF1* and *ORF2*, encode a putative transposase and a putative high light–inducible chlorophyll–binding protein, respectively. It is not clear why these proteins are also co–transcribed with the *ndaS* gene cluster. *ORF2* has been identified in all strains of toxic *Nodularia* and the association between *ORF2* and nodularin biosynthesis may suggest a physiological function associated with high–light stress in the cells producing it. A putative heat shock repressor protein, encoded by the gene *ORF3*, was also identified downstream of ORF2, which may be involved in the transcriptional regulation of the *ndaS* genes in response to heat stress.

Growth experiments using *N. spumigena* batch cultures also suggest a link between light quality and nodularin production. The batch cultures preferred higher irradiances for growth (45 to 155 μmol of photons $m^{-2}.s^{-1}$) and these cultures produced the highest concentration of intracellular and extracellular toxin, although there appeared to be no significant difference between these values (Lehtimaki et al. 1994; Lehtimaki et al. 1997). *N. spumigena* BY1 batch cultures have been shown to grow best at temperatures between 25°C and 28°C (Lehtimaki et al. 1997). The level of in-

tracellular and extracellular nodularin was lower in batch cultures grown at temperatures of 19°C and 28°C. Temperature had a similar effect on the production of microcystin by *Microcystis* and *Anabaena* with highest production rates at around 25°C (Rapala et al. 1998; Codd et al. 1988). Similarly, the growth rate of *N. spumigena* and nodularin production is higher in moderate salinities between 0.5% and 2% NaCl when compared to lower (0%) or higher (3%) salinities. The concentration of intracellular nodularin was highest in the cultures with the highest growth rate (0.5% to 2% NaCl), although the extracellular concentration of nodularin was higher in the cultures maintained in media containing 0.5% to 1% NaCl (Blackburn et al. 1996; Lehtimaki et al. 1997).

The presence of ammonium appeared to have a negative effect on the growth of *N. spumigena* BY1 (Lehtimaki et al. 1997). The growth rate of batch cultures was lowest in media supplemented with the highest concentration of nitrate, and the nodularin concentration also decreased. A batch culture study of *N. spumigena* strain GR8b found that the total nodularin concentration was highest at the end of the experiment, under low nitrate levels (Repka et al. 2001). The opposite was found true for the non–nitrogen fixing genera *Oscillatoria* and *Microcystis* which produced higher levels of microcystin when grown in the presence of high levels of nitrogen (Sivonen et al. 1990; Utkilen et al. 1995; Watanabe et al. 1985). Analysis of the effect of phosphate levels on nodularin production indicated that batch cultures of *N. spumigena* BY1 grown at high concentrations of phosphate produced the highest levels of nodularin per cell dry weight. Recent studies of batch and chemostat cultures found that phosphate had no effect on the total nodularin concentration. Analysis of microcystin–producing cultures *Microcystis*, *Anabaena*, and *Oscillatoria* indicated that there is, in general, an increase in the cellular concentration of microcystin when cultures were grown in media containing higher levels of phosphorus (Utkilen et al. 1995; Rapala et al. 1997; Sivonen et al. 1990).

Saxitoxin and paralytic shellfish toxin expression

The regulation of saxitoxin production in cyanobacteria and the metabolic role of PSP toxins within the producing microorganisms is poorly understood. Historically, factors influencing the biosynthesis of saxitoxin and related compounds in cyanobacteria have also been the subject of sub–optimal studies, both in the laboratory and in the environment. Since the genes for saxitoxin production are presently unknown, this section will focus on what influences PSP toxin accumulation.

One of the most detailed reports in the literature concerning the physico–chemical features associated with saxitoxin production, describes the environmental conditions during one of the largest PSP toxin producing blooms on record (Bowling and Baker 1996). The cyanobacterial bloom, dominated by up to half a million cells of *A. circinalis* per millilitre, affected 1000 km of river waters in the Barwon–Darling basin, New South Wales, Australia. Numerous livestock deaths were reported during this occurrence, and the neurotoxicity of *A. circinalis* samples was demonstrated by mouse bioassay. This cyanobacterial bloom was attributed to reduced river flow as a result of intense drought. Under such environmental conditions nutrient concentrations, especially phosphorus, were found to be very high. Alkaline pH (>8.5) and very high ammonia values, in some instances more than 1 mg L^{-1}, also characterized the majority of sampling sites along the river. Additionally, most of the neurotoxic *A. circinalis* samples were collected from water with high electrical conductivity (Bowling and Baker 1996). Similar environmental parameters, in particular elevated pH and high conductivity, have also been described in Australia for another potentially toxic bloom of *A. circinalis* in Lake Cargelligo, New South Wales, during 1991 (Bowling 1994).

In the laboratory, changes in toxicity or in toxin concentration of cyanobacterial isolates can be induced, although such variations range from only two to four–fold. Among the environmental parameters tested on growth and PSP toxin accumulation were culture age, temperature, light, principal nutrients, salinity, and pH. These investigations indicated that cyanobacteria produce PSP toxins under conditions that are most favourable for their growth as has been reported for other cyanobacterial secondary metabolites (Sivonen and Jones 1999).

Cyanobacteria produce more PSP toxins during their late exponential growth phase, and it has been reported that saxitoxin biosynthesis in *Aphanizomenon flos–aquae* NH–5 was inversely proportional to its growth rate (Gomaa 1990). Comparable results were also described for PSP–producing strains of *Lyngbya wollei* and *Cylindrospermosis raciborskii* (Yin et al. 1997; Castro et al. 2004). High light intensity was shown to result in reduced toxicity in *L. wollei* cultures, coupled to an increase in biomass (Yin et al. 1997). On the other hand, temperature seems to have a marked effect on PSP toxin production. Yin and colleagues (1997) documented a net decrease in PSP toxin content of *L. wollei* cultures with increasing temperature. Similarly, *A. circinalis* was found to produce more saxitoxin and gonyautoxin 2/3 per cell at low temperatures (15°C) compared to values above 30°C (Rossetti and Pomati unpublished data). Data consistent with these results have been recently described in *C. raciborskii*, where changes in the extracellular levels of PSP toxins were noted (Castro

et al. 2004). Modifications in the toxin profile between 19 and 25°C were also detected. These documented effects have been explained by either a lower stability or higher biodegradation rate of saxitoxins at high temperatures, as well as by significant changes in the rate of cell division (Yin et al. 1997; Castro et al. 2004).

Alkalinity is a feature that often characterizes the environment of toxic cyanobacterial blooms, in particular those dominated by PSP–producing species, such as *A. circinalis* (Bowling and Baker 1996). High salinity, recorded as increased conductivity, has also been correlated with neurotoxic blooms of *A. circinalis* in Australia (Bowling 1994; Bowling and Baker 1996) and *C. raciborskii* blooms in Brazil (S. Azevedo personal communication). Intracellular saxitoxin levels were recently found to respond to the alkalinity and Na^+ content of culture media (Pomati et al. 2004b), and by influencing Na^+ fluxes it was possible to induce a corresponding modulation of saxitoxin accumulation (Pomati et al. 2003; Pomati et al. 2004b). Since maintenance of pH and sodium homeostasis are strictly correlated in cyanobacteria (Horikoshi 1991; Maestri & Joset 2000; Waditee et al. 2001), sodium transport and cycling seem to play a vital role in the regulation of intracellular saxitoxin levels. A Na^+–dependent transporter gene, specific to PSP toxin–producing strains of *A. circinalis,* has also recently been cloned and may somehow be involved in the sodium–coupled transport of saxitoxins (Pomati et al. 2004a).

Certain nutrients, in particular light, nitrogen and phosphorus, are essential for cyanobacterial growth. Phosphorus is usually the limiting factor for autotrophic growth in freshwaters, and hence small changes in this nutrient's concentrations may affect toxin production merely as a result of influencing growth. In *L. wollei*, optimum growth levels for PO_4 also correspond to the optimal conditions for PSP toxin production (Yin et al. 1997). Most microbial secondary metabolites are molecules that are rich in nitrogen. Nitrogen–fixing species, such as PSP toxin producing cyanobacteria, are not dependent on fixed nitrogen in their growth media for toxin production (Rapala et al. 1993; Lehtimäki et al. 1997), although very high concentrations of this element were found to inhibit PSP toxin accumulation in cultures of *L. wollei* (Yin et al. 1997). Rising levels of calcium have been shown to increase PSP toxin synthesis in *L. wollei* (Yin et al. 1997), however, no other trace element has been investigated for their effect on the production of saxitoxin in cyanobacteria.

Regulators of cylindrospermopsin production

Direct studies on the expression of cylindrospermopsin–biosynthesis genes have not been performed, however the growth rates and cylindrospermopsin production rates of *C. raciborskii* in response to different levels and types of nitrogen sources were examined in the study by Saker and Neilan (2001). It was found that cultures grown in the absence of a fixed nitrogen source provided the highest concentration of cylindrospermopsin on a per dry weight basis, with a corresponding lowest growth rate. Cultures supplemented with nitrate were intermediary with regard to growth rate and toxin production, while cultures supplemented with ammonia provided the lowest toxin concentrations, but the highest growth rates.

Regulation of anatoxin production

Different media and culture duration have been shown to effect the anatoxin–a content in *Anabaena flos–aquae* (Gupta et al. 2002). Rapala et al. (1993) found that temperature reduced the levels of anatoxin–a regardless of growth rate. In addition, growth in nitrogen–free media has been correlated to anatoxin–a production, however, results in these studies were strain specific and varied widely in response to the different growth conditions employed.

Multiple genera of cyanobacteria share common biosynthetic processes

Microcystin producers

Comparative studies of the *mcyS* gene clusters from *M. aeruginosa*, *P. agardhii* (Christiansen et al., 2003) and *Anabaena* sp. (Rouhiainen et al. 2004) have noted variations in the arrangement of *mcyS* genes between these different species of cyanobacteria, although the proposed toxin biosynthetic processes are thought to be similar (Fig. 1). The *M. aeruginosa* and *Anabaena* sp. *mcyS* clusters are arranged into two divergently transcribed operons, however, the arrangement of genes within these operons differs between the two species. In *P. agardhii*, the *mcyS* cluster also has a distinctive arrangement and lacks *mcyF* and *mcyI*. Furthermore, the *P. agardhii mcyS* cluster contains an additional gene, *mcyT*, upstream of the central promoter region. This gene is thought to encode a putative type–II thioesterase enzyme, which was proposed to have an editing role by re-

moving mis–primed amino acids from the NRPS and PKS enzymes. The characterization of *mcyS* in *M. aeruginosa*, *P. agardhii*, and *Anabaena* sp. has enabled the study of the origins and evolution of hepatotoxin biosynthesis in cyanobacteria. Identification of transposases associated with the *mcyS* and *ndaS* gene clusters and subsequent phylogenetic analysis has led to the theory that horizontal gene transfer and recombination events are responsible for the sporadic distribution of the *mcyS* gene cluster throughout the cyanobacteria and the various microcystin isoforms that have been identified to date (Tillett et al. 2001; Mikalsen et al. 2003, Tanabe et al. 2004). Recently, a phylogenetic study by Rantala et al. (2004 has contradicted this theory, suggesting that the genetic associated with hepatotoxicity was acquired early in evolution and then lost over time. Collectively, these results suggest that recombination, gene loss, and horizontal gene transfer can explain the distribution and variation regarding microcystin production in different cyanobacterial genera.

Nodularin–producers

Nodularin is produced by all strains of the species *N. spumigena* and no other cyanobacterial species, however, the structural analog motuporin, has been isolated from a sponge. Recent genetic studies suggest that the *ndaS* cluster evolved from the *mcyS* cluster through deletion of two NRPS modules (Mikalsen et al. 2003; Moffitt and Neilan 2004; Rantala et al. 2004).

Cylindropsermopsin producers

The production of cylindrospermopsin has been reported in *Cylindrospermopsis raciborskii*, *Aphanizomenon ovalisporum*, *Anabaena bergii*, *Umezakia natans*, and *Raphidiopsis curvata* (Banker et al. 1997; Harada et al. 1994; Li et al. 2001; Schembri et al. 2001). Apart from *C. raciborskii*, which was classified based on both morphology and 16S rRNA gene sequencing, other cylindrospermopsin–producing cyanobacteria were initially only classified by morphology. Subsequent molecular phylogenetic analyses have revealed that the Israeli and Australian *A. ovalisporum* strains reported to produce cylindrospermopsin may in fact represent a species of the genus *Anabaenopsis*, while the cylindrospermopsin–producing *U. natans* and *A. bergii* were closely related to each other and phylogenetically intermediary between the genera *Anabaenopsis* and *Nodularia* (Kellmann et al. 2005). Interestingly, the phylogenetic distance between cylindrospermopsin–producing cyanobacterial species was greater than between the corresponding cylindrospermopsin biosynthesis genes

(NRPS, PKS, and amidinotransferase), which was strong evidence for horizontal transfer of "toxin genes" between these organisms.

Saxitoxin and other PSP–producers

According to the feeding experiments (Shimizu et al. 1984; Shimizu 1986a, 1986b), both the dinoflagellate *Alexandrium tamarense* and the cyanobacterium *Aphanizomenon flos–aquae* synthesize saxitoxin via an identical sequence of biochemical reactions. It is therefore feasible that all PSP toxin–producing microorganisms share a homologous biosynthetic process. Whether putative biosynthetic enzymes for PSP toxin production are common to different strains of the same species, or if the genes are conserved among phylogenetically distant PSP toxin–producing species, is open to question. Because the molecular basis for saxitoxin production is unproven, the origin and the evolution of PSP toxin production remains a mystery.

The toxin profiles of cyanobacteria are consistent traits that appear to have a genetic basis (Castro et al. 2004; Negri et al. 2003; Negri et al. 1997; Velzeboer et al. 2000). Isolates of the same cyanobacterial species from geographically distant locations provide different toxin profiles (Velzeboer et al. 2000), which remain constant for each strain over many generations. The production of PSP toxins is geographically segregated in *A. circinalis* and *C. raciborskii*. Only Australian isolates of *A. circinalis* (Beltran and Neilan 2000) and Brazilian isolates of *C. raciborskii* produce PSPs (Neilan et al. 2003). Unique analogs of saxitoxin have been found in *L. wollei*, a species that is phylogenetically unrelated to other PSP–producing cyanobacteria (Onodera et al. 1997). PSP toxins have been isolated from a broad range of microorganisms, including dinoflagellates and heterotrophic bacteria. This scattering of saxitoxin–producing microorganisms across two of the three kingdoms of life again suggests the potential for the lateral exchange of saxitoxin biosynthetic genes between dinoflagellates, cyanobacteria and other bacteria, probably via a prokaryotic ancestor. This hypothesis is validated by the fact that the synthetic capability of PSP toxin production is limited to certain strains and is not universal to a species. Evidence has previously been found for possible lateral gene transfer events between toxic strains of *A. circinalis* (Pomati and Neilan 2004). This would further support the fact that the basic molecular and genetic machinery underlying saxitoxin biosynthesis is shared by all PSP–producing micro–organisms.

Preferential expression of one toxin over another in certain cyanobacteria

The biological or ecological controls for the preferential production of a particular toxin by any given cyanobacterial strain are not known. Although a rare occurrence, individual strains of cyanobacteria have been shown to produce more than one type of toxin and, therefore this preferential expression is under–pinned by the genes required to produce these compounds. Contentious in this area are reports that describe multiple toxins isolated from a natural bloom sample that is described as having a single species composition. In this situation, minority members of the bloom may be contributing to the toxin profile but are not detected by standard microscopic methods. This highlights another critical area of research, the need for highly skilled microbiologists that can achieve and maintain culture axenicity for future genetic and chemical analyses.

Expression of multiple congeners of a single type of cyanotoxin

Microcystin isoforms

More than 65 isoforms of microcystin are produced by five genera of cyanobacteria. While current data suggest that *Oscillatoria* strains produce only one major toxin at any time, *Anabaena* spp. and *Microcystis* spp. are capable of producing two to four microcystin isoforms simultaneously (Luukkainen et al. 1993). The structure of microcystin differs primarily at the two L–amino acids, and secondarily on the presence or absence of the methyl groups on D–MeAsp and/or Mdha (Namikoshi et al. 1998), however, substitutions of all moieties within microcystin have been reported (Rinehart et al. 1994; Sivonen et al. 1996; Chorus et al. 1999). Varying levels of toxicity have also been reported for each microcystin isoform analysed (Rinehart et al. 1994).

The large number of microcystin variants existing in nature has been attributed to the relaxed substrate specificity of the adenylation domains in the *mcyS* NRPS modules (Mikalsen et al. 2003). In addition to this, toxin variation is also caused by genetic differences in the microcystin synthetase genes themselves. For example, variation within the *mcyB1* module (proposed to result from recombinations between modules *mcyB1* and *mcyC*), has been correlated with the production of different microcystin

isoforms. It has been postulated that variation in *mcyS* genes may be due to several different evolutionary processes, including horizontal gene transfer, lateral recombination and genetic deletions (Mikalsen et al. 2003; Moffitt and Neilan 2004; Rantala et al. 2004; Tanabe et al. 2004). Since the production of microcystin is not restricted to distinct evolutionary clades of species, transposition of the *mcyS* gene cluster across cyanobacterial genera is a strong possibility. Indeed genes encoding transposases have been identified within the flanking regions of *mcyS* (and *ndaS*) clusters, strongly supporting this theory.

While variation at the genetic level appears to be the primary explanation for the plethora of microcystin variants identified, environmental factors may also play a role. Rapala et al. (1997) observed that growth of *Anabaena* cultures at temperatures less than 25°C resulted in the preferential production of microcystin–RR rather than microcystin–LR, which occurred at higher temperatures. Nitrogen added to the growth medium and increasing temperatures also increased the proportion of microcystin variants demethylated in amino acid 3.

Nodularin and related compounds

In contrast, to the wide range of microcystin isoforms present in nature, only seven naturally occurring isoforms of nodularin have been reported. Two of these isoforms, produced by a New Zealand *Nodularia* sp. bloom, have variations within the Adda residue, which reduces or abolishes the toxicity of the compound (Rinehart et al. 1994). The D–Glu residue is essential for the toxicity of nodularin, as esterification of its free carboxyl abolishes toxicity. However, substitutions at position 1 have little effect on toxicity. The other two isoforms, nodularin–Har and motuporin are variable at position 2. Nodularin–Har is produced by the strain *N. harveyana* PCC7804, with the L–Arg, replaced with L–homoarginine (L–Har) (Saito et al. 2001; Beattie et al. 2000). Motuporin has been isolated from the Papua New Guinea sponge *Theonella swinhoei*, and may be synthesized by a commensal cyanobacterium. The L–Arg residue of nodularin is replaced by L–Val in motuporin (deSilva et al. 1992). The L–Val residue is responsible for the additional cytotoxicity of motuporin against cancer cell lines.

The many forms of PSPs

Strains of certain cyanobacterial species, including *L. wollei,* produce a variety of PSP toxin congeners (Onodera et al. 1997). More than thirty dif-

ferent saxitoxin analogs and derivatives are known to date (Sivonen and Jones 1999). There is no indication, however, of a PSP toxin that is preferentially produced by toxic cyanobacterial strains. In addition, each isolate can be characterized by its particular toxin profile (Velzeboer et al. 2000), which is putatively determined by the genetic information carried by its genome. The reason why a particular PSP toxin dominates the profile of a given cyanobacterial strain is unknown. It can be proposed that both the PSP toxin genetics of a cyanobacterium, together with the availability of primary metabolic substrates, is crucial for the production of one PSP toxin over another. Different cyanobacterial strains may also have endogenous enzymes that apply modifications to the basic saxitoxin structure, leading to the production of a range of congeners. Certain environmental conditions are known to favour the stability or the interconversion of individual saxitoxin analogs, although these physical processes mainly occur in the extracellular environment. Transformation reactions may also occur in living cyanobacterial cells, as a result of the aging of both laboratory cultures and natural blooms. Under these conditions, reduction and hydrolysis of carbamate and N–sulphocarbamoyl toxins can yield products of greater toxicity, such as saxitoxin and gonyautoxin, which may become dominant over their original precursors (Jones and Negri 1997). Additionally, high temperatures (> 25ºC) may play a role in the preferential accumulation of decarbamoyl–saxitoxin in *C. raciborskii* (Castro et al. 2004). However, the transformation of saxitoxin into gonyautoxin 2/3 due to a shift to 19ºC from 25ºC has also been reported.

Structures of the anatoxins

Anatoxin–a has one known naturally occurring analogue, homoanatoxin–a. Homoanatoxin–a possesses an extra C–12 methyl group that is probably derived from S–adenosylmethionine and has a similar toxicity to anatoxin–a. So far homoanatoxin-a has only been detected in *Oscillatoria formosa* (Skulberg et al. 1992) and *Raphidiopsis mediterranea* (Namikoshi et al. 2003). The simultaneous production of homoanatoxin–a and anatoxin–a in these species has recently been reported (Namikoshi et al. 2003; Aráoz et al. 2005). As the biosynthetic pathway for both congeners are believed to be almost identical, it is plausible that these organisms posses only a single gene cluster for toxin biosynthesis and the selective action of a tailoring enzyme(s) results in the production of the two variants.

Evolutionary advantages conferred by cyanotoxin production

Toxin biosynthesis by cyanobacteria, is energetically expensive. Toxic species typically commit about 2% of their genomes in order to produce a metabolite that may constitute 2% of the cell's dry mass. In addition, mutation of the genes encoding these pathways does not reduce the viability of cultures in the laboratory and there appears to be a mosaic distribution of toxigenic strains globally in the environment. The ecophysiological basis for cyanotoxin production is a paradox that has directed most of the studies described so far in this paper. Elucidating their role in the life history of the producing organisms is a critical issue in water quality management.

There is evidence that cyanobacteria and their toxins may have an effect on zooplankton (cladoceran and rotiferan) population structure, and that this in turn may guide ecological processes responsible for cyanobacterial success. Cyanobacterial cells are generally a poor food source for zooplankton and are often selectively avoided (DeMott 1991). As a result, zooplankton feed on phytoplankton that are otherwise in competition with cyanobacteria. It would be premature to postulate that toxic cyanobacterial dominance is planned and guided by cyanotoxin production, however, feeding deterrence has been one of the earliest roles suggested for these metabolites. Whether the compounds causing toxicity and deterrence are one and the same has recently been questioned (Rohrlack et al. 1999; Kaebernick et al. 2001; Reinikainen et al. 2001).

Heterotrophic eubacteria, fungi, phytoflagellates and protozoans, commonly associated with cyanobacteria, may also be affected by cyanotoxin production. Many bloom–forming cyanobacteria exhibit optimal growth in the presence of contaminant heterotrophic bacteria. For example, *Pseudomonas aeruginosa* was found to be chemotactically attracted to the heterocysts of *Anabaena oscillarioides*, which subsequently led to the establishment of a mutualistic relationship between the two bacteria sharing the fixed N_2 (Pearl 1984; Pearl and Gallucci 1985). Similarly, *M. aeruginosa* cells exhibit greater cell specific rates of CO_2 fixation when in association with bacteria and protozoan grazers (Pearl and Millie 1996). It has been suggested that the production and secretion of extracellular metabolites, such as cyanotoxins, may play a role in attracting these beneficial hosts while at the same time repelling antagonistic microbes and higher order grazers (Pearl and Millie 1996). An allelopathic function for microcystin has also been suggested as a result of the toxin's growth inhibition of vari-

ous algal species of the genera *Chlamydomonas*, *Haematococcus*, *Navicula* and *Cryptomonas* (Keating 1978).

Advantages conferred by microcystin biosynthesis

Microcystin's affinity for iron and other cations such as copper, calcium and zinc, indicates the molecule's siderophoric properties (Humble et al. 1994) and suggests a putative role as an iron scavenging molecule (Utkilen and Gjolme 1995). Extracellular siderophores bind Fe^{2+} from the environment for use by the cell under conditions of low iron availability (Lukac and Aegerter 1993). Intracellularly, however such siderophoric properties may have a negative effect on cell functions due to competition with primary metabolites for limited essential iron. Alternatively, under conditions of high intracellular iron, an intracellular siderophore may have a protective function by chelating ferrous ions, forming iron–microcystin complexes and thus keeping the cellular level of free radicals low (Utkilen and Gjolme 1995). Such iron–microcystin complexes may also be stored until low iron conditions allow the release of Fe^{2+} for cellular processes. However, microcystin or complexes thereof have not been identified as stored substances in cellular inclusions, and are more commonly found on the thylakoid and plasma membranes (Shi et al. 1995; Young et al. 2005). It is believed that the Adda moiety of the toxin may bind to the thylakoid, leaving the polar peptide ring to bind metals from the cytoplasm (Orr and Jones 1998). This association with the photosynthetic apparatus of the cell may also indicate a putative function in light harvesting and chromatic adaptation mechanisms exhibited by some cyanobacteria.

Putative roles of cylindrospermopsin

Only one study has examined the effects of cylindrospermopsin on a filter–feeding zooplankter, *Daphnia magna* (Nogueira et al. 2004). Upon exposure to a cylindrospermopsin–producing strain of *Cylindrospermopsis raciborskii* Cylin–A, *D. magna* experienced high mortality, significantly reduced individual body growth and reduced fecundity. A second, and non–cylindrospermopsin producing strain also exhibited toxic effects on *D. magna*, due to an unidentified toxin, however the effects were less severe than those observed for cylindrospermopsin. The response of zooplankton to cyanotoxins is very species–specific. In the case of *D. magna* a bloom of cylindrospermopsin–producing cyanobacteria may reduce the grazing–pressure, and therefore give these cyanobacteria a survival advan-

tage over non–producing strains. However, comprehensive ecological studies are required to verify this hypothesis.

The convergent evolution of saxitoxin and PSPs

PSP toxins have been long considered among the most enigmatic of all microbial natural products, for several reasons. These neurotoxins represent one of the most potent classes of venoms, with a highly specific effect targeting voltage–gated sodium channels in excitable cells. Few stimuli induce or repress saxitoxin production in these microorganisms, and the metabolic role of PSP toxins has historically thought to be related to a possible defense strategy against unknown predators. It is a general and reasonable principle that metabolites of all kinds should play a beneficial role in the producing microorganism. The biosynthesis of toxins requires significant cellular energy and it seems unlikely that evolution would tolerate such wasted metabolism. There is limited evidence, however, to suggest, that PSP toxins confer an evolutionary or survival advantage to the producing microorganism.

As stated, the non–phylogentic distribution of saxitoxin production suggests the lateral acquisition of PSP toxin biosynthetic genes by toxic strains. This is considered to be a relatively rare event in the evolution of microorganisms, especially if it involves the exchange of genes between phylogenetically distant species (Eisen and Fraser 2003) or even kingdoms. This could be fostered under particular or critical growth conditions, as the laterally transferred genes could be maintained by the recipient microorganism if they encoded a function that was essential for cell survival. For example, the production of PSP toxins may confer an advantage under adverse environmental conditions. Recently, evidence was found linking saxitoxin production to the maintenance of cyanobacterial homeostasis under alkaline pH or Na^+ stressed conditions (Pomati et al. 2004b). The blockage of Na^+ uptake by saxitoxin was demonstrated in bacterial and cyanobacterial strains (Pomati et al. 2003b), suggesting that this inhibition could represent a possible mechanism that PSP toxin–producing cyanobacteria employ to cope with conditions of elevated pH and salinity. As mentioned previously, these environmental features are not uncommon in rivers and water–bodies characterised by the seasonal occurrence of saxitoxin–producing cyanobacterial blooms. Other results strongly suggest that the genetic differences between saxitoxin–producing and non–toxic *A. circinalis* strains are due to a distinctive adaptation to specific environmental conditions (Beltran and Neilan 2000; Pomati and Neilan 2004). The genetic heterogeneity of *A. circinalis* is explained, to some extent, by

genes associated with the maintenance of Na^+ homeostasis (Pomati et al. 2004a). Taken together, these considerations support the hypothesis that the production of PSP–toxins could represent a potential evolutionary advantage.

The microorganisms producing PSP toxins, and often most of those living in their immediate environment, do not possess nerves or any of the molecular systems that characterize neural transmission, the target of the saxitoxin channel–blocking effect. Ion channels, however, are a common feature that distinguishes all biological membranes. The function of ion channels range from motility or nutrient uptake, in bacteria, to the highest levels of complex neural transmission in the animal central nervous system. While commonly having different structures across phylogenetically unrelated organisms, ion channels often share similar specificity and cellular function. Interactions between saxitoxin and bacterial ion channels have been investigated (Pomati et al. 2003b). The identification and characteristics of the prokaryotic channel(s) inhibited by saxitoxin are yet to be described. The hypothesis, however, of an interaction in the environment between this natural neurotoxin with the ion channels of cyanobacteria, as well as other eukaryotic and prokaryotic planktonic competitors is possible.

Conclusion

While much progress has been made over the past ten years regarding the genetic basis for cyanobacterial toxin biosynthesis, there is now a crucial need to focus investigations on the regulation of toxin production. The toxin biosynthesis pathways have been elucidated for the major cyanobacterial threats to drinking water quality. Microcystin synthetase has been characterized at the genetic and enzyme level, and the public availability of the *M. aeruginosa* genome should be imminent. Together, the knowledge of toxin biosynthesis and the ability to perform global analyses of transcription and translation in *Microcystis* will afford the most complete investigation of the ecophysiological function of microcystin. These systems biological approaches may also be performed on samples in the environment, providing a real–time appraisal of the metabolism of cyanobacteria as they occur in harmful blooms.

However, numerous problems still challenge the success of research into the molecular biology of toxic cyanobacteria. These include the availability of a wide range of quality toxin standards, axenic cultures, methods for genetic transformation and mutagenesis, and the broad use of standardized

protocols for reporting toxin levels, production rates, and gene expression. The genomes of other model toxic cyanobacteria are also required if major advances are to be made, including those of the main producers of cylindrospermopsin (*C. raciborskii*) and saxitoxin (*A. circinalis*).

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