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Invited Paper

Hepatotoxin Biosynthesis and Regulation in Cyanobacteria – the Putative Involvement of Nitrogen and Iron Homeostasis Mechanisms

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ABSTRACT

Cyanobacteria are recognised globally as a human health threat due to their proliferation into toxic blooms. Of particular concern are strains that produce the hepatotoxins, microcystin and nodularin. Research over the past decade has revealed the biochemical and molecular mechanisms behind hepatotoxin production. However, there is still much to learn regarding the regulation of these biologically active metabolites. This review provides an overview of cyanobacterial hepatotoxin research to date and additionally, elaborates on the putative involvement of nitrogen and iron homeostatic mechanisms in cyanotoxin regulation.

Key words: Cyanobacteria, microcystin, nodularin, iron stress, nitrogen stress, NtcA, Fur, non-ribosomal peptide, polyketide, regulation.

1. INTRODUCTION

Cyanobacterial Toxic Blooms

Cyanobacteria are oxygenic photosynthetic bacteria that have developed adaptive mechanisms to enable their survival in a vast range of habitats. The combination of their capacity to form blooms in freshwater, brackish and coastal marine environments and their ability to produce potent toxins, has resulted in cyanobacteria remaining the focus of many international research groups for several decades. This interest has been sustained by the accelerating frequency and intensity of toxic blooms due to anthropogenic eutrophication of water systems. Cyanobacterial blooms and scums are unsightly and also produce odour and taste compounds, however, it is toxin production that causes the deepest concern.

Poisoning of wild and domestic animals and fish occur intermittently as a result of toxins produced by algal blooms in water bodies. This has caused immeasurable cost to agriculture, farming and fisheries as well as caused concern for the quality of limited and precious water resources. Humans have historically avoided acute intoxication by abstaining from consuming water containing high cell concentrations (13). However, in 1996 scores of human fatalities were caused by acute liver failure when patients in Brazil received intravenous dialysis treatment with cyanotoxin contamination (88). It is also likely that less extreme cases of human fatalities or illness have occurred but remain unreported.

Water intended for human consumption is usually treated to reduce the probability of

cyanotoxin contamination, although high quality water treatment is not always available, or as demonstrated by the tragedy in Brazil, may fail. A connection between the chronic presence of waterborne cyanotoxins and the occurrence of cancer has also been predicted (12). Persistent low levels of cyanotoxin contamination of drinking water is of particular concern in developing countries such as China, with continued consumption of untreated stream and well water in areas undergoing rapid industrialisation. In addition to health concerns, the strong odours and visible scums produced by cyanobacterial blooms reduces the recreational use of affected water bodies. Sizeable blooms occur regularly, particularly in the Northern European Baltic region and Australia, as can be seen by the substantial bloom in the Warragamba Dam, Sydney, over the second half of 2007, at a severe social and economic cost to local communities.

Overall, these issues indicate the far reaching environmental and public health impact of toxic cyanobacterial blooms. There is a pressing need to further understand, predict and manage toxic cyanobacterial bloom events, including the comprehensive study of the genetic mechanisms of toxin production and regulation.

Cyanotoxins are classified according to their chemical structures and include cyclic peptides, alkaloids, and lipopolysaccharides (45). These toxins have been shown to be highly poisonous to humans and animals and have displayed bioactivity against algae, bacteria, fungi and mammalian cell lines (12). Toxins are commonly referred to as either neurotoxins or hepatotoxins in reference to their mode of human and animal toxicity.

2. Microcystin

Microcystin is a cyclic toxin that mainly targets the liver, and is produced by some but not all species from the genera *Microcystis*, *Planktothrix* and *Anabaena*. Toxicity occurs through an association with hepatocytes due to the active transport of the toxin to the liver *via* bile acid, multi-specific, organic anion transporters (101). Microcystin is toxic to eukaryotes and higher plants, specifically inhibiting the protein phosphatases 1 and 2A. In humans and animals this results in degradation of the hepatocyte cytoskeleton, resulting in liver haemorrhage and death (13). The presence and level of toxicity can be measured in a variety of ways, including immuno- and phosphatase inhibition- assays, and analytical techniques such as MALDI-TOF mass spectrometry (1, 25, 69).

There are approximately 65 isoforms of the toxin microcystin that have been characterised to date. The common structure of the toxin is cyclic (D-Ala – L-X – D-MeAsp – L-Z – Adda – D-Glu – Mdha) where L-X and L-Z are variable L amino acids, Adda is 3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyl-4,6-decandienoic acid, D-MeAsp is 3-methylaspartic acid and Mdha is *N*-methyl-dehydroalanine (13). The modified β-amino acid Adda appears to be conserved in all hepatotoxins and is found in all known toxic microcystins. Isoforms of microcystin occur due to variations in methylation, hydroxylation, epimerisation and, peptide substitutions.

The unusual amino acids found in microcystin isoforms, in conjunction with its small size and cyclic structure, led to the discovery that the toxins were produced non-ribosomally (113). In a system similar to fatty acid synthesis, non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) genes are made up of modules that are built up from individual domains. Each module is comprised of an adenylation, thiolation and condensation domain and corresponds to one amino acid that will be

incorporated into the growing peptide chain. The adenylation domain is responsible for amino acid recognition and activation, following which the aminoacyl adenylate is transferred to the 4′-phosphopantetheine carrier within the thiolation domain. Ultimately, peptide bond formation of the activated amino acid to the previous residue in the chain is *via* the condensation domain (50, 63). Variations in microcystin isoforms have been explained by relaxed adenylation domain specificity (72).

Many toxic microorganisms proliferate in eutrophic conditions to cause blooms (92), however, *Microcystis aeruginosa* PCC 7806, a unicellular photosynthetic cyanobacteria, is the major producer of microcystin. The *Microcystis* genera include both toxic and non-toxic species as well as toxic and non-toxic strains within species groups. The presence of the hepatotoxin microcystin, produced by *M. aeruginosa* PCC 7806 has been identified as the causative factor in a number of cyanobacterial poisoning investigations. As the cellular role of microcystin is yet to be established, and a need exists to understand microcystin from both an environmental and molecular biology perspective, microcystin has been the focus of most recent studies.

3. The *mcyA/D* Promoter

The non-ribosomal mechanism of microcystin synthesis was initially confirmed by the disruption of a NRPS gene, which formed a non toxic mutant, thus establishing the role of this gene in microcystin production (24). Tillett *et al.*, (2000) further characterised the surrounding gene cluster describing the complete non-ribosomal synthesis of microcystin. It was revealed that the genes were arranged in two operons, the *mcyA-C* and *mcyD-J* clusters, which are responsible for the incorporation of the precursor amino acids which form microcystin (113). The gene cluster is 55 kb in length and involves 10 open reading frames. The genes *mcyA* to *mcyC* encode 5 NRPS modules, *mcyD* encodes two type 1 PKS modules and *mcyE* and *mcyG* encode hybrid NRPS-PKS modules. Genes *mcyF*, *mcyH*, *mcyI* and *mcyJ* encode a racemase, an ABC transporter, D-3-phophoglycerate dehydrogenase (D-3-PGDH) and *O*-methyl-transferase (OM), respectively. The function of these genes includes post-translational tailoring (*mcyJ*, *mcyF* and *mcyI*) and toxin transport (*mcyH*) (113). The gene cluster responsible for microcystin synthesis in *Planktothrix* (17) and *Anabaena* (100) have similarly been characterised (17).

All genera with microcystin producing strains also possess related strains that are non toxic, and this is generally attributed to the absence or presence of the gene cluster (68). Lateral gene transfer has been suggested to explain the sporadic occurrence of the gene cluster in both closely and distantly related strains, with transfer events also presumed to be recent (72), although little experimental evidence has succeeded in supporting this hypothesis. Rantala *et al.,* (2004) suggest that transfer events were not possible due to the coevolution of microcystin synthetase genes and marker housekeeping genes such as *16S* rRNA and *rpoC1*, which indicates the ancient origin of microcystins. The non toxic strains were therefore assumed to have lost the genes and the ability to produce toxin. Alternatively, those non microcystin producing strains may have the ability to produce different non-ribosomal peptides which at least may function similarly to microcystin (91). A 750 bp promoter region which bidirectionally initiates the transcription of *mcyA-C* and *mcyD-J* is located within the gene cluster. This promoter is of particular interest as it is the main regulatory tool thus far identified within the gene cluster. The *mcyA/D* promoter may enable transcription attenuation leading to

variation in microcystin production, and therefore indicating a potential target for an approach for toxin management systems. Characterisation of transcription regulation in microcystin may yield a clearer picture of factors that may effect toxin production. The promoter region may be connected to a signal transduction pathway, and therefore greater understanding of the *mcyA/D* promoter may resolve a role or function for microcystin.

Thus far, some work is available detailing the *mcyA/D* promoter from *M. aeruginosa* PCC 7806. This includes the work of Kaebernick *et al*., (2002) in which two transcription start sites were identified for both *mcyA* and *mcyD*, the two genes flanking the *mcyA/D* bi-directional promoter. Utilising reverse transcription and rapid amplification of cDNA ends, or RACE, the transcription start sites were mapped under high and low light intensities (44). The use of the two sites appeared dependent on light conditions, and also resulted in a relatively long untranslated leader region upstream of the transcription start point in each transcript. In addition, intercistronic promoter regions were putatively identified, although on closer inspection showed low identity to the conserved –10 (TATAAT) and –35 (TTGACA) hexamers recognised by the *E. coli* RNA polymerase σ^{70} subunit. Due to the predicted low efficiency of these promoters, it was suggested they may only be active under certain cellular conditions, or to ensure adequate transcription of distal genes as part of a very large gene cluster (44). Due to similarly low identity of suggested promoter motifs within the *mcyA/D* promoter it was also proposed activator proteins may act in increasing the efficiency of transcription under varying growth conditions or cellular climates, other than light conditions. Although these factors were not detailed in the study, it was suggested as an interesting area for further investigation.

Following this theory, Martin-Luna *et al*., (2006) described the putative binding of the iron-dependant transcription factor Fur to regions within the *mcyA/D* promoter (65), using the Fur protein they previously identified, characterised, and demonstrated to be autoregulatory, from *M. aeruginosa* PCC 7806 (64). This was suggested to indicate the regulation of microcystin transcription may be iron dependent, or also linked to light availability, as a link between cellular iron uptake levels and light intensities was previously suggested (115). The possible Fur binding sites, identified in these communications, within both the *fur* (64) and *mcyA/D* (65) promoters were unclear. In addition, the DNA probes utilised in the electromobility gel shift studies published were not labelled with radioactive nucleotides, yielding results far less sensitive than may be achieved otherwise. It therefore may be suggested further results may still be achieved using a more sensitive method in the experimental design and leaves scope for further investigation of this interesting topic. The DNA binding of *M. aeruginosa* PCC 7806 Fur to the intercistronic promoter regions upstream of *mcyE*, *mcyG*, *mcyH* and *mcyJ* was also indicated, although putative Fur binding motifs and images of electromobility gel shifts were not shown (65).

The *mcyA/D* promoter region will undoubtedly prove to be central in discerning the process of microcystin synthesis, function and regulation.

4. Biological Role of Microcystins

Many attempts have been made to determine a possible function for microcystin, in the hope of understanding the cellular role of this toxin and its purpose in cyanobacterial blooms. Understanding the function of microcystin may lead to improved toxin management systems with the possibility of controlling or avoiding toxic blooms.

An ecophysiological role has been hypothesised for microcystin as an ion chelator. To acquire iron in limited conditions, many bacteria secrete small, soluble iron chelators known as siderophores, which compete with environmental iron binding molecules (10). A siderophore function was suggested for microcystin due to its high affinity for cations such as iron, copper and zinc (115). In addition, the unique Adda component of microcystin has been suggested to bind to the thylakoid membrane leaving the polar peptide ring to bind metals from the cytoplasm (85, 104). On reporting immunogold localisation of up to 70-80 % of total microcystin on and between thylakiod membranes, Young *et al.,* (2005) suggested microcystin may be involved in photosynthesis or other light related processes. However, microcystin also associated with polyphosphate bodies, which are phosphate storage granules also able to trap and detoxify metals such as zinc (124). As microcystin has been seen to bind divalent metals (42), it may act as a ligand to remove and detoxify metals from the cell. Many other bacterial siderophores are synthesised non-ribosomally, such as mycobactin in *Mycobacterium tuberculosis* (22) and yersiniabactin in *Yersinia pestis* (30).

Microcystin, in early speculation, was hypothesised to play a role in allelopathy. Rohrlack *et al.,* (1999) suggested microcystin was produced as a feeding deterrent defence mechanism against grazing zooplankton such as *Daphnia gelaeta* (99). However, earlier work by Dittman (1997) displayed that predominantly cell bound microcystin was ingested by *D. gelaeta* in similar quantities to a non microcystin producing mutant (24). Further feeding work with microcystin producing and non-producing strains of *M. aeruginosa* PCC 7806 revealed that both strains were lethal when ingested by *D. gelaeta.* This also led to the discovery of microviridin-J, a potent protease inhibitory compound of *Microcystis* (98).

Phylogenetic evidence was recently presented by Rantala *et al.,* (2004), to suggest the microcystin synthetase gene cluster from *Microcystis* is ancient, and not widespread due to mobile genetic elements and lateral gene transfer (91). This finding disputed the theory attempting to explain the seemingly random presence and absence of microcystin genes in similar strains, based upon the observation of mobile insertion sequences in cyanobacterial genomes (73). In addition, microcystin gene mutants and rearrangements caused by transposons and insertion sequences have been shown to occur frequently and is surprising as this also indicates microcystin may not be essential to the cell (18). The high degree of congruence between the microcystin marker genes and housekeeping genes observed by Rantala *et al.,* (2004), indicated that gene transfer events were unlikely to have take place, thus indicating the ancient origins of microcystin. It was also suggested that the ability of cyanobacteria to produce hepatotoxins predated the metazoan lineage, and therefore was probably not adapted as a means of defence against grazers (91). This interesting topic is still under debate.

In a recent communication by Schatz *et al.,* (2007), the release of microcystin by broken cells to the extracellular environment was seen to be sensed by the cellular population resulting in the up regulation of microcystin synthesis (102), which was proposed to enhance the fitness of the remaining cells. The presence or addition of alternative biologically active secondary metabolites such as the protease inhibitors micropeptin and microginin has also been observed to increase the level of microcystin expression (95). The mechanism of up regulation at a transcriptional level was not explored in the study by Schatz *et al.,* (2007), and the interaction

with extracellular microcystin and other polypeptides remains unclear. Ultimately, incorporating evidence to suggest that microcystin may be actively transported out of the cell (86), Schatz *et al.* (2007) suggested an intercellular intraspecies communication role for microcystin, as a signalling molecule particularly to signal cell lysis (102).

Advances in cyanobacterial molecular genetics are gradually revealing the complex elements of microcystin function and regulation. In 2001, Dittmann *et al.*, described MrpA, a microcystin related protein and showed that expression of this protein was affected by differing light intensities and wavelengths. This evidence, in conjunction with the similarity between MrpA and proteins controlled by quorum sensing mediators in *Rhizobium* species, was used to suggest microcystin expression may be affected by light (23) and also have a quorum sensing function. Recently, Kehr *et al.,* (2006) broadened the hypothesis involving microcystin as a signalling molecule, with the putative involvement of microcystin with an isolated lectin, termed MVN, in *M. aeruginosa* PCC 7806 (49). Normally possessing carbohydrate binding sites to allow cell-cell cross linking *via* surface binding, prokaryotic lectins were demonstrated in the formation of biofilms and host recognition (112), and due to their ability to preferentially bind specific cell types, a cyanobacterial lectin has been pursued as an anti-HIV drug candidate (7). Kehr *et al.,* (2006) identified binding partners of MVN in the outer cell membrane and in the sheath of *M. aeruginosa* PCC 7806, suggesting the mechanism by which aggregation of single *Microcystis* cells form colonies, a phenomenon which was simulated experimentally in this study when large colonies formed on the addition of MVN to MVN deficient cells (49). In combination with the observation of an 84 bp region of homology between the *mcyA/D* promoter and the upstream region of the MVN lectin protein, an inter-relationship between MVN and microcystin was postulated, resulting in the conclusion that microcystin may be accepted as a signal after which the expression of both MVN and its binding partners are influenced (49).

Interestingly, it was suggested by Orr and Jones, (1998) in an early study that microcystin is not a secondary metabolite, as they identified a linear relationship between toxin production and cell division (85). The authors concluded that this relationship may be applicable regardless of any environmental factor limiting cell division, such as light or substrate availability. Although this theory has largely been indirectly disproven experimentally, this highlights the scope of deliberation currently on the topic of the natural role, function and dynamics of microcystin. Ultimately due to variation in culturing techniques, growth conditions as well as experimental design and analysis, no consensus has been reached regarding the function of microcystin as results are incomparable. As advances in cyanobacterial research include the sequencing of entire genomes, interesting and novel findings further indicate the complexity and uniqueness of toxin producing cyanobacterial genera. Due to the recent partial sequence of the *M. aeruginosa* PCC 7806 genome, the discovery of the coexistence of two proteins related to the large subunits of Rubisco, $RbcL_I$ and $RbcL_{IV}$, indicated the presence of a methionine salvage pathway, not yet described in cyanobacteria (14), which may aid in the survival of *Microcystis* cells in variable bloom conditions. Overall, the field of cyanobacterial research still offers unlimited potential, with microcystin production, regulation and function remaining intriguing concepts at the forefront of *Microcystis* research.

5. NODULARIN

Similar to *M. aeruginosa* PCC 7806, *Nodularia spumigena* NSOR10 produces a non-ribosomally encoded toxin (83), nodularin. The nodularin synthetase gene cluster encoding nodularin, a cyclic hepatotoxin, is similar to microcystin but encodes a condensed five carbon ring (74). Many *N. spumigena* NSOR10 blooms produce nodularin and have caused death in livestock (82), and has been attributed to the production of the pentapeptide hepatotoxin nodularin.

Nodularia is a bloom forming, filamentous cyanobacteria found in brackish water worldwide. *N. spumigena* NSOR10 has been classified as the most common bloom forming species of the genus *Nodularia*, with blooms frequently reported in summer months around the world (94, 106, 107). Regular intensive cyanobacterial blooms occur in the Baltic Sea environment where many of the studies on *Nodularia* are focused. Nodularin, resulting from toxic *Nodularia* blooms, is of particular concern due to toxin bioaccumulation in shellfish and other seafood products (116).

Nodularin is a cyclic pentapeptide consisting of 3-amino-9-methoxy-2,6, 8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda), D-glutamic acid (D-Glu), *N*-methyldehydrobutyrine, D-*erythro*-β-methylaspartic acid (MeDhb) and L-arginine (L-Arg). There are seven isoforms of nodularin, with varying levels of toxicity. Similar to the mode of microcystin toxicity, nodularin is associated with the inhibition of eukaryotic protein phosphatases catalytic subunit types 1 and 2A (40). Nodularin is potentially carcinogenic *via* liver tumour promotion in cases of chronic low level exposure, with acute doses leading to liver haemorrhage (97). The amino acid Adda, present in both nodularin and microcystin, blocks protein phosphatase enzyme activity by obstructing substrate access to the active site (62).

A major concern exists regarding the bioaccumulation of nodularin in fish and other marine organisms. Nodularin has been demonstrated to cause oxidative stress in the brown alga *Fucus vesiculosus*, or Phaeophyceae, with up to 45.1 μg per kilogram of nodularin taken up by cells observed (87). As filter feeders, the blue mussels *Mytilus edulis* are particularly prone to accumulation of chemical compounds present in seawater, both in soluble form and those associated with particles, and are an important source of food to other organisms such as fish and birds (48). Nodularin was demonstrated to accumulate in mussels up to 2200 μg per kilogram (105), clearly indicating the extent of potential hazard. Kankaanpaa *et al.,* (2007) observed the blue mussel *Mytilus edulis* accumulate nodularin quickly, throughout all tissues, however then observed a rapidly induced depurination, or detoxifying state, in which tissue bound toxin was excreted or altered. The complete removal of nodularin was not achieved, which is suggested to be the case *in situ*, as mussels commonly fail to fully detoxify following a toxic bloom event (48). The majority of total nodularin was found located in the digestive gland of the mussels, the main organ of active metabolism and absorption of food, with rapid loss of accumulated toxin through faeces demonstrated (110). An applied aspect of toxin research for management of bloom events is the dynamics of toxin degradation, including such as *via* the bacterial community (93) or by physical means such as UV radiation (66). This focus would be of far greater effect if the true role of hepatotoxins was known, and as yet the natural physiological and ecological functions of nodularin are not well understood and are yet to be elucidated.

6. The *ndaA/C* Promoter

The gene cluster responsible for nodularin synthesis consists of polyketide synthase modules and non-ribosomal peptide synthetase modules, which form a large enzyme complex encoded by a 48 kb operon (74). Since the characterisation of the nodularin synthetase gene cluster, highly sensitive methods of toxin detection such as real-time PCR have been made available for use (51). As seen in the microcystin gene cluster, the nodularin operon centres around a bidirectional promoter and contains two large operons, *ndaA-B* and *ndaC-I*. The *ndaA/C* promoter is approximately 700 bp in length.

Thus far, little experimental work has focused on the transcriptional regulation of the nodularin synthetase gene cluster *via* the *ndaA/C* promoter. Nodularins are solely produced by strains of the genus *Nodularia*, however similarities in structure and biological function to microcystin are obvious. It is not yet clear if the structural similarities found between microcystins and nodularins would echo in their modes of transcriptional regulation, although a similar biosynthetic pathway of the two hepatotoxins was assumed due to the structural similarities (75). Rantala *et al.,* (2004) proposed the nodularin gene cluster was the result of a recent divergence of the microcystin gene cluster due to mutation and deletion events. This theory is seen as consistent with the fact that nodularins are solely produced by the single cyanobacterial genus *Nodularia,* with few known isoforms (91).

7. The Biological Role of Nodularin

It has been suggested that the nodularin gene cluster was derived from the microcystin synthetase gene cluster *via* recent deletion and mutation events of three peptide synthetase modules (91), with nodularin regarded as an outlying isoform of approximately 65 variants of microcystin. Similarly with microcystin, the role and function of nodularin remains unidentified. There have been many studies aimed at ascertaining the effect of various environmental parameters on growth and toxin production in *Nodularia* species. Although commonly from an environmental management or bloom dynamic perspective, these studies can be seen as contributing to the eventual aim of clarifying the natural role of nodularin through research in growth and toxin production.

As with similar experiments on microcystin, results are contradictory. An increase in toxin production has been observed during favourable growth conditions, such as moderate salinity or higher irradiances (55). However, it appears that nodularin concentrations may be higher under low nitrate levels (55) and phosphate levels had no effect on nodularin in batch and chemostat cultures (94). Traditional growth and toxin analyses have been updated with the use of real-time PCR (51), which in turn moves towards a molecular approach to the study of nodularin. In addition, molecular studies such as described by Copp *et al.,* (2007) detailing the *N. spumigena* NSOR10 phosphopantetheinyl transferase elevate the biotechnological interest of such studies (20). Due to the putative relationship between microcystin and nodularin, and in combination with their similar structure, it is reasonable to suggest that current hypotheses regarding the natural function of microcystin would apply to nodularin. As previously outlined, these hypotheses include functions of siderophoric trace metal scavenging (115), signalling and intercellular communication (102), feeding deterrence from grazers (99) and a photosynthetic or light harvesting role (124). Overall, as with microcystin, the true function of nodularin remains unclear, with new and more detailed evidence arising supporting each hypothesis.

8. Promoter Regions in Cyanobacteria

Advances in cyanobacterial research have led to the development of a knowledge base on promoters, sequences found upstream from genes, that regulate and initiate transcription. Cyanobacteria can be found in a large range of habitats, many of which are dynamic and therefore not always conducive for optimum growth. The short term challenges of surviving are met by rapid adjustments in metabolic pathways (21). The key step in this adjustment is at the transcriptional level. A typical example of this flexible control is the up and down regulation of photosystem and light-harvesting antenna complexes in response to light limiting and light saturating conditions respectively (39). Suggested to be regulated at a transcriptional level, Muramatsu *et al.,* (2007) reported recently the analysis of the promoter regions upstream from PSI genes, and *via* promoter deletion studies, observed an AT-rich sequence located just upstream of the –35 motif crucial for the light response seen by PSI genes (78). It has been shown that the mode of regulation of secondary metabolites, such as microcystin and nodularin, is similar across all bacteria, in order to control an appropriate level of expression (121). Accordingly, cyanobacterial promoters display similarities to other bacterial promoters.

The σ subunit of *E. coli* RNA polymerase (RNAP) recognises and binds to conserved hexamers located at positions –35 (TTGACA) and –10 (TATAAT) with respect to the transcription initiation site in most micro-organisms. These promoter hexamers are mostly at a distance of 17 ± 1 nucleotides from one another (67), with promoter strength or level of activity commonly predicted by the degree of sequence identity to these hexamers. A promoter is weakened by changes that result in a decrease of consensus identity or alter the consensus spacer length (32). From databases formed by the alignment of *E. coli* promoters, it has been recognised that although variations are observed in all bases within the hexamers, the first three bases at –35 (TTGNNN) and the first two and last bases at –10 (TANNNT) are conserved in up to 90% of promoters (36, 37). In addition to hexamer sequences, other motifs have been identified that are important to the overall efficiency of a promoter. These include the TG motif often found situated upstream of the –10 region and also called the extended –10 motif (3), as well as the A and T rich region upstream from the –35 region, known as the UP element (11).

Prokaryotic promoters commonly follow the aforementioned patterns, however the growing amount of information on cyanobacterial promoters has elucidated new cyanobacterial specific information. A conserved region is observed at the –10 position from the transcriptional start point in sequence alignments of cyanobacterial promoters and therefore adheres to the *E. coli* paradigm. Transcription initiation is 7 ± 1 bases downstream from the conserved –10 regions and generally this nucleotide is a purine (21). The majority of cyanobacterial promoter alignments lack strong –35 regions, which is hypothesised to indicate a dependence on alternative transcription factors that may increase the affinity of RNA polymerase to initiate transcription (21). As mentioned, analyses of cyanobacterial promoters have revealed that they share a core element with *E. coli* promoters, however statistical and functional analyses must await a larger database of promoters on which to work. Characterisation of the promoter region of a newly identified gene is of particular interest if the gene may be of use in molecular biotechnology. This aids in the gathering of sequence data to elucidate core element

consensus motifs, allowing a clear picture of the mode of regulation of a gene to be resolved.

Transcription studies on cyanobacterial gene regulation are appearing with increasing frequency. Sjöholm *et al.,* (2007) reported the transcription of *Nostoc* sp. PCC 7120 *hox* genes, encoding a bidirectioanl hydrogenase, may in part be regulated by the transcription factor LexA, which as an activator may function as a general regulator of redox-responsive genes, or alternatively, in the SOS response (108). In addition, by searching for transcription factor binding motifs, putative recognition sites for FNR (fumarate and nitrate reduction) binding was identified, a global transcription factor that may respond to levels of environmental oxygen. Resulting from this finding, gene candidates in *Nostoc* sp. PCC 7120 are currently under investigation to identify the FNR protein (108). It therefore may be observed that to pursue a line of enquiry regarding gene regulation may facilitate the discovery of entirely new cyanobacterial pathways.

The majority of toxin production in cyanobacteria appears to be constitutive, although proliferation in bloom conditions may affect the level of toxin produced. Therefore the transcription of genes and thus production of proteins involved with the regulation of toxin transcription may also alter through the progression of an algal bloom. Through the activity of transcription factors, the expression of a range of genes specific to certain metabolism may change in response to environmental flux.

9. The Global Nitrogen Regulator NtcA

Nitrogen homeostasis and assimilation in cyanobacteria is subject to fine control. The prokaryotic global nitrogen regulator NtcA facilitates regulation of nitrogen responsive genes, and belongs to the CAP family of transcription factors (28, 38, 117, 118, 120). NtcA acts as an activator in the majority of cases, however it has also been observed as a repressor (43). The classical behaviour of a transcriptional activator is to increase or initiate transcription by binding to the promoter upon recognising a certain cellular indicator.

The *nir* (nitrate assimilation) operon in *Synechococcus* sp. PCC 7942 was shown activated by NtcA. Three binding sites were found upstream of the operon, allowing for N-regulated expression of the genes. In this strain and in all others observed to date, the *ntcA* transcript is synthesised from a N-regulated promoter and therefore NtcA is positively autoregulated (60). N-regulated genes which are proceeded by NtcA-type promoters in *Synechocystis* sp. PCC 6803 include *amt1* (76), *glnA* (96), *glnB* (29) and *rpoD2-V*, which is a sigma factor affecting survival under nitrogen stress (79).

NtcA possesses the C-terminal helix-turn-helix motif consistent with the DNA binding domain of transcription factors (122) and binds to the consensus region GTAN_8TAC. Some variation in the length of binding regions between the palindromic bases, have been observed. The consensus is generally 22-23 bp upstream from an accompanied σ^{70}-like –10 hexamer of TAN_3T (38, 61), and multiple NtcA binding sites have also been shown to occur in a single promoter. The autoregulated *ntcA* gene is transcribed at a basal level in the presence of ammonium, and elevated in conditions of nitrogen stress (56, 80, 90, 120). NtcA was first isolated from *Synechococcus* PCC 7942 (117) and has now been characterised from a wide range of cyanobacterial genera. Due to the large number of cyanobacterial genes that are regulated by NtcA, it appears that NtcA activity is not solely related to ammonium, but also to the C to N ratio within the cell (61).

10. NITROGEN STRESS

Cyanobacteria live in diverse and dynamic environments thus displaying their highly evolved adaptive mechanisms. In large bloom settings, the cyanobacterial community reaches a critical mass, where available nutrients become scarce. Cyanobacteria respond to environmental stress by pre-determined physiological adaptations. The adaptive mechanisms in response to environmental change have been well studied in cyanobacteria, include adaptation to changes in temperature, salinity, nutrient availability osmotic pressure and desiccation. Stress responses include modifications to cell membrane constituents, alterations in metabolic pathways, accumulation of compatible solutes and induction of homologous stress-inducible proteins. In general, a change in transcription and translation of genes enables bacteria to change their metabolic processes to better achieve growth or survival in dynamic conditions.

Acclimation responses to nutrient availability can be characterised as either specific to the nutrient that is limiting, or those that are general and occur during any of a number of different nutrient limitations (103). Specific responses include increasing production of high affinity transport systems in order to scavenge for remaining nutrients and the production of hydrolytic enzymes that enable the alternative use of an alternative metabolite (84, 89). General adaptations include reduction in cell division, and dramatic alteration in cellular structure and metabolism (19, 123).

The cell will enter a stage of nutrient deprivation once all external nitrogen sources conducive for growth are exploited. Growth may continue transiently during cell adaptation due to many physiological changes that occur, including the specific degradation of phycobiliprotein which results in chlorosis (33). This nitrogen recycling allows for synthesis of proteins necessary for survival (34). As described earlier, NtcA activates N-responsive genes and therefore is a useful tool in field studies regarding ammonium sufficiency and nitrogen deprivation, due to the sensitivity of *ntcA* expression to ammonium and nitrogen levels (57). It has been suggested that nitrogen concentration directly influences the toxin production and growth of hepatotoxic *Microcystis* strains (119).

As with transcription signals activated under iron and oxidative stress, cold stress in *Anabaena* PCC 7120 was found to result in a transient decrease in the expression of *ntcA* (77). However, after acclimation to cold, expression levels rose significantly, suggesting that long-term cold adaptation limits nitrogen availability (77). Alternative transcription factors also play a role in nitrogen starvation, observed by the absence of NtcA binding sites upstream of differentially expressed genes under nitrogen depletion (9, 15, 81, 114). This highlights the complexity of cyanobacterial specific and general responses to environmental stress.

11. THE GLOBAL IRON REGULATOR FUR

Iron is one of the most abundant elements found in the Earths crust. However, in an aqueous oxygenic environment, ferrous iron is quickly oxidised to ferric iron, which forms insoluble hydroxides at physiological pH (10). The resultant low bioavailability necessitates the fine control of iron within microorganisms, as this element serves as a major component in processes such as photosynthesis and respiration. In particular, primary production in cyanobacteria requires iron for electron transport in Photosystem II, however cells must also prevent the deleterious effect of excess iron due to its ability to catalyse Fenton reactions. In part, molecular regulation of iron metabolism operates in microorganisms such as cyanobacteria through a DNA-protein

binding interaction with the transcription regulator Fur (Ferric Uptake Regulator) (2, 31, 47).

Bacterial Fur family proteins range from 14 to 20 kDa and have highly conserved, internal histidine rich areas. Under high iron conditions, Fur acts as a repressor protein by binding to the 19-bp consensus sequence GATAATGATAATCATTATC. Fur acts as a dimer once complexed with ferrous ions, however the mechanism under which Fur binds the Fur box remains unclear. Many Fur homologues have been identified in cyanobacterial genera, including *Synechocystis* (47), *Synechococcus* (31), *Anabaena* (5) and most recently *Microcystis* (64).

When Fur acts as a repressor, transcription of iron acquisition genes is attenuated by binding the target genes when the intracellular concentration of Fe(II) exceeds a certain threshold. Although Fur regulates iron responsive genes via this DNA binding activity, this protein has also been suggested to regulate genes involved in oxidative stress defences (111), acid shock responses (35) and chemotaxis (26). A clear example of how the complex and dynamic methods through which cyanobacteria survive in adverse environments and rely upon attenuating metabolic pathways, remains unclear.

As observed with NtcA, Fur may be involved with the transcription regulation of the hepatotoxins microcystin and nodularin, due to the putative Fur binding sites found on the *mcyA/D* and *ndaA/C* promoters. Therefore, the peptides microcystin and nodularin may play a role in iron and nitrogen homeostasis in the cell.

12. IRON STRESS

Cyanobacteria represent the largest group of photoautotrophs on Earth, and with iron rich photosynthesis apparatus, cyanobacteria are vulnerable in iron limiting conditions (4, 27). The essential role of iron in cell growth and replication is due to many iron-containing proteins which catalyse key reactions in photosynthesis, respiration, nitrogen assimilation and DNA synthesis (8, 109). The redox properties that make iron an important cofactor also result in oxidative reactions that lead to harmful radicals. Therefore iron accumulation is tightly regulated to reduce that amount of free iron in the cell (46).

The iron rich PSI reaction centre (12 irons) and the terminal electron acceptor ferredoxin (2 irons) are the most susceptible photosynthetic elements in the event of iron deprivation (27). Adaptations to iron deprivation in the photosynthetic apparatus include a specific PSI antenna complex (6) and the replacement of ferrodoxin by flavodoxin in the electron transport chain (54). The *isiA* gene, encoding the stress inducible chlorophyll binding protein IsiA, is up regulated in conditions of iron deprivation and oxidative stress, suggesting a link between these two responses through a multiple input signal pathway (52). It has been suggested that iron deprivation causes oxidative stress, which as mentioned, is the main signal for *isiA* activation (53).

A close interrelationship has been suggested to exist between iron homeostasis and oxidative stress (71) and further research may help us to clarify the roles of microcystin in the maintenance of homeostasis in the hepatotoxic cell. Photosynthetic autotrophs may often be subjected to active oxygen species which are abundant in conditions of iron deprivation and oxidative stress. Many iron stress response proteins have been observed to contain Fur boxes in their promoters (70) hypothesised to be repressor sites, although an activator role for Fur has also been described (41).

One remaining aspect of the regulation of transcription *via* DNA binding proteins such as NtcA and Fur, is the suggested

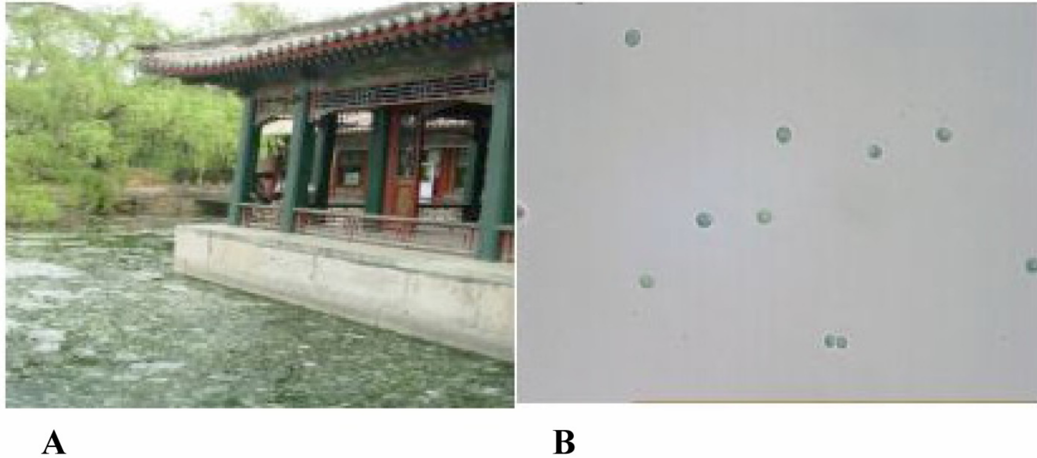

Figure 1. (A) A persistent cyanobacterial bloom in a small man-made lake at the Summer Palace, Beijing, China, 2007. (B) Unicellular *Microcystis aeruginosa* PCC 7806 cells, magnification x100.

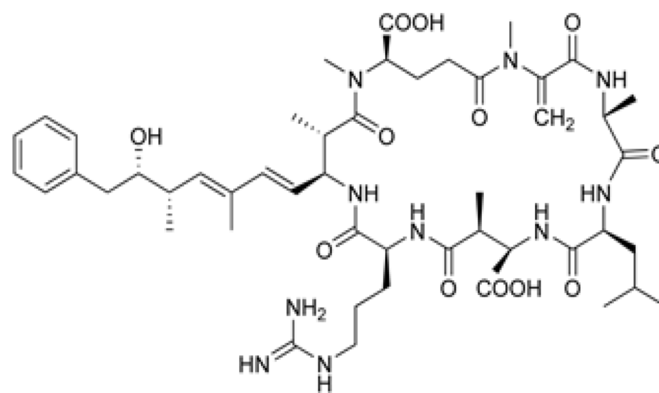

Figure 2. Chemical structure of the heptapeptide cyanotoxin microcystin-LR.

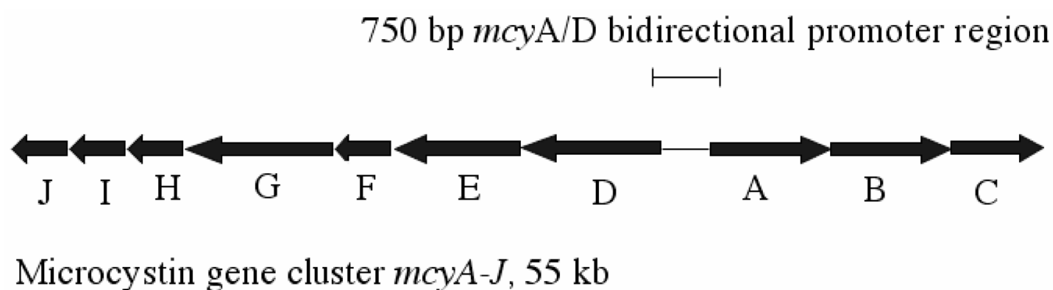

Figure 3. Schematic of the 55 kb microcystin gene cluster, *mcyA-J* in *M. aeruginosa* PCC 7806. Genes are transcribed in two transcripts, *mcyA-C* and *mcyD-J*, from the central bidirectional promoter, between *mcyA-D* and hence forth termed as the *mcyA/D* promoter.

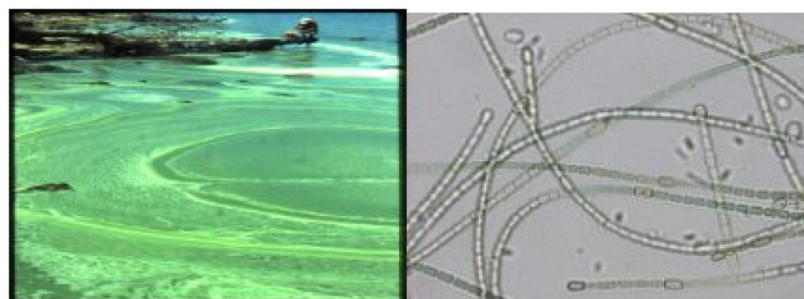

Figure 4. (A) Commonly observed *Nodularia* bloom. (B) Filamentous *Nodularia spumigena* NSOR10 cells, magnification x100.

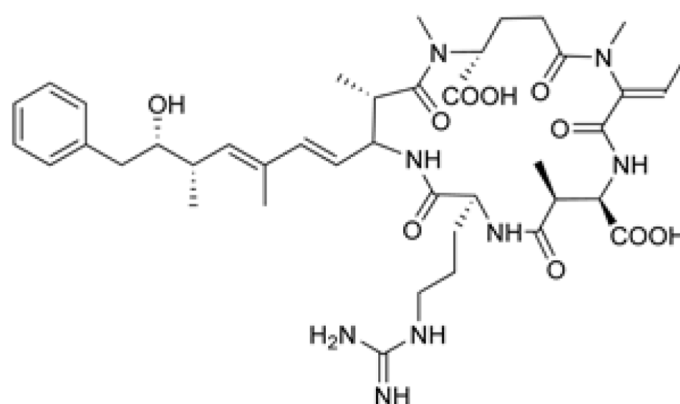

Figure 5. Chemical structure of the pentapeptide cyanotoxin nodularin-R.

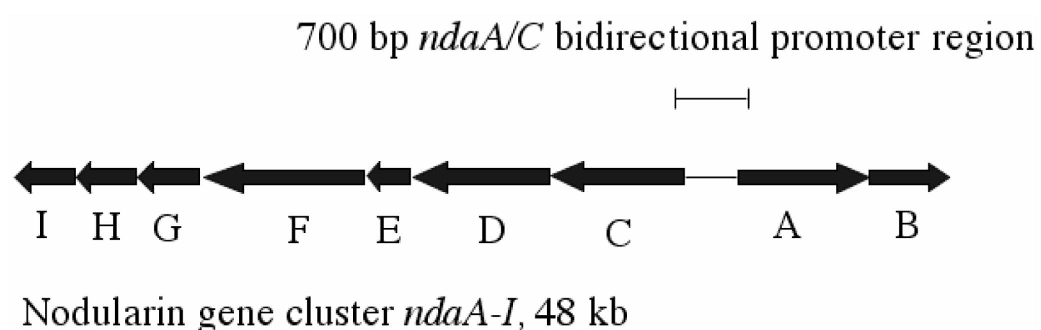

Figure 6. Schematic of the nodularin gene cluster in *Nodularia spumigena* NSOR10, *ndaA-I*. Genes are transcribed in two transcripts *ndaAB* and *ndaC-I*, highlighting the 700 bp promoter region between *ndaC* and *ndaA*, termed the *ndaA/C* promoter.

interaction of the two in cyanobacteria, creating a coupling between the nitrogen and iron regulatory pathways. Existing examples are limited only to nitrogen fixing *Anabaena* PCC 7120, however these may be a starting point from which a broader theory may develop. Cheng *et al.,* (2006) observed that NtcA bound to the promoter regions of two genes involved with the regulation of iron content within cells under severe iron limitation, suggesting that NtcA is involved in both the coordination of nitrogen metabolism and iron homeostasis (16). It was suggested that due to the fact that iron is a critical component of nitrogen regulation processes, and the fact that iron stress symptoms in cyanobacterial cells resemble those of nitrogen stress, it was reasonable to predict the two responses may share common components (16). An example of the Crp regulatory protein from *E. coli*, a member of the same family of transcription factors as NtcA, interacting with Fur in the regulation of carbon and iron utilisation genes involved with the Krebs cycle in *E. coli* (125) was suggested as further evidence to support the possibility of the interaction of nitrogen and iron regulatory pathways in cyanobacteria. Similarly, López-Gomollón *et al.,* (2007) suggested that the expression of *furA*, encoding the ferric uptake regulator in *Anabaena* PCC 7120, was greatly affected by cellular nitrogen deprivation, with specific differences in the expression of FurA within an *ntcA* mutant strain (59). Due to observed up regulation of Fur proteins localised in proheterocyst and mature heterocyst cells, it was suggested that Fur may coordinate the availability of iron, with responses to oxidative stress. This in turn was due to the need for anoxic conditions to allow for nitrogenase components to function in the specialised cells (59). Expanding on this preliminary work, the close link between iron and nitrogen metabolism was investigated further by the bioinformatic survey of genes putatively coregulated by NtcA and Fur in *Anabaena* PCC 7120 (58). Examples of NtcA regulated genes with Fur binding sites include genes involved with photosynthesis, heterocyst differentiation, transcriptional regulation and redox balance (58), indicating that the putative cross-talk between iron and nitrogen regulatory systems may prove to be extensive. This investigation correlated computational results with electromobility gel shift assays predicting a definable and experimentally supported overlap between transcription regulation of iron and nitrogen responsive genes (58).

13. Concluding Remarks

Toxic cyanobacterial blooms occur regularly in water bodies around the world, at great cost to effected communities. The cyanotoxins have been attributed to illnesses and deaths associated with cyanobacteria contaminated water. The motivation for much of the research reviewed above stems from the fact that the cellular role and function of hepatotoxins produced in bloom events is still unknown. It is hypothesised that further understanding of the transcriptional regulation of microcystin and nodularin may elucidate the ecological and physiological role of the toxins, which in turn, may yield novel management strategies.

Preliminary sequence analysis of the *mcyA/D* and the *ndaA/C* promoter regions, centrally located within the microcystin and nodularin synthetase gene clusters, has revealed putative regulatory protein binding sites recognised by the transcription factors, NtcA and Fur. The hypothesised mode of transcription regulation of the two toxins is therefore further linked to cellular levels of nitrogen and iron, the two main determinants of NtcA and Fur activity, respectively. As the two toxins are hypothesised to act in similar cellular roles due to their analogous structure

and biosynthetic origin, a common regulation system is expected. The future characterisation of the transcription regulation mechanism in hepatotoxic cyanobacteria should therefore address the synergistic influences of both nitrogen and iron.

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