ENVIRONMENTAL CONDITIONS THAT INFLUENCE TOXIN BIOSYNTHESIS IN CYANOBACTERIA

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MICROCYSTIN
NODULARIN
CYLINDROSPERMOPSIN
SAXITOXIN
CONCLUSION
Microcystins

- Non-ribosomal heptapeptides
- Hepatotoxin and tumour promoter due to protein phosphatase inhibition
- Produced by various cyanobacteria including Microcystis, Planktothrix, Synechocystis, Anabaena, Nostoc.
Environmental regulators of microcystin production

• High nitrogen and phosphate availability correlates with high microcystin production – or due to indirect effects on growth rate?
• Low iron availability correlates with high microcystin concentration
• Light intensity, temperature and pH also have been reported to affect MC production

Microcystin synthetase promoter

ATTAAATGATTATTACTAAT
Fur binding site

GTAN₈TAC
NtcA binding site

DNA: dnaN mcyJIIH G F E D A B C uma1-6
High Light Iron Deficiency

1. MC-dependent extracellular proteins, e.g. MVN, MrpC
2. Released MC infochemical
3. Redox Control C/N Metabolism
   - RbcL
   - McyH
   - stably active
   - stably active?
   - ?
   - Receptor?

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Released MC infochemical

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Summary –
Regulation of microcystin biosynthesis

• MC advantageous in severe iron starvation and oxidative stress.
• High nitrogen and phosphate availability may not have a direct impact on MC production.
• Instead regulation is complex with no single parameter regulating production in on-off manner
• MC has a complex global role related to C/N metabolism and oxidative stress
MICROCYSTIN
NODULARIN
CYLINDROSPERMP PRESIN
SAXITOXIN
CONCLUSION
Nodularin

- Light, nitrogen and phosphate may play a role but studies are inconclusive
- Promoter analysis implicates light, nitrogen and iron but not phosphate
- Influence of nitrogen and phosphate availability different to microcystin – need to study nodularin regulation separately

MICROCYSTIN
NODULARIN
CYLINDROSPERMOPSIN
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CONCLUSION
Cylindrospermopsin

- General cytotoxic effects & potential carcinogen
- Produced by various filamentous cyanobacteria, e.g. *Cylindrospermopsis raciborskii*
Environmental regulators of cylindrospermopsin production

- Nitrogen source available for growth:
  - $N_2$ +++ CYLN
  - $NO_3^-$ ++ CYLN
  - $NH_4^+$ + CYLN
- High light increases CYLN production
- Phosphate starvation decreases CYLN production

Cylindrospermopsin synthetase promoter

Cylindrospermopsis synthetase gene expression

• Effect of nitrogen source on gene expression?

• High light decreased gene expression

Biological role of cylindrospermopsin in phosphate procurement?

- Cylindrospermopsin stimulates phytoplankton to secrete an enzyme, which then frees up phosphate in the environment for uptake by the cyanobacterium.

Summary – Regulation of cylindrospermopsin biosynthesis

• Nitrogen source (\(\text{NO}_3^-\), \(\text{NH}_4^+\), \(\text{N}_2\)), light intensity and phosphate availability are the only environmental factors investigated so far

• Conflicting results - lack of standardization between experiments
MICROCYSTIN
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CYLINDROSPERMOPSIN
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CONCLUSION
Saxitoxin

- Paralytic shellfish poison, ~60 analogues
- Neurotoxicity due to inhibition of voltage-gated sodium, potassium and calcium channels
- Produced by eukaryotic dinoflagellates as well as cyanobacteria (*Anabaena, Cylindrospermopsis, Lyngbya*...)

![Chemical structure of saxitoxin]

![Image of saxitoxin-producing dinoflagellates]
Environmental regulators of saxitoxin production

- Temperature – contradicting results
- STX accumulates linearly in response to increasing extracellular Na\(^+\) concentration
- High fixed nitrogen availability, low STX production
- STX production highest in intermediate light and decreased in the dark

Genetics of saxitoxin production

- *sxtY*, *sxtZ* and *ompR* similar to regulatory proteins that respond to phosphate starvation

Saxitoxin synthetase promoter

Effect of phosphate on STX synthetase gene expression

Chapter 5

Effect of phosphate on STX synthetase gene expression

5.3.4 Growth of phosphate depleted cultures

As mentioned previously, the growth of cultures exposed to phosphate depleted media were monitored from lag to stationary phase and normalised by the total protein content. Figure 5.4 shows the growth of cultures in various concentrations of phosphate depleted media. As it can be seen, the growth at 20 \(\mu\)M phosphate severely limited the culture growth, with a constant decrease in biomass from lag to stationary phase. The other phosphate concentration (20 \(\mu\)M) did not affect the growth when compared to the control. Interestingly, STX production in the 70 \(\mu\)M phosphate conditions constantly increased.

Figure 5.3

Bar graphs showing intracellular STX levels over time in C. raciborskii T3 phosphate deprived cultures. Values were normalised to the protein content of the cultures. 1, 7, 15, 22, 30 represent the time points at which samples were taken, starting from lag to stationary phase. Data are means of biological and technical triplicates. Error bars are ± SE.

Figure 6.7

Effects of phosphate levels on the transcription of a putative phosphate uptake regulator (\(\text{phoU}\)), Hystidine kinase (\(\text{phoR}\)), transcriptional regulator (\(\text{phoB}\)) and \(\text{stxA}\), under phosphate depleted conditions.

6.4 Discussion

Cyanobacteria possess a large repertoire of TCSs in order to sense and respond to changes in the environment (Ashby & Houmard, 2006). Transcriptional analysis and regulation studies conducted in Chapter 5 revealed that phosphate is an important nutrient with regards to STX production in cyanobacteria. Sequence analysis of the 3' region adjacent to the STX gene cluster in C. raciborskii T3 revealed a putative PhoR-PhoB TCS. In this thesis we hypothesised that the presence of such a system, homologous to that in E.coli, is intermediate to STX biosynthesis in this cyanobacterium. It is also proposed that this TCS

Summary –
Regulation of saxitoxin biosynthesis

• Strong evidence that phosphate starvation stimulates STX production
• Potential effects of temperature, salinity, light and nitrogen need further investigation
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CYLINDROSPERMOPSIN
SAXITOXIN
CONCLUSION
Conclusion

• Regulation is complex and affected by more than one factor – global role for toxins?
• Light and the availability of nitrogen, phosphate and iron seem to be the main factors but it is never a single parameter that regulates biosynthesis
• Often a mismatch between changes in transcript abundance and toxin concentration – post-translational regulation?
• Conflicting results due to lack of standardization
ACKNOWLEDGEMENTS

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Cyanobacteria

- Oxygenic phototrophs
- Some nitrogen-fixing & spore-forming
- Morphologically (and habitat) diverse but phylogenetically conserved
- Potential for toxic blooms

Lake Taihu, China
Iron regulates microcystin synthesis

Table 1. Summary of transcription changes in selected genes of three *M. aeruginosa* strains in iron starvation (10 nM Fe) and iron limitation (100 nM Fe).

<table>
<thead>
<tr>
<th></th>
<th>PCC 7806 - toxic</th>
<th></th>
<th>PCC 7005 - non-toxic</th>
<th></th>
<th>PCC 7806 <em>mcyH</em> -</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exponential</td>
<td>Stationary</td>
<td>Exponential</td>
<td>Stationary</td>
<td>Exponential</td>
<td>Stationary</td>
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<tr>
<td></td>
<td>10</td>
<td>100</td>
<td>10</td>
<td>100</td>
<td>10</td>
<td>100</td>
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<tr>
<td><em>isiA</em></td>
<td>35.92</td>
<td>9.55</td>
<td>152.74</td>
<td>7.63</td>
<td>13.67</td>
<td>12.09</td>
</tr>
<tr>
<td><em>futA</em></td>
<td>9.31</td>
<td>1.16</td>
<td>5.54</td>
<td>-1.79</td>
<td>-4.19</td>
<td>-1.80</td>
</tr>
<tr>
<td><em>feoB</em></td>
<td>3.24</td>
<td>1.92</td>
<td>-14.00</td>
<td>-20.00</td>
<td>-1.78</td>
<td>-2.94</td>
</tr>
<tr>
<td><em>mcyA</em></td>
<td>-1.24</td>
<td>-1.16</td>
<td>-117.36</td>
<td>-2.28</td>
<td>-4.39</td>
<td>-1.14</td>
</tr>
<tr>
<td><em>mcyH</em></td>
<td>-10.12</td>
<td>-2.15</td>
<td>-1003.61</td>
<td>-18.5</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td><em>mcnC</em></td>
<td>-7.92</td>
<td>-3.81</td>
<td>-957.64</td>
<td>-14.42</td>
<td>-3.71</td>
<td>-1.18</td>
</tr>
<tr>
<td><em>furA</em></td>
<td>2.32</td>
<td>-1.05</td>
<td>-4.44</td>
<td>-4.60</td>
<td>-1.10</td>
<td>1.31</td>
</tr>
<tr>
<td><em>furB</em></td>
<td>47.51</td>
<td>-1.68</td>
<td>-3.95</td>
<td>-16.90</td>
<td>14.50</td>
<td>-1.57</td>
</tr>
<tr>
<td><em>furC</em></td>
<td>2.73</td>
<td>2.19</td>
<td>-6.25</td>
<td>1.51</td>
<td>3.15</td>
<td>1.79</td>
</tr>
</tbody>
</table>

Up- and downregulation for each gene at the exponential and stationary growth phases are given relative to transcript levels of cells grown in 1000 nM Fe. Values in bold represent significant changes after a one-tailed *t*-test (*P* < 0.05, *n* = 3). n/d, no transcript detected.

Environmental regulators of nodularin production

• With regards to light intensity, nitrogen and phosphate availability, nodularin concentration increases under conditions that promote optimal growth.

• Contrary, no change in nodularin concentration was reported for chemostat cultures under phosphate starvation and ammonium supplementation.

Nodularin synthetase promoter

TTTAAATGTTGATTTAAT
Fur binding site

TAATTCTGGTAATTGCAA
Fur binding site

TGTGGCATCTTCACA
NtcA binding site

TTGTGAATGATTGTTA
RcaA binding site

TGTTAAAAGGCAACA
NtcA binding site

ACACTAATCGTAGTGT
NtcA binding site

TTGTGAATGATTGTTA
RcaA binding site

ndal ndaG
ndaH
ndaF
ndaE
ndaD
ndaC
ndaA
ndaB

Polyketide synthase
NRPS/PKS hybrid
Non-ribosomal peptide synthetase
Tailoring

Nodularin synthetase gene expression

- *nda* gene expression:
  - increases two-fold during phosphate starvation
  - decreases two-fold in the presence of ammonium
- Nodularin concentration remained stable - post-translational regulation?

Summary –
Regulation of nodularin biosynthesis

- Not extensively studied – light, nitrogen, phosphate, iron and temperature may play a role but studies are inconclusive
- Influence of nitrogen and phosphate availability on transcript abundance different to microcystin – need to study nodularin regulation separately

Genetics of cylindrospermopsin production

Cylindrospermopsin synthetase gene expression

- *Aphanizomenon ovalisporum*:
  - Two different transcription start points active under different growth conditions (nitrogen and light availability) - one constitutive promoter, another for up- or down-regulation under specific environmental conditions?
  - Binding of the transcriptional regulator AbrB
mcyB transcription in response to light (early to late growth)

(A) mcyB transcripts

(B) 16S rRNA

low (16 µmol photons m\(^{-2}\)s\(^{-1}\))
medium (31 µmol photons m\(^{-2}\)s\(^{-1}\))
high (68 µmol photons m\(^{-2}\)s\(^{-1}\))
Transcript levels of ABC transporter in response to light

*mcyH* probe

16S rRNA probe
Transcription studies of *ntcA* under excess, limited and starved nitrogen

Relative expression of *ntcA* and *mcyB* under acclimatised conditions of excess, limited or starved nitrogen

Relative expression of *ntcA* and *mcyB* under newly transitioned conditions of excess, limited and starved nitrogen
Comparative proteomic analysis of non-toxic mutant

- Homologues to RhiA/B (nodulation) found to be down-regulated
- In *Rhizobium* quorum-sensing mediated
- In *Microcystis* by light-sensing regulation (but opposite to effect of light on toxin production)
Effect of Iron on Growth and Toxin Production

Toxin assay results
(PP2A pmol / mg protein):

Control = 20017
Half iron = 24843
No iron = 401887
Effect of iron on microcystin production

Nodularin synthetase gene expression

The expression of both copies declined to their pre-starvation levels of expression by day 21. The \( nda \) cluster in the \( N. spumigena \) strain AV1 was continuously expressed under these conditions (Figs 2a and 3a). The expression of the \( nda \) cluster responded to phosphate starvation (Fig. 2a), and the observed increase in expression of the nine \( nda \) genes was 2.73-fold \( /C6 \) \( 0.53 \) \((n = 8)\). Although this increase is relatively small, it was reproducible and consistently observed. The results established an effect of phosphate availability on the expression of the \( nda \) cluster in the \( N. spumigena \) strain AV1, and demonstrated that the nine genes exhibit an identical phosphate-dependent pattern of expression, likely due to transcription from the single bidirectional promoter. The results also showed that individual genes had different transcriptional levels, because the PCR efficiencies of all the \( nda \) genes were similar (see Materials and methods); this indicates that transcription from alternative promoters probably occurs similar to what was shown previously for microcystin (Kaebernick et al., 2002).

Analysis of nodularin concentration under these experimental conditions revealed that the intracellular and extracellular concentrations of nodularin did not vary significantly in response to phosphate starvation (Fig. 2b). These results are in accord with the results of Repka et al. (2001), which demonstrated that in chemostat cultures of \( Nodularia \) strain GR8b, the phosphate concentration did not have a statistically significant effect on nodularin production rate measured either as nodularin cell quotas, or normalized to dry weight or to protein concentration.

Our results suggest that the cells maintain a threshold level of intracellular nodularin concentration, and that any excess nodularin (resulting from the continuous expression of the \( nda \) cluster and biosynthesis of nodularin) above the threshold level is downregulated possibly by either intracellular degradation or excretion from cells by means of a transport system, rather than leakage due to cell lysis. However, no intracellular degradation mechanism has been discovered for nodularin or microcystin thus far. In addition, the possibility that there is an unknown transcription-independent regulatory mechanism acting on the enzyme-activity level and regulating the biosynthesis and/or the maturation of nodularin cannot be excluded.

Expression of the \( nda \) cluster and toxin concentration during ammonium supplementation

As shown in Fig. 3a, expression of \( nifH \) decreased in response to ammonium supplementation in Z8X, reaching an undetectable level at day 6. When the cells were collected,
Genetics of microcystin production

*Microcystis aeruginosa* PCC7806

- dnaN
- mcy J-H

*Anabaena* sp. 90

- mcyH I F
- J D G A B C atn1

*Planktothrix agardhii* CYA126

- mcyT
- D E G H A B C J ORF1

*Nodularia spumigena* NSOR10

- ORF6-8 I H G F E D C A B ORF1-5

- Nonribosomal peptide synthetase (NRPS) genes
- Polyketide synthase (PKS) genes
- Tailoring genes
- Putative transposases
- Toxin flanking genes
FeoB, futA and isiA) and has been shown to bind to the mcy gene cluster promoter (Martin-Luna et al., 2006a). Therefore, changes in the expression of this transcription factor may provide explanation to the link between the iron stress response and toxicity in M. aeruginosa. As expected, given the role of iron as a cofactor for FurA, furA transcription also decreased in M. aeruginosa PCC 7806 and the mcyH strain with decreasing iron availability. However, despite the similar transcript levels of FurA in PCC 7806 at 10 nM and 100 nM Fe relative to iron-replete conditions, an increase in toxicity was only measured at 10 nM Fe. This is indirect evidence for an additional level of toxin synthesis regulation besides transcription repression by FurA, possibly mediated by other Fur homologues or redox-sensitive transcriptional regulators such as NtcA.

FurA regulates the transcription of iron uptake proteins and IsiA, therefore differences in photosystem modification and iron uptake that were observed in the wild-type strains studied here could be attributed to differential expression of FurA (Fig. 5). The lack of a significant change in transcription in PCC 7005 furA may also explain the failure of this strain to upregulate isiA, futA and feoB transcription to levels similar to those in PCC 7806. On the other hand, although furA was downregulated in mcyH as expected under iron-starved conditions, the increased transcription of Fe$_2^+$ rather than Fe$_3^+$ transporters in this strain, or the lower levels of isiA transcription, cannot be attributed to FurA regulation alone. These differences of response to iron stress in PCC 7005 and PCC 7806 mcyH may be the result of the adaptation of naturally occurring non-toxic strains to the environmental niche that they occupy allowing their survival in a nutrient-limited environment.

A recent detailed study of a cyanobacterial FurB protein found that transcription of furB in iron starvation was influenced by oxidative stress but not iron limitation, which may only induce ROS formation if severe (López-...
Genetics of nodularin production

A. Nodularin synthetase *N. spumigena*

B. Microcystin synthetase *M. aeruginosa*

C. Microcystin synthetase *P. agardhii*
Genetics of nodularin production

A. Nodularin synthetase *N. spumigena*

B. Microcystin synthetase *M. aeruginosa*

C. Microcystin synthetase *P. agardhii*

- Putative repressor protein flanks the gene cluster and may be involved in transcriptional regulation of *nda* genes in response to heat stress