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Fur from *Microcystis aeruginosa* binds in vitro promoter regions of the microcystin biosynthesis gene cluster

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Abstract

Promoter regions of the *mcy* operon from *Microcystis aeruginosa* PCC7806, which is responsible for microcystin synthesis in this organism, exhibit sequences that are similar to the sequences recognized by Fur (ferric uptake regulator). This DNA-binding protein is a sensor of iron availability and oxidative stress. In the presence of Fe^{2+}, a dimer of Fur binds the iron-boxes in their target genes, repressing their expression. When iron is absent the expression of those gene products is allowed. Here, we show that Fur from *M. aeruginosa* binds in vitro promoter regions of several *mcy* genes, which suggests that Fur might regulate, among other factors, microcystin synthesis. The binding affinity is increased by the presence of metal and DTT, suggesting a response to iron availability and redox status of the cell.

Keywords: *Microcystis aeruginosa* PCC7806; Cyanobacteria; Fur; Iron stress; *mcy* Operon; Microcystin; Oxidative stress

1. Introduction

Toxic cyanobacterial blooms are increasingly widespread in surface waters of the Earth, and a serious health concern in many areas due to the production of several toxins, such as high levels of microcystins. Freshwater eutrophication has lead to frequent occurrence of blooms. However, there is general debate about the effect of the environment on microcystin production, since the toxicity of blooms can vary between years under apparently the same environmental conditions (Kaebernick and Neilan, 2001).

Iron availability and biolimitation by iron are important subjects discussed by oceanographers. After IronExII (Behrenfeld et al., 1996; Kirchman, 1996), we know that iron availability limits the rate of cell division and the abundance and production of phytoplankton in the equatorial Pacific and in other high nutrient, low chlorophyll regions (Frost, 1996; Boyd et al., 2000, 2004). In freshwater, Twiss et al. (2000) showed using enrichment experiments, that iron availability may be limiting phytoplankton productivity in certain conditions. Although iron is one of the most abundant elements on Earth, its deficiency is a constant source of stress in many biological systems, because iron is not always freely available in its soluble form (Lewin, 1984). Paradoxically, the uptake and incorporation of iron must be tightly regulated because iron catalyses the formation of reactive oxygen species through the Fenton reaction (Halliwell and Gutteridge, 1984). Often, the ability of a microorganism to capture and incorporate iron determines its success. Indeed, cyanobacteria have evolved very efficient mechanisms to maintain iron homeostasis (Strauss, 1994). In this way, iron limitation induces the expression of several proteins to overcome the stress. The response to limited iron

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includes the synthesis of secondary metabolites as siderophores (Wilhelm and Trick, 1994), new chlorophyll-binding proteins, and the replacement of proteins containing iron cofactors by non-iron proteins, such as flavodoxin (Strauss, 1994).

Bacterial pathogens, which are responsible for a wide range of infectious diseases, compete with host iron-binding proteins for available iron. To maximize the probability of establishing a successful infection, bacterial pathogens have an intricate network of virulence factors that are coordinately expressed and regulated (Crosa, 1997). Low iron concentrations induce a wide range of well-documented iron-regulated virulence genes (Litwin and Calderwood, 1993; Crosa and Walsh, 2002). One of the key factors in the response is a DNA-binding protein called Fur (Ferric uptake regulator). The current model indicates that when complexed to ferrous ions, a dimer of Fur binds to a specific DNA sequence (known as Fur-box) located in iron-responsive gene promoters (Escolar et al., 1999). Even though Fur is an iron-dependent repressor, the interest in Fur is not limited to its role as a modulator of genes related to iron homeostasis. The relevance of Fur in the oxidative stress response is known (Nunoshiba et al., 1999) and also its role in overcoming situations of acidic stress has been proposed (Hall and Foster, 1996). Moreover, Fur is known to be an essential element in triggering the expression of virulence factors in heterotrophic bacteria (Crosa and Walsh, 2002).

Cyanobacteria produce a broad range of secondary metabolites known as cyanotoxins that have toxic effects on eukaryotes. Among the cyanotoxins, there is a group of potent hepatoxins called microcystins. Several genera of cyanobacteria, such as *Microcystis*, *Anabaena*, *Planktothrix*, and *Nostoc* can produce the cyclic heptapeptide microcystin (Carmichael, 1994). Microcystins are potent inhibitors of protein phosphatases 1 and 2A in eukaryotes and affect seriously animal and human health (Carmichael et al., 2001). Studies of the regulation and function of cyanotoxins are interconnected and have focused on local ecosystems and the effect of environmental parameters on toxin production (Kaebernick and Neilan, 2001). In Nature, cyanobaterial blooms can be unpredictable toxic or non-toxic from one year to the next (Kaebernick and Neilan, 2001), and even under laboratory conditions, results are highly variable. The effects of environmental factors such as light intensity, temperature, nitrogen, phosphorous, and trace metals on microcystins production have been studied under field and laboratory. Some studies show that iron starvation increases the toxicity of *Microcystis aeruginosa* (Lukac and Aegerter, 1993; Lyck et al., 1996) although other studies appear to be contradictory (Utkilen and Gjolme, 1995).

Microcystins are synthesized in a mixed polyketide synthase/nonribosomal peptide synthetase system called microcystin synthetase. Tillett et al. (2000) identified and sequenced the gene cluster called *mcy* operon, which encodes the microcystin synthetase complex in *Microcystis aeruginosa* PCC7806. This 55-kb gene cluster consists of 10 open reading frames bidirectionally transcribed from a central 732-bp intergenic region between *mcyA* and *mcyD*. Interestingly, both of the polycistronic transcripts have alternate transcription start sites that appear to be light-dependent (Kaebernick et al., 2002). When cells were exposed to high or low light intensities, two transcriptional start sites were detected for both *mcyA* and *mcyD*, which indicates long untranslated leader regions. Putative transcription start sites were identified for *mcyE*, *mcyF*, *mcyG*, *mcyH*, *mcyI* and *mcyJ*, but not for *mcyB* and *mcyC* (Kaebernick et al., 2002).

Here, we show that promoter regions of several genes of the *mcy* operon exhibit putative iron-boxes, similarly to bacterial peptide synthetases. Moreover, Fur from *M. aeruginosa* binds in vitro, with different affinity, promoter regions of microcystin biosynthesis genes.

2. Results and discussion

To identify the possible Fur-binding sequences in the promoter regions of the genes of the *mcy* operon, we used a consensus sequence identified for cyanobacteria, GATAATGATAATCATTATC, previously described in *Anabaena* PCC7120 (Hernandez et al., 2006). Two putative iron-boxes were located in the 732-bp locus between *mcyA* and *mcyD* (Fig. 1). Furthermore, promoters from *mcyE, mcyH* showed two A-T rich regions with high scores in the alignment with the previously described consensus sequence (Hernandez et al., 2006). *mcyJ* and *mcyG* showed also an A-T rich region, each supposed a putative iron-box, in their promoter regions. In this case, the sequences exhibit lower scores matching with the consensus. To study the possible regulation of the microcystin synthesis by Fur from *M. aeruginosa*, the recombinant protein was tested for functional studies by electrophoretic mobility shift assays (EMSA) using DNA fragments containing the identified putative iron-boxes. Several *Fur* family members can be present in cyanobacterial genomes, and the *Fur* product used in this work, had high identity (70%) with FurA from *Anabaena* PCC7120, belonging to the class of Fur proteins involved in response to oxidative stress and iron availability (Martin-Luna et al., 2006). In a first experiment, we incubated the bidireccional *mcyDA* promoter region with Fur from *Microcystis* and analysed its binding activity. We detected a high affinity of the protein for the 732 bp bidireccional promoter (data not shown). Since EMSA is not recommended for fragments over 500 bp, the promoter was divided into two fragments, a 438 bp *mcyA* upstream sequence and a 331 bp *mcyD* gene upstream sequence. Fig. 2 shows the EMSA with the two fragments using increasing Fur concentrations, and in the gel is possible to see that Fur binds specifically to the two promoter fragments, with more affinity for the *mcyD* upstream sequence. Two Fur-DNA complexes were observed in both cases, one of them with very slight shift. In other bacteria, high

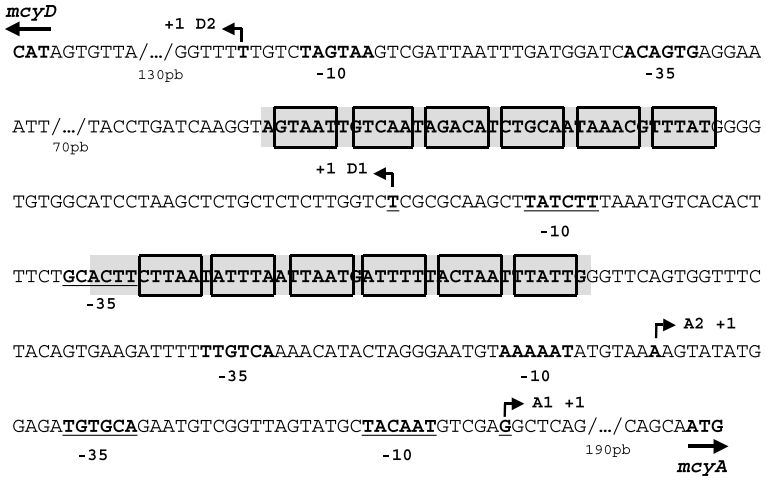

Fig. 1. Putative iron boxes identified in the bidireccional promoter region of the *mcyDA* gene from *M. aeruginosa*. The A-T rich regions are indicted shadowed in grey. A possible distribution of Fur boxes is shown in bold boxes. Bold underlined sequences and bold regions indicated the site of transcription initiation and −35/−10 boxes described by Kaebernick et al. (2000) for high and low light, respectively. GATAATGATAATCATTATC was the consensus sequence (Hernandez et al., 2006) used for the in silico prediction of putative iron-boxes.

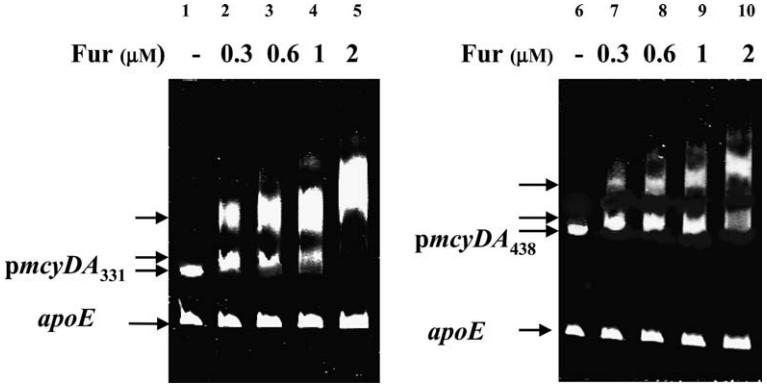

Fig. 2. Fur-*mcyDA* binding assays. The EMSA were performed with recombinant *M. aeruginosa* Fur, a fragment of exon IV from the human *apoE* gene as non-specific competitor DNA, and the corresponding promoter, as indicated in the figure. Fur concentrations are expressed in μM. Lane 1: free 331 bp fragment. Lanes 2, 3, 4 and 5, contain Fur 0.3 μM, 0.6 μM, 1 μM and 2 μM, respectively. Lane 6: free 438 bp fragment. Lanes 7, 8, 9 and 10, contain Fur 0.3 μM, 0.6 μM, 1 μM and 2 μM, respectively. Assays were performed in the presence of 100 μM $MnCl_2$ and 10 mM DTT. Arrows without label correspond to Fur–DNA complexes.

mobility complex and low mobility complex were identified as the occupancy by a single dimer and two dimers or a tetramer, respectively (Friedman and O'Brian, 2003). DNA-binding activity of Fur was assayed using *mcyE*, *mcyG*, *mcyH* and *mcyJ* promoter regions (data not shown), with binding in all of them, with variable affinity. Table 1 contains the estimation of apparent K_d, defined as the Fur concentration at which 50% of the DNA is associated with the protein. We evaluated the EMSA by estimating the unbound DNA remaining in each sample. Free DNA was taken as reference, assigning arbitrarily a value of 100%, and the obtained values should be considered only as indicative. The apparent K_d, in the case of the bidireccional promoter are not very different of those described for other well-known Fur-regulated promoter genes, as *isiB* (flavodoxin gene) from *Anabaena* PCC7120, 0.052 μM (Hernandez et al., 2006). It is interesting to notice that the affinity of Fur for the different promoters observed by EMSA is in good concordance with the scores of the sequences of the putative iron-boxes identified *in silico*, when compared to the iron-box used as consensus. In order to analyse the binding, the formation of Fur–DNA complexes was carried out in the presence or absence of a co-

Table 1
Apparent K_d of Fur from *M. aeruginosa* binding several promoter regions from *mcy* operon

Promoter region	$K_{d(app)}$ (μM)
$mcyDA_{331}$	0.28 ± 0.03
$mcyDA_{438}$	0.36 ± 0.04
mcyH	0.48 ± 0.03
mcyG	0.60 ± 0.07
mcyE	0.77 ± 0.03
mcyJ	1.53 ± 0.1

repressor metal (Mn^{2+}instead of Fe^{2+} due to the low stability of iron under the conditions used). DTT was also tested to study the influence of the redox status of the cysteines on binding activity. Binding of Fur to the 331 bp sequence of *mcyDA* upstream sequence decreases in the absence of metal or DTT (Fig. 3), suggesting a role of *M. aeruginosa* Fur in a potential response to the redox status of the cell. Similar results were obtained with the *mcyDA* 438 bp fragment and the other promoter regions tested (data not shown).

When *Microcystis* cells grown in iron deficient conditions (0.18 μM) were supplemented with iron (30 μM), a dramatic decrease of microcystin LR relative to total protein was observed after 2 h, remaining only 12% of the initial toxin. Moreover, an increase of microcystin LR was found when *Microcystis* cells growing in iron-replete conditions (30 μM) were treated with 50 μM 2,2′-dipyridyl, an iron chelator. These cells exhibit 125% more microcystin LR (relative to total protein) after 30 min of the iron depletion caused by the chelating agent. Both experiments were carried out simultaneously with a control aliquot. In supplementation experiments, the supplemented cells exhibit in 2 h a slight increase in chlorophyll while the 2,2′-bipyridyl treated samples showed similar chlorophyll values after 2 h of treatment.

Based on those results, we would not be surprised to find out a role for Fur in the regulation of microcystin synthesis genes, either related to iron and/or to response to oxidative damage. Likewise, non-ribosomal peptide synthetases involved in the synthesis of peptidic siderophores in heterotrophic bacteria are Fur regulated (Crosa and Walsh, 2002). The presence of several putative Fur-binding sequences, as well as Fur binding activity to the promoter regions of the *mcy* genes suggest its possible participation in the regulation of the synthesis of microcystins. It is important to consider that Fur is not only responsive to iron availability, and members of the Fur family play key roles in the response to oxidative stress (Thompson et al., 2002).

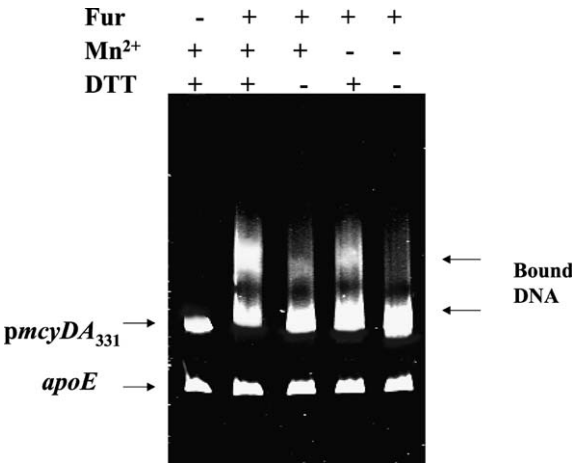

Fig. 3. Effect of $Mn^{2+}$and DTT in the interaction of *M. aeruginosa* Fur with the 331 bp *mcyDA* fragment. The EMSA contained 0.6 μM recombinant Fur, a fragment of exon IV from the human *apoE* gene as non-specific competitor DNA, the 331 fragment of the *mcyDA* promoter, and 10 mM DTT and/or 100 μM $MnCl_2$ as indicated in the figure. Lane 1: control without Fur. Lane 2: DTT and $MnCl_2$. Lane 3: $MnCl_2$. Lane 4: DTT. Lane 5 without DTT and $MnCl_2$. Arrows without label correspond to Fur–DNA complexes.

Transcriptional analysis of the *mcy* operon showed that transcription is light-dependent (Kaebernick et al., 2000, 2002), so it is interesting to note the relationship between iron uptake and light intensity because high light intensities increase cellular iron uptake (Utkilen and Gjolme, 1995). That might be explained if iron acquisition systems were expressed because of the liberation of Fur from iron-boxes, as consequence either for more iron needs or by the redox status of the photosynthetic chain, affecting to Fur cysteines and its DNA-binding capability. Another factor to consider is that light might cause oxidative damage and, perhaps, it might be ultimately responsible for *mcy* derepression and higher toxin production, with Fur as oxidative stress sensor.

The ecostrategy or physiological meaning of microcystin production is unknown, but several observations suggest a link between microcystin production and iron metabolism. Lyck et al. (1996) showed that, during iron-depletion, toxic strains of *Microcystis* maintained cell vitality much longer than non-toxic strains. Moreover, Utkilen and Gjolme (1995) found that the rate of iron uptake in toxic strains was higher than non-toxic strains, which suggests a possible simultaneous derepression of microcystin production and iron uptake systems. In most of the microorganisms studied to date, the regulation of the synthesis of bacterial peptidic siderophores involves the protein Fur, which senses the iron concentration in the cell cytosol, working as a negative regulator (Litwin and Calderwood, 1993). In toxic strains, iron starvation would turn on *mcy* gene expression producing microcystin, as well as other responses to manage stress. If microcystin could act as an intracellular iron chelator (Utkilen and Gjolme, 1995), Fur-regulated target promoters such as the bidireccional *mcyDA* promoter would be derepressed, even in iron-sufficient conditions. Microcystins might be part of the set of responses to compete for iron availability. If it could be active as an intracellular iron captor and thereby maintaining Fur free from DNA, the presence of microcystins might amplify the response of iron acquisition systems even if the cell is not really starved.

3. Experimental

3.1. Growth conditions

M. aeruginosa PCC7806 was provided by the Pasteur Culture Collection and growth in BG11 media with 2 mM of $NaNO_3$ and 10 mM of $NaHCO_3$ (Rippka et al., 1979).

Table 2
Oligonucleotides used for cloning *mcy* promoter regions

Promoter regions	Primer designations	Primer sequences (5′-3′)	Amplified fragments (pb)
pmcyDA$_{331}$	DA-N	CATAGTGTTAGAATCGACTTGG	331
	DA$_{331}$-C	GAGCTTAGGATGCCACACCC	
pmcyDA$_{438}$	DA$_{438}$-N	GTGTGGCATCCTAAGCTCTG	438
	DA-C	CCAGATGTGCTTCCATTGCTG	
pmcyE	ED-N	CTCTCAACCATGCCCTATTCTC	203
	ED-C	CGGAGCTTGATCAATATGGAGGTT	
pmcyG	GF-N	CCTCCAAGACTGATGGAATGC	175
	GF-C	CCAAAAAGCCCTTGACCATCG	
pmcyH	HG-N	GGAGGAGAAACAGCATTCCC	278
	HG-C	CAAGAACTTACTGATTTGGAGG	
pmcyJ	JI-N	CTGATCTTGCAAAAACGCCATT	218
	JI-C	GATTAATCCGGAGGCGTGGA	

Sequences of the amplified fragments were identical to the expected sequences according to the data available in the GenBank (Accession Number: AF183408, http://www.ncbi.nlm.nih.gov/), and the pair of oligonucleotides used.

3.2. Cloning and overexpression of Microcystis Fur; purification of the recombinant protein

Cloning and overexpression of the *Fur* gene from *M. aerugionosa* PCC7806 were performed according to Martin-Luna et al. (2006) using inverse-PCR. Purification of the recombinant protein was performed according to Hernandez et al. (2002), using Heparin-Sepharose chromatography followed by a metal affinity column.

3.3. Analytical methods

Total protein was quantified using the bicinchoninic acid method (BCA™ Protein Assay Reagent Kit from Pierce). The extinction coefficient used for *Microcystis* Fur was of 22,781 M^{-1} cm^{-1}. This value was calculated based on Gill and von Hippel (1989). Quantification for binding assays was confirmed by densitometry measures in gel using FurA from *Anabaena* sp. PCC7120 as standard. SDS–PAGE was carried out using 15% (w/w) polyacrylamide gels. Microcystin LR in cells was determined by HPLC according to the procedure described by Lawton and Edwards (2001).

3.4. Protein–DNA gel retardation assays

Binding assays were performed in 10 mM Tris, pH 7.5, 40 mM KCl, 2 mM $MgCl_2$, 0.1 mg/ml BSA and 5% glycerol as binding buffer. DTT (10 mM) and 100 μM Mn^{2+} were used as indicated. To avoid oxidation to Fe^{3+}, manganese was used instead of Fe^{2+}, as usual in this kind of experiments. Promoter fragments used in EMSA were obtained by PCR. Primer design (Table 2) was performed according to the sequences described by Tillett et al. (2000) and available in GeneBank (AF183408). To demonstrate the specificity of the DNA-binding activity of the proteins to the promoters, reactions were performed in the presence of an equal concentration of a 224 bp non-specific competitor DNA (a fragment of the fourth exon of human *apoE* gene). Each electrophoretical mobility shift assay was performed five times, and the gels shown in the figures are representative of the results of the assays. We evaluated binding activity by estimating the unbound DNA remaining in each sample. Free DNA was taken as reference, assigning arbitrarily a value of 100% (Gel Doc 2000 Image Analyser from BioRad). For estimation of $K_{d(app)}$, percentage of unbound DNA data were used.

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