2-oxoglutarate enhances NtcA binding activity to promoter regions of the microcystin synthesis gene cluster

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\textbf{ABSTRACT}

The binding affinity of NtcA towards promoter regions of the microcystin gene cluster from Microcystis aeruginosa PCC 7806 has been analyzed by band-shift assay (EMSA). The key nitrogen transcriptional regulator exhibits affinity for two fragments of the bidirectional mcyDA promoter, as well as for promoter regions of mcyE and mcyH. The presence of 2-oxoglutarate increased by 2.5 fold the affinity of NtcA for the mcyA promoter region. The 2-oxoglutarate effect peaked at 0.8 mM, a physiological concentration for this compound under nitrogen-limiting conditions. The results suggest that the 2-oxoglutarate level, as a signal of the C to N balance of the cells, regulates the microcystin gene cluster.

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\section{1. Introduction}

Microcystins (MC) are the most frequently reported cyanotoxin class to cause outbreaks of mass poisoning \cite{1,2}. MCs are cyclic heptapeptides produced nonribosomally by microcystin synthetases \cite{3}. In Microcystis aeruginosa PCC 7806, genes of microcystin synthetase have been identified and sequenced \cite{4}. This is a 55-kb gene cluster consisting of 10 open reading frames bidirectionally transcribed from a central promoter of 732-bp located in an intergenic region between mcyA and mcyD. Two transcriptional start sites (tsp) for the bidirectional promoter region have been identified and appear to be dependent on light conditions \cite{5}. However, other start sites have been detected for mcyA and mcyD \cite{6,7}. Putative internal promoters have also been determined for the genes mcyE, mcyF, mcyG, mcyH, mcyJ and mcyL employing RACE \cite{5}.

The environmental and nutritional factors involved in the expansion of harmful algal blooms in surface waters have remained largely unresolved. Multiple laboratory culture or field sample studies have shown conflicting results in relation to the effects of several nutrients or physical factors. For instance, published results concerning phosphorus \cite{8,9} nitrogen \cite{10,11} and iron \cite{12,13} in microcystin synthesis are frequently contradictory. Recent findings using molecular methods have indicated that Fur, the Ferric uptake regulator from Microcystis aeruginosa PCC 7806 binds in vitro promoter regions of genes of the mcy operon \cite{14} and that in batch cultures, iron starvation conditions result in both mcyD transcription and an increase in microcystin synthesis \cite{6}. NtcA, a key regulator for nitrogen metabolism control in cyanobacteria, exhibits binding activity to the bidirectional promoter mcyDA \cite{15,16}. In apparent contradiction, under laboratory conditions, nitrogen availability correlated positively with \textit{M. aeruginosa} growth rate without increasing the mcyD transcription and MC production per cell \cite{7}.

Many studies have shown the key role of the master regulator NtcA in the regulation of genes involved in nitrogen metabolism, carbon metabolism, photosynthesis and stress response, performing as an activator and also as a repressor \cite{17–20}. NtcA has a C-terminal binding DNA domain helix-turn-helix which interacts with a palindromic sequence signature GT\textasciitilde{TAC} \cite{21} or TG\textasciitilde{TN} or \textasciitilde{TAC} \cite{22}. It has been suggested that NtcA activates promoters carrying a vegetative-type sigma factor (SigA), similar to the -10 box TAN\textasciitilde{T}Escherichia coli \textasciitilde{\sigma} \textasciitilde{70} \cite{20} and a consensus GT\textasciitilde{TAC} variably located between 20 and 24 bp upstream from the -10 hexamer \cite{20}. In \textit{Synechocystis} sp. PCC 6803, NtcA represses \textit{gifA} and \textit{gifB} expression and the NtcA-binding site is located closer to the tsp than in the case of NtcA activated promoters \cite{23}. It has been
suggested that the position of the NtcA binding site related to tsp determines the NtcA repressor or activator action [22]. A number of NtcA dependent promoters have also been described which do not match the canonical NtcA-activated structure, and it has been suggested that another factor is required to stimulate the gene expression [20,24]. The signalling function of 2-oxoglutarate (2-OG) has been observed in *Anabaena* PCC 7120 and *Synechococcus elongatus* PCC 7942 performing as a positive effector of NtcA binding activity [26,27]. Tanigawa et al. [25] found that in *Synechococcus* the binding of NtcA to *glnA* and *nctA* promoters in vitro was enhanced by 2-oxoglutarate, and they described that 2-oxoglutarate was required for transcriptional initiation mediated by NtcA [25]. 2-Oxoglutarate levels in cyanobacteria are directly related to nitrogen availability in relation to the carbon metabolism and they provide feedback information to the cells of the status of the C/N balance. Analysis of the crystal structure of the complex 2-OG and NtcA from *Anabaena* sp. has revealed that the presence of this metabolite enhances the DNA-binding activity of NtcA due to the fact that the complex conformation is more suitable for recognizing successive DNA major grooves [28]. Here we report new checkpoints in the *mcy* cluster from *M. aeruginosa* PCC 7806 potentially operated by NtcA. Furthermore, we report that the presence of 2-oxoglutarate enhances NtcA binding activity in vitro suggesting that the nitrogen and carbon nutritional status in the cyanobacteria may have an important role in microcystin synthesis.

### 2. Materials and methods

#### 2.1. Strains and culture conditions

This study was carried out with *M. aeruginosa* PCC 7806 provided by the Pasteur Culture Collection. Cells were photoautotrophically grown in a BG11 medium supplemented with 2 mM of NaNO₃ and 10 mM of NaHCO₃, as described previously by Rippka et al. and as recommended by the Pasteur Institute [29]. The culture medium was air bubbled at 25–30 °C with continuous illumination (25 μmol of photon m⁻² s⁻¹).

#### 2.2. DNA extraction and promoter region amplification

Total DNA from *M. aeruginosa* PCC 7806 was isolated using 0.03 g of dry weight culture as described previously [30]. The amplification of *mcy* promoters was performed using primers listed in Table 1, based on the *mcy* operon sequence published by Tillett et al. [4]. PCR products were purified using GPX™ PCR DNA Kit (GE Healthcare). As non specific competitor DNA, a 224 bp DNA fragment of the fourth exon of the human apoE gene and the promoter region of the *mcy* operon intergenic region. Total DNA from *M. aeruginosa* PCC 7806 was isolated as described in [30].

#### 2.3. Protein–DNA gel retardation assays

Binding assays were performed using a core binding buffer containing 12 mM HEPES–NaOH (pH 8), 4 mM Tris–HCl pH 8, 60 mM KCl, 1 mM dithiothreitol, 50 μg ml⁻¹ of bovine serum albumin, and 8% glycerol as described by Vazquez-Bermudez et al. [26]. A mixture of 40 ng of each DNA fragment and different concentrations of *Anabaena* PCC 7120 recombinant NtcA (from 125 to 500 nM) were incubated at room temperature for 30 min and subsequently loaded on a non-denaturing polyacrylamide gel (3.75–5%). MgCl₂ and 2-oxoglutarate were added when indicated. The gel was developed using as running buffer 25 mM Tris–Cl pH 8, 150 mM of glycine and 13 V cm⁻¹. 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 non-specific competitor DNA. Gels were stained with SYBR Safe DNA (Invitrogen). 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 Kd (app), unbound DNA percentage data were used as described previously [14,19]. NtcA from *Anabaena* sp. PCC7120 was obtained as described in [31].

#### 3. Results

#### 3.1. Identification of NtcA-binding sites

In previous studies, two palindromic partially overlapping signature sequences TGTN₉ or 10ACA and GTAN₈TAC were described as the consensus NtcA binding site [20,22]. Using biocomputational approaches, several sequences with high homology to the NtcA consensus were found in the *mcy* operon intergenic region. Fig. 1A shows additional putative NtcA-target sites to those previously described [16]. In the case of *mcyA* promoter, multiple NtcA boxes were found at flanking regions of tsp. Recent data, as result of cyanobacteria genome comparisons and NtcA affinity assays revealed that the nucleotides “GT” of the first triplets and “AC” from the second are more important for NtcA binding [32,33]. In Fig. 1A we identify these nucleotides in the *mcyA* boxes. In Fig. 1B, other putative NtcA boxes are identified in silico in *mcyE* and *mcyH* promoters. The NtcA binding site was also located in *mcyG* and *mcyJ* (data not shown).

#### 3.2. NtcA binds several mcy promoters with high affinity

Once the NtcA boxes were identified in silico, band-shift assays (EMSA) were carried out using recombinant NtcA protein from *Anabaena* sp. PCC 7120 (with 67% similarity to the NtcA from *M. aeruginosa* PCC 7806), and several DNA probes of *mcy* promoters. Since EMSA is not recommended for fragments over 500 bp, the

### Table 1

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>Primer sequences (5’–3’)</th>
<th>Description</th>
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<tbody>
<tr>
<td>DA 438-N</td>
<td>GTGGCCATCTAAGCTTG</td>
<td>Forward primer for cloning mcyA promoter</td>
</tr>
<tr>
<td>DA 438-C</td>
<td>CCAAGTGTGCTACTGCTGCTG</td>
<td>Reverse primer for cloning mcyA promoter</td>
</tr>
<tr>
<td>DA 331-N</td>
<td>CATAGCTGTAATGCGACTTG</td>
<td>Forward primer for cloning mcyB promoter</td>
</tr>
<tr>
<td>DA 331-C</td>
<td>AAGCTTAGATGACCAACCC</td>
<td>Reverse primer for cloning mcyB promoter</td>
</tr>
<tr>
<td>pmcyE-N</td>
<td>CTCTCAACCGCTGCTT</td>
<td>Forward primer for cloning mcyE promoter</td>
</tr>
<tr>
<td>pmcyE-C</td>
<td>CGGACCGTATCTATAGGCTG</td>
<td>Reverse primer for cloning mcyE promoter</td>
</tr>
<tr>
<td>pmcyG-N</td>
<td>CTCCTCAAGACTGTTGGAAGC</td>
<td>Forward primer for cloning mcyG promoter</td>
</tr>
<tr>
<td>pmcyG-C</td>
<td>CCAAAAGCCCTTGACTCG</td>
<td>Reverse primer for cloning mcyG promoter</td>
</tr>
<tr>
<td>pmcyH-N</td>
<td>GGAGGAGAACAGCTACCC</td>
<td>Forward primer for cloning mcyH promoter</td>
</tr>
<tr>
<td>pmcyH-C</td>
<td>CAAGAAGCTACTGTTGGAGG</td>
<td>Reverse primer for cloning mcyH promoter</td>
</tr>
<tr>
<td>pmcyJ-N</td>
<td>CTCTAACTATGATTGAGG</td>
<td>Forward primer for cloning mcyH promoter</td>
</tr>
<tr>
<td>pmcyJ-C</td>
<td>CTGAACTCTGGTCTT</td>
<td>Reverse primer for cloning mcyJ promoter</td>
</tr>
<tr>
<td>pisB-N</td>
<td>CTCTTGATGTTGCG</td>
<td>Forward primer for cloning isb promoter</td>
</tr>
<tr>
<td>pisB-C</td>
<td>CATCATGACACCTGTATTAG</td>
<td>Reverse primer for cloning isb promoter</td>
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</table>
Fig. 1. Additional putative NtcA binding sites identified in silico in mcy operon promoter regions of M. aeruginosa PCC 7806. The previously described NtcA boxes [16] are indicated underlined. Panel A shows the mcyD/A promoter region with bold boxes indicating the new putative NtcA-binding sites. Tsp (bold) described by Kaebernick et al. [5] are marked as D1, D2, A1, and A2 while the described in preceding work of our group are indicated with an asterisk [6,7]. The boxes in dotted line are not perfectly matched with the consensus. The bidirectional promoter was fragmented in two sequences for EMSA and two arrows indicate the cut of the fragments, 438 bp upstream mcyA and 331 bp upstream mcyD. Panel B shows other identified putative NtcA-binding sites of internal promoters of mcy gene cluster.
bidirectional mcyAD promoter was divided into two fragments, a 438 bp mcyA upstream sequence and a 331 bp mcyD gene upstream sequence. Fig. 2A shows the bands shifts obtained with the 438 bp fragment using increasing NtcA concentrations, while Fig. 2C shows the activity of NtcA using the 331 bp fragment. DNA-binding activity of NtcA was also assayed using the complete promoter regions of mcyE, mcyG, mcyH and mcyJ genes (data not shown) and only promoter regions of mcyE (Fig. 3) and mcyH (data not shown) showed affinity for the protein. In the case of the mcyE upstream region (203 bp), strong DNA-binding activity of NtcA was found using 200 nM of NtcA. The complexes were similar in the presence and absence of 4 mM of MgCl2 (Fig. 3). The presence of Mg2+ did not appear to affect binding activity.

3.3. 2-Oxoglutarate enhances NtcA binding activity to mcy promoters

The metabolite 2-oxoglutarate (2-OG) is the carbon skeleton for the assimilation of nitrogen in cyanobacteria, and it has been reported that 2-OG is a signalling molecule of nitrogen starvation, which could act as an effector on NtcA-dependent transcription activation [25–27]. When 2-OG was present in the binding assays, NtcA exhibited enhanced affinity for pmcyA (Fig. 2A). We evaluated the EMSA by estimating the unbound DNA remaining in each sample (Fig. 2B). Free DNA was taken as reference, assigning arbitrarily a value of 100%, and the obtained values should be considered only as indicative. The apparent Kd of the NtcA-DNA complex was calculated in the presence (Kd 0.625 μM) and absence (Kd 0.354 μM) of 2-oxoglutarate. The effect of 0.6 mM 2-OG stimulated the binding activity of NtcA by two and a half times more than that obtained in the absence of this metabolite (Fig. 2B). In order to characterize the effect of 2-OG, the influence of several concentrations of 2-OG was tested on the binding affinity of NtcA to the 448 bp mcyA upstream fragment. The data are shown in Fig. 4. The 2-oxoglutarate effects peaked at about 0.6–0.8 mM, a physiological concentration for this compound.

4. Discussion

In the present work, we have located other putative NtcA binding sites in the intergenic region of mcy operon in addition to that described previously [16] as well as some boxes included in the internal promoter regions of the mcy gene cluster. Multiple NtcA boxes have also been described in Anabaena sp. PCC 7120 [22] and Serrasalmus elongates [21] which display a complex regulation of NtcA affinity dependent on the binding site position with respect
Changes in our group shows that nitrogen availability in itself does not induce 2-OG, can increase its affinity. This would be a bizarre physiological hypothesis since an imbalance of C/N, signalled by increased levels of tsp, increasing amounts of 2-OG, namely 0.3, 0.4, 0.6, 0.8, 1 and 1.5 mM respectively.

Effect of different concentrations of 2-OG on NtcA- p

Our results indicate that the binding of NtcA to the mcyA upstream region is enhanced in the presence of 2-OG. The latter compound is a commonly accepted key indicator of the carbon/nitrogen balance in the cell, suggesting that microcystin synthesis responds to the equilibrium between both metabolisms [34]. In Synechococcus, 2-OG acts as a positive effector of the NtcA binding to the glnA promoter, with an observed five-fold increase in the EMSA [26]. Studies related to the optimum 2-OG concentration in vitro in NtcA binding experiments have revealed different results [25–27]. In Anabaena sp. PCC 7120, the binding of both promoters of ntcA and glnA was enhanced with increasing concentrations of 2-OG, being obtained a maximal effect at 5 mM [27]. However, in glnA promoter from Synechococcus sp., NtcA binding activity was more effective at 0.6 mM of 2-OG, and at higher levels of this metabolite a decrease in the electrophoretic retardation was observed [26]. This order of magnitude coincides with our results. The accumulation of 2-OG acts as a nitrogen starvation signal and therefore it is possible that with a specific C/N ratio in cyanobacteria there would be a maximum NtcA activity state for each promoter. Consistent with this notion, the binding of NtcA to pmcyA has a maximal enhancement at 0.8 mM of 2-OG and a lower stimulation was observed at increasing concentrations of this effector. Crystal structure studies of the NtcA apoform and 2-OG–NtcA complex indicate that the presence of this metabolite is not essential to the DNA-binding capacity of this regulator [28], which could have interesting physiological implications. The complex NtcA/2-OG does not have an on-to-off state, but subtle changes in the distance between the two F-helices for DNA recognition due to the presence of 2-OG results in a stronger DNA-binding activity of NtcA [28], which could have interesting physiological implications. The complex NtcA/2-OG does not have an on-to-off state, but subtle changes in the distance between the two F-helices for DNA recognition due to the presence of 2-OG results in a stronger DNA-binding activity of NtcA [28].

[| [NtcA] 2μM | - | + | + | + | + | + |
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<tr>
<td>[2-OG] mM</td>
<td>0</td>
<td>0.3</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
<td>1.0</td>
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Fig. 4. Effect of different concentrations of 2-OG on NtcA- pmcyA binding assay. NtcA was used 200 nM. Lane 1: free mcyA promoter. Lanes 2, 3, 4, 5 and 6 contain increasing amounts of 2-OG, namely 0.3, 0.4, 0.6, 0.8, 1 and 1.5 mM respectively.

putative NtcA boxes to the start sites. NtcA-binding sites in repressed promoters usually overlap with -35 or -10 boxes, or even with the tsp, and bound NtcA blocks the access of the RNA polymerase to the promoter [34]. This suggests that in our case NtcA can repress the mcyA gene as described in the case of nrcB in Anabaena sp. [22] and gfpA and gfpB in Synechocystis sp. [23]. All the data taken together suggest that NtcA acts in this case as a repressor of microcystin synthesis, responding to the C/N balance. Indeed, the microcystin synthesis undoubtedly involves different nutritional and environmental factors, sensed by different transcriptional factors, and at the very least NtcA and Fur [14] are involved in the microcystin gene cluster regulation.

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References


