Cross-talk Between Iron and Nitrogen Regulatory Networks in *Anabaena (Nostoc)* sp. PCC 7120: Identification of Overlapping Genes in FurA and NtcA Regulons

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Received 1 June 2007; received in revised form 30 August 2007; accepted 4 September 2007

Nitrogen signalling in cyanobacteria involves a complex network in which the availability of iron plays an important role. In the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120, iron uptake is controlled by FurA, while NtcA is the master regulator of nitrogen metabolism and shows a mutual dependence with HetR in the first steps of heterocyst development. Expression of FurA is modulated by NtcA and it is enhanced in a hetR− background. Iron starvation in cells grown in the presence of combined nitrogen causes a moderate increase in the transcription of *glnA* that is more evident in a ntcA− background. Those results evidence a tight link between the reserves of iron and nitrogen metabolism that leads us to search for target genes potentially co-regulated by FurA and NtcA. Using a bioinformatic approach we have found a significant number of NtcA-regulated genes exhibiting iron boxes in their upstream regions. Our computational predictions have been validated using electrophoretic mobility shift assay (EMSA) analysis. These candidates for dual regulation are involved in different functions such as photosynthesis (i.e. *psaL, petH, rbcL, isiA*), heterocyst differentiation (i.e. *xisA, lanA, prpJ, nifH*), transcriptional regulation (several alternative sigma factors) or redox balance (i.e. *trxA, ftrC, gor*). The identification of common elements overlapping the NtcA and FurA regulons allows us to establish a previously unrecognized transcriptional regulatory connection between iron homeostasis, redox control and nitrogen metabolism.

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Edited by J. Karn

Keywords: *Anabaena*; FurA; NtcA; cross-talk; regulatory networks

**Introduction**

Limitation of iron is one of the most common stress factors in cyanobacterial communities that restricts nitrogen fixation. In many bacteria, iron homeostasis is controlled by the ferric uptake regulator protein (Fur), a global regulator that links iron metabolism to the oxidative stress response and modulates a large number of genes involved in central metabolism, electron transport and defence against several stresses. Fur binds specifically to arrays of A/T-rich sequences, known as iron boxes, usually found in proximity to the −10 and/or −35 promoter elements of target genes. Functional interactions between Fur and Crp, the primary sensor of C availability, unravel a complex transcriptional regulatory connection between iron and carbon metabolism in *Escherichia coli*.

In the nitrogen-fixing cyanobacterium *Anabaena (Nostoc)* sp. PCC 7120, iron homeostasis is controlled by FurA, while NtcA is the master regulator of...
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nitrogen metabolism. NtcA is a transcriptional regulator that belongs to the Crp family, which controls the expression of an important number of genes, mainly related to nitrogen metabolism. In response to 2-oxoglutarate levels, NtcA binds to gene operator regions at sites that contain the consensus sequence GTA\textsubscript{N8}TAC.\textsuperscript{7} In filamentous cyanobacteria, NtcA is essential for heterocyst formation and shows a mutual dependence with HetR, a DNA-binding protein that exhibits autoenzymatic activity and is essential in the early stages of heterocyst development.\textsuperscript{8,9}

Cyanobacterial NtcA regulons have been predicted by computational approaches as an ample set of genes involved not only in nitrogen metabolism but also in several stages of photosynthesis, suggesting that this regulator couples both biosynthetic processes.\textsuperscript{10} This finding is in good agreement with previous genetic and physiological studies showing that photosynthetic electron transport influences the levels of expression of ntcA.\textsuperscript{11} Redox regulation of NtcA has also been proposed based on \textit{in vitro} analysis of DNA binding.\textsuperscript{12}

A close interrelationship between iron and nitrogen metabolism can be inferred from different reports. Many of NtcA-regulated genes encode iron-rich proteins.\textsuperscript{7} Razquin \textit{et al}.\textsuperscript{13} showed that transcription of the nifHDK operon and excision of the 11 kb DNA fragment takes place in iron-starved \textit{Anabaena}, even though cells grew in the presence of combined nitrogen. Besides, several iron-responsive genes in cyanobacteria, such as \textit{nblA}, \textit{petH}, \textit{pkn41}, \textit{pkn42}, among others, are also modulated by NtcA.\textsuperscript{14-16} We have recently shown that NtcA modulates the expression of FurA, that is strongly enhanced in the heterocyst,\textsuperscript{17} providing a very strong evidence of the link between those regulators.

In recent years, a lot of computational strategies have been developed to predict transcription factor binding sites in the regulatory regions of genes. Most of them are based on the construction of probabilistic models, in the form of position-specific weight matrices, from multiple alignments of experimentally characterized binding sites for a given transcription factor.\textsuperscript{18-21} Position-specific weight matrices characterize the relative binding preference of each base in every position of the alignment. The resulting values have been postulated to be a good statistical representation of the binding energy contributions of each base to the total site recognition energy.\textsuperscript{22} In many cases where binding sites for transcription factors are known from experiments, these programs have been shown to yield the known binding site patterns, indicating that the results of these methods can be useful in discovering unknown binding sites from a collection of sequences believed to contain a common binding site pattern.\textsuperscript{23} As stated before these methodologies have produced fairly decent results over the years and have been applied to predict the complete regulons of transcription factors and the transcriptional regulatory network organization in a broad group of complete sequenced genomes.\textsuperscript{24-28}

Here we have used biochemical, genetic and computational approaches to gain a better understanding of the cross-talk between iron homeostasis and nitrogen metabolism. To this end, we have analyzed the expression of FurA in vegetative cells in \textit{hetR} and \textit{ntcA} backgrounds. Moreover, we have also investigated the iron-dependency of the expression of \textit{glnA}, given the fact that it constitutes a key regulatory enzyme linking the nitrogen and carbon metabolisms in cyanobacteria. Finally, previously unidentified common elements from the NtcA and FurA regulons have been predicted using a bioinformatic approach to locate their correspondent binding sequences in the upstream regions of a wide set of genes. Results derived from this \textit{in silico} analysis were validated using a gel-shift assay with excellent correlations between our computational predictions and the experimental binding assays.

All the results unravel a significant overlapping between FurA and NtcA regulons. The identification of co-regulated genes allows us to define a transcriptional regulatory connection between iron homeostasis, redox control and nitrogen metabolism, where FurA and NtcA are the main nodes.

Results

Expression of FurA is enhanced in \textit{ntcA}\textsuperscript{−} and \textit{hetR}\textsuperscript{−} backgrounds

In a previous work we have shown that under nitrogen step-down, NtcA activates the expression of \textit{furA} in the heterocyst. Moreover, in a \textit{ntcA}\textsuperscript{−} background, changes in the translation of \textit{furA} caused by nitrogen deprivation are very small.\textsuperscript{17} Recently, we have consistently observed that in the presence of ammonium, the expression of \textit{furA} in CSE2 cells is around 1.5 times higher than in \textit{Anabaena} sp. PCC 7120 (Figure 1). Those results support a model in which NtcA would act as a repressor of \textit{furA} expression in nitrogen-repleted cultures and not as an activator in the heterocyst.

Since the expression of some key proteins in the heterocyst exhibits a double dependence on NtcA and HetR,\textsuperscript{29} we sought to investigate the effect of a \textit{hetR}\textsuperscript{−} mutation on the expression of \textit{furA}. The Western analysis shown in Figure 1 indicates that in nitrogen-repleted cultures, the level of \textit{furA} expression in a \textit{hetR}\textsuperscript{−} background is 1.6 times higher than that in wild-type (WT) \textit{Anabaena}. This higher level is similar to the amount of FurA detected in those WT cultures after 6 h of nitrogen deficiency. However, when HetR-deficient cells are transferred to nitrogen-depleted medium, like in \textit{ntcA}\textsuperscript{−} cultures, there was no increase in the level of \textit{furA}.

Role of FurA in \textit{glnA} expression

Because glutamine synthetase (GS) is the gateway of ammonium to carbonated skeletons, we examined the effect of iron deficiency in the expression of \textit{glnA}. Northern blot analyses were performed in
nitrogen and iron-deprived cultures of *Anabaena* PCC 7120 and the *ntcA*− strain CSE2. In accordance with previous reports, Figure 2(a) shows that in WT *Anabaena*, expression of *glnA* is induced after 3 h of nitrogen step-down. As expected, absence of NtcA abolishes transcriptional activation of *glnA* from its major tsp that generates RNAI.30 Figure 2(b) shows the effect of iron deprivation in the levels of *glnA* mRNA. A slight increase in the amount of *glnA* transcript after 5 h of iron deficiency is observed in *Anabaena* PCC 7120, followed by a decrease of the *glnA* message at 10 h. Transcription of *glnA* in CSE2 cells dropped to 40% after 10 h of iron deprivation. However, this decrease is followed by a sequential recovery of the levels of *glnA* mRNA. Though transcription of *glnA* is influenced by iron availability, the transcription start sites of *glnA* determined by primer extension exhibited the same pattern in cells from iron-repleted cultures or after 48 h of iron deprivation (not shown).

Changes in the levels of *glnA* mRNA could be due either to a secondary nitrogen stress caused by iron starvation, to the modulation of P*glnA* by an iron-responsive regulator or both. In order to assess the

![Figure 1](image1.png)  
**Figure 1.** Immunodetection of FurA in *Anabaena* sp. PCC 7120, the *ntcA*− strain CSE2 and the *hetR*− strain DR884a. Whole-cell extracts containing 30 μg of total protein were prepared from ammonium-grown cells and deprived of combined nitrogen for the number of hours indicated in each lane. The level of expression of FurA (defined as the ratio of the signal in each lane to the corresponding signal at 0 h and expressed in arbitrary units) was obtained from two independent experiments and the mean values are depicted in the graphs.

![Figure 2](image2.png)  
**Figure 2.** Northern blot analysis of *glnA* expression in *Anabaena* sp. PCC 7120 and the *ntcA*− strain CSE2 under nitrogen and iron deficiency. Samples containing 40 μg of RNA were hybridized to a *glnA* probe (upper panel) or to an *rnpB* probe (bottom panel) used as loading and transfer control. The relative amount of *furA* mRNA in each case (defined as the ratio of the signal in each lane to the signal in the corresponding *rnpB* hybridization lane, normalized to the value at 0 h, and expressed in arbitrary units) is shown. Approximate size corresponding to the observed signal is indicated. (a) RNA was isolated from ammonium-grown cells (lane 0 h) or from ammonium-grown cells incubated in the absence of combined nitrogen for 3, 6, 9 and 24 h. (b) RNA was isolated from ammonium-grown cells (lane 0 h) or from ammonium-grown cells incubated in iron deficiency for 5, 10, 24, 34 and 48 h.
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Figure 3. Electrophoretic mobility shift assay of increasing amounts of FurA to a 497 bp DNA fragment containing the Anabaena sp. PCC 7120 glnA promoter. A fragment of exon IV from the human apoE gene was used as non-specific competitor DNA. The assay was performed in the presence (a) and the absence (b) of 100 μM MnCl₂. Complexes were separated through 4.8% (w/v) polyacrylamide gels. Lane 1, free DNA; lane 2, 50 nM FurA; lane 3, 100 nM FurA; lane 4, 200 nM FurA; lane 5, 300 nM FurA; lane 6, 500 nM FurA; and lane 7, 750 nM FurA. (c) Electrophoretic mobility shift assay of increasing amounts of FurA to a 497 bp DNA fragment containing the Anabaena sp. PCC 7120 glnA promoter and to a 360 bp DNA fragment containing the Anabaena sp. PCC 7120 furA promoter. Lane 1, free DNA; lane 2, 250 nM FurA; lane 3, 500 nM FurA; lane 4, 750 nM FurA; lane 5, 1000 nM FurA.

direct involvement of FurA in the modulation of glnA, we studied whether FurA binds to P<sub>glnA</sub> using electrophoretic mobility shift assays (EMSA). Figure 3 shows that FurA binds to the UPSR of glnA in a metal-dependent fashion. Affinity of FurA to P<sub>glnA</sub> is lower than that to its own promoter as it can be observed in the competition assay shown in Figure 3(c).

The promoter region of the glnA gene in Anabaena has a complex structure. Alignment of P<sub>glnA</sub> with the FurA-protected sites in P<sub>furA</sub> and P<sub>asiB</sub>, unravels the presence of several candidate FurA-binding sites in the upstream region (UPSR) of this gene. The scanning of this region using the position weight matrix built for Fur did not result in any putative binding site passing the strong cut-off defined by us. However, as shown in Figure 4, the distribution of the potential iron boxes along P<sub>glnA</sub> suggests that FurA participates in the modulation of this gene. To test the contribution of the Fur-binding sites exhibiting lower score values to FurA–P<sub>glnA</sub> interaction, EMSA was performed with different DNA fragments of P<sub>glnA</sub>. Results shown in Figure 5 indicate that deletion of a part of the region containing two contiguous binding sites (i.e. fragments B and C) does not significantly impair FurA–DNA interaction. FurA binds to the three fragments from the UPSR of the glnA gene. The affinity of this regulator for fragment B, consisting of P<sub>II</sub> and P<sub>III</sub>, is higher than that for the rest of the fragments. This result suggests that the significant binding sites excluded by the strong cut-off are false negatives, and that FurA contributes to modulate the expression of glnA in Anabaena.

Identification of common genes in NtcA and Fur regulons

The results shown above, together with prior work from our laboratory, suggest a tight relationshipship between nitrogen and iron-regulated genes. In order to identify the genes involved in a potential regulatory network coordinated by FurA and NtcA, 106 UPSRs from the Anabaena sp. PCC 7120 genome previously reported as NtcA-regulated were scanned to identify iron-boxes. Regulatory sites of genes related to the thioredoxin family, that were predicted as NtcA-targets in other cyanobacteria, as well as the UPSRs of petH and hetR were included in this computational analysis. The sequences of the Fur-regulated promoters P<sub>furA</sub>, P<sub>furB</sub>, P<sub>furC</sub>, P<sub>asiA</sub> and P<sub>asiB</sub> were also scanned and considered as positive controls. On the other hand, the presence of the NtcA GTA[N8]TAC consensus motif was checked in a series of genes known to be either iron-dependent or Fur-regulated. Finally, the binding of FurA and NtcA to a selection of 30 UPSRs was verified experimentally by EMSA analysis.

Computational predictions for iron boxes in NtcA-modulated genes

Predictions obtained using the PATSER program as described in Materials and Methods are available as Supplementary Data (Table S1). The high-scoring sequences were located in the genome of Anabaena. In the few cases that a predicted sequence layed into the open reading frame (ORF) of the gene upstream, this putative Fur-binding site was not considered. Moreover, when overlapping sites were predicted, only the sequence exhibiting the highest score was considered. Applying those restrictions, a set of 54 selected genes was identified as probably co-regulated by FurA and NtcA (Table 1). According to the definition for a global regulator, high-scoring FurA-binding sites are present in genes belonging to diverse functional categories, such as photosynthesis and respiration, carbon metabolism and transcriptional regulation among others. We have

† http://www.ebi.ac.uk/clustalw/ ‡ http://bacteria.kazusa.or.jp/cyano/
Figure 4. Sequence of *Anabaena* sp. *glnA* promoter showing the putative Fur-binding sites (shadowed in grey) and the NtcA box. Transcription start points of each mRNA are indicated with black triangles along with the following features:

−10 boxes for RNA_{i}, RNA_{ii}, RNA_{iv} and RNA_{vi}; −35 box for RNA_{ii} (dotted boxes). The location of the oligonucleotides used to obtain the A fragment (GA5 and GA6), B fragment (GA3 and GA4) and C fragment (GA1 and GA2) of *P_{glnA}* are indicated with arrows. The translation start codon is indicated in bold letters.

Figure 5. (a) Gel retardation assays of increasing amounts of FurA to the A, B and C fragments of the *glnA* promoter. A fragment of exon IV from the human *apoE* gene was used as non-specific competitor DNA. Lane 1, free DNA; lane 2, 250 nM FurA; lane 3, 500 nM FurA; lane 4, 1000 nM FurA. (b) Schematic representation of the UPSR of *glnA*. The location of the transcription start points of RNA_{i}, RNA_{ii}, RNA_{iii} and RNA_{iv} are indicated with black arrows. The location of Fur-binding sites (grey boxes) and NtcA binding site (white box) is depicted. The limits of the A, B and C fragments (shown below) used in the gel retardation assay are indicated.
| Gene Location in computational sequence | Score | P-value | Putative FurA binding site | Location from ATG
<table>
<thead>
<tr>
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<td>8.25</td>
<td>–10.84</td>
<td>ATTCAGAGTTTTGAGGATCTTTT</td>
<td>–68</td>
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<tr>
<td>all1017 Photosystem I subunit XI; Psal (psal) 234</td>
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<td>–10.11</td>
<td>TTAATGTCCTTATATTAATGCTAA</td>
<td>–231</td>
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<td>all1030 Two-component sensor histidine kinase 150</td>
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<td>–9.77</td>
<td>CTTGATGAAGAGTGTGAATTAAATATT</td>
<td>–129</td>
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<tr>
<td>all0911 ABC phosphate transport system phosphate-binding periplasmic protein 413</td>
<td>8.63</td>
<td>–11.13</td>
<td>TTTCTATAACCCCTAGTATATGCTCA</td>
<td>–392</td>
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<tr>
<td>all0911 ABC phosphate transport system phosphate-binding periplasmic protein 193</td>
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<td>–10.87</td>
<td>CTCCATGAATTATGCAAGGATTTAC</td>
<td>–172</td>
</tr>
<tr>
<td>all1021 Probable proteinase 130</td>
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<td>–11.23</td>
<td>ATACGGCAGTTTTTATATAACGTTAGT</td>
<td>–109</td>
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<tr>
<td>all1071 Hypothetical protein 214</td>
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<td>TGGTAGCATAATATATAGAAAGAAAT</td>
<td>–193</td>
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<tr>
<td>all1071 Hypothetical protein 212</td>
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<td>–10.25</td>
<td>GTGATAAATGGTAAAGAAAGAAAGAA</td>
<td>–191</td>
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<tr>
<td>all1127 NADH dehydrogenase 90</td>
<td>6.28</td>
<td>–9.44</td>
<td>ATTTGACATTTTTGACAAAGTTGTA</td>
<td>–69</td>
</tr>
<tr>
<td>all1125 Photosystem II 11 kD protein PsbZ (psbZ) 41</td>
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<td>–8.66</td>
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<td>–20</td>
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<tr>
<td>all1145 Nitrogenase iron protein NiII (mifB) 254</td>
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<tr>
<td>all1169 Ferric uptake regulator (furA) 186</td>
<td>5.66</td>
<td>–9.03</td>
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<tr>
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<td>CAAAATGTGATATCTTCAATACAA</td>
<td>–16</td>
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</table>
found iron boxes in the UPSRs of genes coding for key regulatory proteins, such as PII and ntcA itself. The ferredoxin-NADP+ reductase gene, encoded by petH, is another NtcA-target that exhibits one high-scoring iron-box.

Genes involved in heterocyst maturation and nitrogen fixation (i.e. prpJ, nifH), nitrogen transport, such as the urea permease urtA, and the ammonium transporter alr0992, as well as elements from different ABC transport systems, exhibited high-scoring UPSRs. However, the score for the UPSR region of hetR was not high enough to be recovered in our restrictive conditions.

It is noticeable that most of the alternative transcription factors encoded by the *Anabaena* genome (i.e. sigC, sigD, sigE and sigF) are Fur-binding candidates. Co-regulation by FurA and NtcA also seems to coordinate the transcription of several two-component response regulators. The presence of iron boxes in the regulatory sites of genes with predicted functions in redox balance maintenance, like trxA, alr3955, gor and ftrC, is in agreement with the participation of Fur proteins in the modulation of the oxidative stress response in different bacteria. 3,32

### Identification of NtcA boxes in iron-dependent genes

We searched potential NtcA-binding motifs in the regulatory regions of several FurA-modulated genes that were not described as NtcA-regulated. We also included in this study the UPSRs of some nitrogen-dependent genes, such as sigC, nifJ2 and dpsA,
whose NtcA-binding sites have not been reported before in *Anabaena*.

Those genes were included in our study for the following reasons: (1) *nif2* is essential for growth on medium depleted of iron and combined nitrogen \(^{33,34}\); and (2) flavodoxin (*isiB*) and DpsA, both shown to be modulated by FurA, have been related to nitrogen metabolism.

Table 2 shows the potential NtcA boxes identified in the UPSRs under study. We failed to find NtcA-binding motifs in P*_{isiB}* and P*_{dpsA}*. Further experimental validation by EMSA of our bioinformatic analysis (Table 3) also shows that NtcA does not bind to those sequences.

### Experimental validation of potentially co-regulated genes

In order to verify our computational approaches, we amplified by PCR the regulatory sequences of 30 genes and checked by EMSA their ability to bind FurA and NtcA. Most of those sequences exhibited statistically significant predictions for iron boxes and contained the NtcA-binding signature. In addition to those high-scoring UPSRs, we included in our EMSA analysis the regulatory regions of some genes that presented lower scores, since because of their functions, these genes could undergo dual regulation. Those UPSRs were the corresponding to the *xisA* and *hanA* genes, as well as those from the thioredoxin-encoding genes *all1893*, *all2367* and *all2205*.

Table 3 lists the UPSRs that were checked by EMSA and recognized by FurA, NtcA, or both. The regulatory regions of the three *fur* homologues and of the *ntcA* gene were recognized by both regulators. With the exception of P*_{isiB}*, all the UPSRs from genes belonging to the functional category of photosynthesis and respiration that were tested also bound to both regulators. Regulation of *isiA* and flavodoxin (*isiB*) by FurA has been reported\(^{35}\).

![Table 2](image)

<table>
<thead>
<tr>
<th>Gene</th>
<th>NtcA box</th>
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<tr>
<td><em>all2473</em> (furB)</td>
<td>GTAGGCCATCGAGCAC-N_{32}-ATG</td>
</tr>
<tr>
<td><em>alr0557</em> (furC)</td>
<td>GTAAATGATCGATAC-N_{32}-ATG</td>
</tr>
<tr>
<td><em>alr3434</em> (glsB)</td>
<td>GTACCAATGAGACATAC-N_{32}-ATG</td>
</tr>
<tr>
<td><em>alr2803</em> (nif2)</td>
<td>GTATTGAGAGATCAGAAT-GATG</td>
</tr>
<tr>
<td><em>all4001</em> (isA)</td>
<td>GTAAAATATGACATAC-N_{32}-ATG</td>
</tr>
<tr>
<td><em>alr4123</em> (prk)</td>
<td>GTGAAAATGGATCTACTATATAT-GATG</td>
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<tr>
<td><em>all1507</em> (psaL)</td>
<td>GATAAAAATGATACATAC-N_{32}-ATG</td>
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<td><em>alr2515</em> (psoZ)</td>
<td>GTATTTTTCGAAC-GATG</td>
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<td><em>alr2205</em> (psbZ)</td>
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<td><em>all1127</em> (psbZ)</td>
<td>GTATTTTTCGAAC-GATG</td>
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<td><em>all1893</em> (trxA)</td>
<td>GTATAAATGAGACATAC-N_{32}-ATG</td>
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<td><em>all2367</em></td>
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<td><em>alr6065</em> (psbZ)</td>
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<td><em>alr2405</em> (psaL)</td>
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<td><em>alr1911</em> (nif1)</td>
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<td><em>alr2803</em> (nif2)</td>
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<tr>
<td><em>alr1911</em> (nif1)</td>
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</table>

Here we show that in *Anabaena*, where those genes are located in different operons, NtcA participates in the modulation of *isiA* but has not influenced the transcription of flavodoxin, whose UPSR does not interact with NtcA. This result is in agreement with our previous work\(^{13}\) which showed that unlike N_{2}-fixing heterotrophic bacteria, *isiB* is not a *nif* gene in cyanobacteria.

UPSRSs of six genes belonging to the functional category of “biosynthesis of cofactors, prosthetic groups and carriers”, which are mainly related to the oxidative stress defence, were also able to bind both FurA and NtcA. It is noteworthy that the *trxA* gene, in addition to the NtcA-box, contains two binding sites for FurA, and one of them exhibits the best score and *p*-value of all the UPSRs scanned in this work (Table 1).

Gel-shift assays showing the interaction of FurA and NtcA with the most relevant UPSRs are presented in Figure 6. NtcA binds to P*_{furB}* and P*_{furC}* although a different pattern was observed with respect to P*_{furA}*. In accordance with our com-
putational predictions, EMSA assays indicate that FurA and NtcA display lower affinity for P_{furB} than for the other fur UPSRs. The interaction between NtcA and P_{furB} yields a single protein–DNA complex, while three discrete complexes are observed in the binding assay of NtcA to P_{furA}. In the case of P_{furC}, the binding to NtcA produces a faint band and a smear, indicative of a continuous association– dissociation of the complex in the gel.

Other genes whose expression has been related to the nitrogen availability in the culture, also seem to be modulated by both regulators, such as sigC, hanA and the alr1690-2foutA message. However, we failed to detect binding of NtcA to the dpsA UPSR even if this DNA-binding protein is overexpressed under nitrogen starvation.35,39

Dual recognition of the xisA and hanA UPSRs, whose expression is required at the first stages of heterocyst differentiation40,41 was also detected by EMSA. Brusca et al.42 identified a regulatory region upstream xisA that blocks its expression in vegetative cells. This region contains part of the NtcA-protected area determined by DNase I footprinting assay, and it also includes sequences more proximal to the xisA open reading frame that are involved in the regulation of xisA.43 We have identified five putative Fur-binding sites, two of them located inside of this regulatory region (Figure 7). Although those binding sites were not detected in the computational analysis when the highest cut-off was chosen, previous results strongly indicate that FurA binds to the UPSR of xisA. The interaction of FurA with P_{xisA} has been confirmed by EMSA assays, pointing to a dual-regulation of this excisase. A similar situation occurs with the regulatory region of hanA, which encodes a histone-like DNA-binding protein essential for the initiation of heterocyst differentiation. The hanA promoter contains two low-scoring iron boxes, and binding of FurA and NtcA was clearly detected by EMSA analysis (Figure 6).

Binding of NtcA to the UPSRs of several genes that have been proposed to contain putative NtcA-binding sites (e.g. nifJ1 or trxA), is also shown in Figure 6. Moreover, it is noticeable that the interaction of FurA occurs with both, NtcA-activated promoters, such as P_{xisA} and P_{sigC}, and NtcA-repressed promoters, such as P_{rbcL} and P_{gor}.

**Discussion**

A tight coordination of regulatory networks is critical for providing cells the resources they need to carry out their metabolism efficiently and face changes in environmental conditions.

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**Figure 6.** Binding of 500 nM FurA and 500 nM NtcA to the UPSRs of furA, furB, furC, ntcA, xisA, sigC, nifJ1, trxA, rbcL, gor, hanA and gltS. A DNA fragment of exon IV from the human apoE was used as competitor to demonstrate that the interaction with each UPSRs is specific.
Identification of Overlapping Genes in FurA and NtcA Regulons in Anabaena

Figure 7. Schematic representation of $P_{\text{xisA}}$ showing the regulatory region identified that contains the NtcA protected region (white box) and the two putative Fur binding sites (brackets). The limits of each sequence are indicated.

tent of iron found in many key proteins involved in nitrogen metabolism conditions this process to iron uptake. Accordingly, the existence of regulatory interactions between iron and nitrogen metabolisms, likely orchestrated by FurA and NtcA, could be anticipated.

There are excellent, full-genome studies concerning the response to iron availability in unicellular cyanobacteria. However, little is known about the transcriptional networks linking iron homeostasis to heterocyst development and nitrogen metabolism. Computational identification of candidate binding sites in regulatory regions is an essential tool in the reconstruction of regulatory networks. Phylogenetic footprinting has become one of the most popular approaches for identification of target sequences in bacterial promoters. We ruled out the use of phylogenetic footprinting in this work for two reasons: (1) the number of completely sequenced genomes from nitrogen-fixing cyanobacteria is small; and (2) the signature of Fur-binding sites determined experimentally in Anabaena is not as conserved as the consensus for other regulators, such as the NtcA-binding sites. Instead, we decided to analyze a lower number of genes that had been predicted as NtcA-regulated and append to our sample some genes that according to our previous work, or to their predicted roles could overlap both regulons. This approach, followed by experimental validation of the bioinformatic predictions provides reliable information about the set of genes coordinately regulated by FurA and NtcA.

We have previously reported that nitrogen status modulates the expression of furA. NtcA binds to the operators present in the UPSRs of furA and $alr1690-x$-furA, resulting in a strong enhancement of $P_{furA}$ transcription in the heterocyst. While NtcA would activate the expression of FurA in heterocysts, it seems to exert a negative regulation in vegetative cells, since FurA presents a higher expression in ntcA$^{-}$ and hetR$^{-}$ backgrounds than in the wild-type (Figure 1). This differential regulation could be exerted by means of several NtcA binding sites in the UPSRs of furA and $alr1690-x$-furA that would allow NtcA to either activate or repress target genes, depending on the status of the cell. Regarding the functional diversity of the genes that conform the NtcA regulon, it seems that modulation will be defined not only for the reserves of fixed nitrogen in the cell, but also for the availability of iron and the redox conditions, which are mainly determined by the photosynthetic activity, among other parameters.

It has been demonstrated that NtcA isolated from heterocysts, offering reducing conditions, exhibited a stronger affinity for DNA than the protein purified from vegetative cells. Moreover, NtcA is able to regulate gene expression by a redox-dependent mechanism. Our results showing that NtcA regulates transcription of $furA$ in a different manner in vegetative cells and in heterocysts could be explained by the stronger activation of NtcA in the heterocyst. However, the effect of other regulatory factors that may accumulate during the developmental process cannot be dismissed. It is worth noting that the amount of FurA from nitrogen-depleted cells is higher in a hetR background than that in wild-type cells, suggesting a down-regulation of FurA by HetR. Further work should be done to elucidate whether HetR exerts a direct regulation on $P_{furA}$ or activates the transcription of a FurA-repressing factor. Alternatively, the mechanism of this regulation could be based on the proteolytic activity of HetR, which could degrade a FurA-activating factor or proteolyze FurA itself. In addition to the regulatory interactions of FurA with NtcA and with HetR, there are several lines of evidence pointing to the participation of FurA in the control of the changes associated to heterocyst development. Bioinformatic predictions display a high scoring iron-box in the UPSR of prpJ, a protein phosphatase involved in the control of heterocyst maturation. Moreover, the ability of FurA to recognize the UPSRs regions of xisA, hanA, nifH and nifF2 links this regulator to the control of heterocyst differentiation at different stages.

Computational predictions and EMSA assays unravels reciprocal, regulatory interactions between FurA and NtcA. Therefore, one could expect that FurA and NtcA regulons share some elements. That is the case of glnA, encoding glutamine synthetase (GS). Northern assays demonstrate that iron availability influences the transcription of glnA. This influence is more evident in the CSE2 strain, which presents a different evolution of the glnA transcript under iron depletion. Those results sug-
gest that, apart from NtcA regulation, there are other factors modulating \( glnA \) transcription under iron starvation. A good candidate is FurA, since EMSA assays show that it binds to \( P_{glnA} \) in a metal-dependent fashion. Glutamine synthetase from \( \text{Anabaena variabilis} \) or \( \text{Prochlorococcus marinus} \) is inactivated and even degraded by reactive oxygen species. As iron deficiency leads to oxidative stress in cyanobacteria, \( \text{FurA} \) could play a role maintaining the level of \( glnA \) mRNA to ensure enough amount of protein under oxidative stress conditions.

The computational analysis of \( P_{glnA} \) using the PATSER program results in no high scoring putative binding sites for Fur. Nevertheless, a group of three statistically significant binding sites were found in the scan, despite not passing the high cut-off defined by us to rescue just the strong binding putative sites. All three predictions score high enough to pass the mean value, and two of them are sufficiently close to be considered as cooperatively interacting binding sites, which is a common feature of this transcription factor. It has been described that where the affinity for a given transcription factor is low, cooperative interaction with another factor placed at an appropriate distance on the \( \text{cis}-\text{regulatory} \) region can increase both complex stability and specificity for the protein–DNA interaction.

When we extend our search for co-regulated genes using bioinformatic approaches, we find that there is a significant number of common elements in FurA and NtcA regulons. UPSR regions from 28 genes interacted with both regulators. Some of the identified genes have been proposed as NtcA targets, despite this interaction has not been demonstrated experimentally. Among the genes studied, it is worthwhile mentioning the role of NtcA in the regulation of the \( \text{fur} \) family. Under nitrogen deprivation, levels of \( \text{furA} \) are enhanced in the heterocyst, whereas levels of \( \text{furB} \) and \( \text{furC} \) remain unaltered. EMSA assays suggest that NtcA could contribute to modulate the transcription of \( \text{furB} \) and \( \text{furC} \), although it does not seem to be the main regulator that controls their levels in the cell.

Interestingly, we also detected co-regulation of genes involved in key metabolic pathways such as photosynthesis or carbon metabolism. Among those genes, a dual-regulation of \( \text{petH} \) was expected on basis of our previous work. Linkage between photosynthesis and nitrogen metabolism has been proposed. The molecular mechanism for this linkage is based on the role of ferredoxin as electron donor for nitrate and nitrite reductase. Moreover, the important function that iron plays in photosynthetic electron transport is well-known.

With respect to the carbon pathways, it is accepted that carbon metabolism is controlled by its own regulators and also by the availability of nitrogen, iron, and other nutrients. Functional interactions between \( \text{Crc} \) and \( \text{Fur} \) have been characterized in heterotrophic bacteria, while the signal transducer \( P_{II} \) is one of the main nodes that integrate carbon and nitrogen metabolism. Binding of FurA to the regulatory region of \( P_{II} \) unravels a new connection in the coordinated regulation of cell metabolism.

The \( \text{sigC} \) gene lies upstream of \( \text{furA} \), and its transcription is induced specifically under sulfur or nitrogen deprivation. \( \text{SigC} \) takes part in a complex regulatory network that involves other group II \( \sigma \)-factors and cooperates in the modulation of several NtcA-dependent genes and NtcA itself. Binding of NtcA to the regulatory region of \( \text{sigC} \) states a reciprocal modulation of those transcription factors. Northern blot analysis failed to detect the \( \text{sigC} \) transcript under iron deprivation. However, using RT-PCR we were able to observe induction of \( \text{sigC} \) transcription in iron-starved cells (our unpublished results). In addition, the interaction of FurA with \( P_{\text{sigC}} \) links the regulatory network connecting the sigma genes to iron metabolism.

For some genes, such as \( \text{rbcL} \) (encoding a subunit of ribulose bisphosphate carboxylase/oxygenase), \( \text{gor} \) (encoding glutathione reductase), \( \text{gls} \) (glutamate synthase) or \( \text{hanA} \) (HU protein), NtcA appears to have a repressor role, since the corresponding products are expressed only in the presence of ammonium or are absent in heterocysts. For \( \text{rbcL} \) and \( \text{gor} \) genes, NtcA binds to NtcA-repressor sites located in the UPSR of those genes. Here we demonstrate that NtcA also binds to the UPSR region of \( \text{hanA} \) and \( \text{gls} \), supporting the hypothesis that NtcA modulated their transcription. Prediction of Fur boxes and EMSA assays show that FurA binds to the UPSR of those NtcA down-regulated genes. More likely, the participation of NtcA and FurA in the modulation of those genes will be related to growth conditions.

To cope with changing environmental conditions, bacteria have to sense the nutrients available and adapt their metabolism to the new situation. The existence of interconnections among the different metabolic routes is crucial for the coordination of cell response. In summary, our genetic, computational and EMSA results provide strong evidence for the existence of a cross-talk between two regulatory networks, one governing iron incorporation and the other controlling nitrogen metabolism, including heterocyst development. These regulatory interactions involve several alternative sigma factors, as well as genes related to photosynthesis, carbon metabolism and the oxidative stress defence. This cooperation between transcription factors conforms a general network that integrates regulatory stimuli for an optimal adaptation to different environmental conditions.

**Materials and Methods**

**Strains and culture conditions**

This study was carried out with the filamentous heterocyst-forming cyanobacterium \( \text{Anabaena} \) sp. (also known as \( \text{Nostoc} \) sp.) strain PCC 7120, and two Het\(^{-}\) derivatives: the strain CSE2, an insertion mutant of the
Anabaena sp. strain PCC 7120 and strain CSE2 was isolated as explained using 40 ml of cells containing ca. 5 μg of chlorophyll a ml⁻¹. Northern analyses were performed using 40 μg of RNA as described. DNA probes for Northern blotting were obtained by PCR amplification of the furA and glnA sequences using the primers listed as Supplementary Data in Table S2. Transcripts were quantified using the Cyclone Storage Phosphor System (Packard).

Computational identification of Fur and NtcA putative binding sites

A position-specific weight matrix was constructed for Fur starting from a set of 16 binding sites obtained from the experimentally characterized binding sites in Anabaena sp. 7120 for this transcriptional regulator. These sequences were used as training set for the CONSENSUS algorithm using a variable matrix width and leaving the program free to select the number of significant sequences to be included in the matrix. Matrices from different widths were filtered out by their expected frequency, and the resulting p-values suggested a matrix of width 29 was significant. The matrix obtained for Fur in this cyanobacterium is rather similar in base distribution and positional information content to the matrix obtained for this transcription factor in E. coli and other gamma-proteobacteria.

The matrix constructed for Fur was used to scan the regulatory regions of 123 genes, 108 of which have been found to bear binding sites for NtcA⁴⁴ and the other 15 were likely to be under the control of these two transcription factors. The complete genome sequence and the gene description table were obtained from the Cyanobase website. The UPSRs were defined from −400 to +50 with respect to putative translation initiation. The sequence scan was performed with the PATSER program from the CONSENSUS package. A strong cut-off, i.e. mean, plus one standard deviation from the mean, was defined to rescue the most relevant putative sites.

Putative binding sites for NtcA were identified using a simple pattern matching search with the regular expression (GTA[N₆]TAC)² and allowing as much as two mismatches in the flanking triplets.

Electrophoretic mobility shift assays

DNA fragments containing the upstream sequences of each gene were obtained by PCR using the primers listed in Supplementary Data. The sequences were processed with a Gel Doc 2000 Image Analyser from BioRad.

Acknowledgements

This research was supported by grant BFU2006-03454/BMC (Programa nacional de I+D, Ministerio de Educación y Ciencia, Spain). We thank Dr A. Válladares for the kind gift of P glnA fragments, L. González for PCR amplification of several UPSRs, Dr J. Sancho for helpful discussion, Drs E. Flores and A. Herrero for the kind gift of hetR and CSE2 strains. V. E.A. acknowledges the support given to him by the Red Iberoamericana de Bioinformática (RIBIO rt VIII), CYTED, Banco Santander Central Hispano, Fundación Carolina and Universidad de Zaragoza.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2007.09.010

References


