Expression of *furA* is modulated by NtcA and strongly enhanced in heterocysts of *Anabaena* sp. PCC 7120

S. López-Gomollón,^{1,2†} J. A. Hernández,^{1†‡} C. P. Wolk,³ M. L. Peleato^{1,2} and M. F. Fillat^{1,2}

Correspondence
M. F. Fillat
fillat@unizar.es

¹Department of Biochemistry and Molecular and Cell Biology, University of Zaragoza, Zaragoza, Spain

²Biocomputation and Complex Systems Physics Institute (BiFi), University of Zaragoza, Zaragoza, Spain

³MSU-DOE Plant Research Laboratory and Department of Plant Biology, Michigan State University, East Lansing, MI, USA

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Fur (ferric uptake regulator) proteins are principally responsible for maintaining iron homeostasis in prokaryotes. Iron is usually a scarce resource. Its limitation reduces photosynthetic rates and cell growth in cyanobacteria in general and especially in cyanobacteria that are fixing dinitrogen, a process that requires the synthesis of numerous proteins with a high content of iron. This paper shows that in the diazotrophic cyanobacterium *Anabaena* sp. strain PCC 7120, levels of *furA* mRNA and FurA protein increase significantly in response to nitrogen deprivation, and that *furA* up-regulation takes place specifically in proheterocysts and mature heterocysts. Great differences in a Northern blot, probed with *furA*, of RNA from an *ntcA* mutant relative to wild-type *Anabaena* sp. were attributable to binding of NtcA, a global regulator of nitrogen metabolism, to the promoter of *furA* and to the promoter of the *furA* antisense transcript *alr1690- α -furA*.

INTRODUCTION

Iron is an abundant element in the Earth's crust. However, due to the low solubility of iron(III) at physiological pH, iron limitation is one of the most common stresses in nature. Photosynthetic organisms need large amounts of iron, because iron is a principal component of the cofactors of proteins that are involved in photosynthesis and nitrogen metabolism. As a consequence, the bioavailability of iron is a major determinant of primary photosynthetic productivity in oceans (Falkowski *et al.*, 1998). In cyanobacteria, iron availability critically influences rates of photosynthesis and nitrogen fixation (Straus, 1994; Kustka *et al.*, 2002). To overcome iron stress, prokaryotes have evolved a number of responses, most of them under genetic control by the Fur (ferric uptake regulator) proteins. These global regulatory proteins are involved in the maintenance of iron homeostasis. They recognize specific DNA sequences denoted

iron boxes, and coordinate iron metabolism with cellular defences against several stresses (Andrews *et al.*, 2003). It is thought that Fur proteins act as classical repressors. Under iron-rich conditions, Fur dimers complexed with iron(II) bind to iron boxes, preventing transcription (Bagg & Neilands, 1987). Fur proteins are constitutive, and are highly abundant compared to other regulators (Zheng *et al.*, 1999). The genome of *Anabaena* sp. strain PCC 7120 (hereinafter, *Anabaena* sp.) encodes three Fur homologues. FurA (the product of ORF *all1691*) shows the highest homology with Fur from heterotrophic bacteria (Hernández *et al.*, 2004a) and is an essential protein in cyanobacteria (Hernández *et al.*, 2006a). The amount of active FurA in *Anabaena* sp. responds to a complex balance between iron availability, redox status of the cell and expression of the antisense RNA α -*furA*, among other effectors (Hernández *et al.*, 2004a, 2006a).

In the absence of combined nitrogen, *Anabaena* sp. is able to reduce atmospheric dinitrogen to ammonium in a process that involves activation of numerous genes. Since nitrogenase is very sensitive to oxygen, nitrogen-fixing cyanobacteria have developed a variety of strategies to allow oxygenic photosynthesis and nitrogen fixation to take place simultaneously. In *Anabaena* sp., nitrogen fixation is confined to specialized cells called heterocysts. To protect nitrogenase

†These authors contributed equally to this work.

‡Present address: Plant and Microbial Biology Department, 211 Koshland Hall, University of California at Berkeley, 94720 Berkeley, CA, USA.

Abbreviation: EMSA, electrophoretic mobility shift assay.

A supplementary figure showing the construction of plasmids is available with the online version of this paper.

from inactivation by oxygen, the heterocyst lacks photosystem II activity, has an elevated rate of respiration and has a thick envelope that minimizes the influx of O₂, thus creating a micro-oxic intracellular environment (Wolk *et al.*, 1994). Some proteins involved in heterocyst development and metabolism, including PatB (Liang *et al.*, 1993), FdxH (Böhme & Haselkorn, 1988) and the constituent proteins of nitrogenase and of the up-regulated respiratory apparatus, are rich in iron. Therefore, the differentiating cells must use much of their iron reserves to construct the machinery required for nitrogen fixation and maintenance of micro-oxic internal conditions.

Surprisingly little is known about the regulation of nitrogen fixation genes. NtcA, the principal transcription factor involved in the regulatory network of nitrogen metabolism, belongs to the CAP family (Herrero *et al.*, 2001). NtcA helps to regulate the initiation and progression of heterocyst differentiation, and is also involved in the activation of numerous genes involved in nitrogen assimilation. NtcA senses the nitrogen status in cyanobacteria via 2-oxoglutarate (Laurent *et al.*, 2005) and positively regulates genes by binding to their promoter regions at sites that exhibit the consensus sequence GTAN₈TAC. By binding to target sites that are located downstream of the transcription start point (*tsp*), NtcA can also play a role as repressor (Herrero *et al.*, 2001; Muro-Pastor *et al.*, 2001). It has been proposed that NtcA responds to redox changes (Jiang *et al.*, 1997; Alfonso *et al.*, 2001). Moreover, NtcA may coordinate iron acquisition and nitrogen metabolism by activating the expression of *pkn41* and *pkn42* (Cheng *et al.*, 2006).

We show here that the nitrogen status of a culture contributes to the regulation of the expression of *FurA*. Nitrogen deprivation strongly activated *P_{furA}* in proheterocysts and heterocysts. In contrast, fusions of reporter genes to *P_{furB}* and *P_{furC}* showed no changes in expression in response to nitrogen deprivation and heterocyst differentiation. NtcA, in addition to other factors previously reported (Bes *et al.*, 2001; Hernández *et al.*, 2004a, 2006a), is involved in the regulation of expression of *furA*, as illustrated by great differences in the transcriptional pattern of the *furA* locus in

an *ntcA* mutant. These results suggest that *FurA* plays a significant role in the metabolism of heterocysts.

METHODS

Strains and culture conditions. The present study was carried out with the heterocyst-forming cyanobacterium *Anabaena* (*Nostoc*) sp. strain PCC 7120 and its *ntcA* derivative, strain CSE2 (Frias *et al.*, 1994). Cells were grown at 30 °C in nitrogen-free medium BG-11₀ or in BG-11 medium supplemented with 6 mM NH₄Cl plus 12 mM TES/NaOH buffer (pH 7.5) (Rippka, 1988). Cultures of CSE2 were supplemented with 2 µg ml⁻¹ each of streptomycin and spectinomycin. *Escherichia coli* strains DH5α and BL21-Gold DE3 grown in Luria-Bertani liquid or agar medium at 37 °C were used for DNA manipulations. Media were supplemented with appropriate antibiotics according to standard protocols (Sambrook *et al.*, 1989). To place cultures under nitrogen-stress conditions, cells from a nitrogen-replete medium were washed three times at room temperature with, and resuspended in, BG11₀C medium (i.e., BG11₀ supplemented with 10 mM NaHCO₃), and incubated under growth conditions for the times indicated.

RNA isolation and Northern analysis. *Anabaena* sp. RNA was prepared as described previously (Hernández *et al.*, 2006a), using 40 ml cells containing ~5 µg chlorophyll *a* ml⁻¹. Northern blot analysis was performed using 40 µg RNA, as described by Hernández *et al.* (2006a). DNA probes for Northern blotting were obtained by PCR amplification of the *furA* sequence using the primers listed in Table 1. Transcripts were quantified using the Cyclone Storage Phosphor System (Packard).

Construction of reporter vectors and visualization of GFP expression. The promoter regions of *furA*, *furB* and *furC*, amplified according to Hernández *et al.* (2004a), were cloned as 338, 457 and 413 bp fragments into pGEM-T (Promega), producing pCZS3, pCZS4 and pCZS5, respectively. Promoterless *gfpmut2* was cloned as a 911 bp *XbaI*-*PstI* fragment from pGFPM2 (Cormack *et al.*, 1996) between the *SpeI* and *PstI* sites of pCZS3, pCZS4 or pCZS5, producing pCZS9, pCZS10 and pCZS11, respectively (see supplementary Fig. S1, available with the online version of this paper). Digestion of the resulting constructs with *SacI* and *PstI* released *P_{fur}-gfp* on fragments that were ligated into *SacI*- and *PstI*-digested pRL2696, producing pCZS25, pCZS26 and pCZS27, respectively. pRL2696 consists of the RSF1010-based vector pRL1383a (Fan *et al.*, 2005) into whose unique *SalI* site is inserted cassette C.C1 from pRL178 (Elhai & Wolk, 1988a). Control plasmid pCZS20 (Fig. S1) was constructed by cloning promoterless *gfp* as an *XbaI*-*PstI* fragment from pGFPM2 into pRL2696 digested with *XbaI* and *PstI*. pCZS20,

Table 1. Oligodeoxyribonucleotides used in this study

No.	Sequence (5'–3')	Description
1	ATTACCATGCGTGTCTACAC	<i>furA</i> sense strand*
2	CTTGTAAGCTTACCAGGTGC	<i>furA</i> antisense strand†
3	CTCGCCTAGCAATTTAACAAC	389 bp and 307 bp <i>P_{furA}</i> coding strand
4	GCCTTGAGCGAAGTATTTGTG	389 bp <i>P_{furA}</i> complementary strand
5	GAGTGATTTGCAACAAACATTGC	307 bp <i>P_{furA}</i> complementary strand
6	GCGAGAAGAAATTGCCAGAG	<i>P_{alr1690-α-furA}</i> RNA coding strand
7	TAGGCGAAAGCTAGGTGCAA	<i>P_{alr1690-α-furA}</i> RNA complementary strand

*Corresponds to bp 2020532–2020513, modified to include an *NcoI* site (Bes *et al.*, 2001).

†Corresponds to bp 2020051–2020071, modified to include a *HindIII* site (Bes *et al.*, 2001).

pCZS25, pCZS26 and pCZS27 were introduced into *Anabaena* sp. by conjugation (Elhai & Wolk, 1988b). Photographs were taken with a Nikon DXM1200F digital camera mounted on a Nikon Eclipse50i microscope. GFP expression was visualized by fluorescence microscopy, with a Nikon Epi-fluorescence filter B-2A (EX 450-490, DM 505, BA520). Images were captured with ACT-1 and edited with Adobe Photoshop 7.0.

Electrophoretic mobility shift assays (EMSAs). DNA fragments of 389 and 307 bp that contain the promoter regions of *furA*, and a 420 bp fragment with the promoter of the *alr1690- α -furA* RNA transcript (Table 1), were amplified by PCR and used in non-radioactive band-shift assays, as described previously (Bes *et al.*, 2001). Binding assays were carried out in a final volume of 20 μ l containing 10 mM BisTris/HCl (pH 7.5), 40 mM KCl, 1 mM dithiothreitol, 0.1 mM MgCl₂, 0.05 μ g bovine serum albumin μ l⁻¹, 75–100 ng of the DNA fragment to be tested, 75 ng of a control DNA fragment and 5% (v/v) glycerol. The DNA fragment used as unrelated, control DNA was a 228 bp fragment from human *apoE* (Bes *et al.*, 2001). Assays were carried out with 5 pmol histidine-tagged NtcA purified from *E. coli* cells bearing pCSAM70 (Muro-Pastor *et al.*, 1999). Results were processed with an Imagestore 5000 image analyser (UVP Inc.).

DNase I footprinting. The DNase I protection assay used was modified from that described previously (Frías *et al.*, 2000). The PCR product containing the promoter region of *furA* used in the EMSAs was cloned into pGEM-T and sequenced to verify the fidelity of the amplification. The fragment excised using enzymes *Nco*I and *Pst*I was 3'-end-labelled with the Klenow fragment of DNA polymerase and [α -³²P]dCTP (3000 Ci mmol⁻¹; 111 TBq mmol⁻¹). Binding reactions were performed in a volume of 20 μ l, with incubation of 0.05 pmol labelled promoter with 1 μ M purified NtcA in the presence of 1.5 μ g salmon sperm DNA as non-specific competitor. Radioactive bands were visualized with a Cyclone storage phosphor system and OptiQuant image analysis software (Packard).

Electrophoresis and immunoblotting. Crude extracts were prepared by ultrasonic treatment, followed by centrifugation to remove cell debris. Protein was quantified using a bicinchoninic acid protein

assay reagent (Pierce). Samples (25 μ g) of total protein were separated by SDS-PAGE (17% polyacrylamide gel). For immunoblotting, proteins were transferred by electrophoresis to PVDF (0.45 mm pore size transfer membrane; Waters), as described previously (Hernández *et al.*, 2006a). Rabbit polyclonal antibodies raised against *Anabaena* sp. FurA protein were used, and the blot was visualized and quantified with a Gel Doc 2000 image analyser (Bio-Rad).

RESULTS

Influence of nitrogen status on expression of *furA*

Since nitrogen-fixing conditions strongly increase iron demand in cells, we investigated the influence of nitrogen status on the expression of *furA*. Northern analysis of *furA* and *alr1690- α -furA* transcripts was performed using RNA from cultures grown with ammonium and then deprived of nitrogen. The results shown in Fig. 1(a) indicate that under nitrogen-fixing conditions, the abundance of the 0.6 kb *furA* transcript increased $\sim$ 2.3-fold with respect to the level of message detected in the presence of ammonium, whereas the amount of the 2.2 kb *alr1690- α -furA* RNA decreased by 30%. As a result, FurA expression increased, as detected by Western blot analysis (Fig. 1c).

NtcA affects expression of *furA*

We sought to identify possible NtcA-binding sequences in the promoter regions of *furA* and of *alr1690- α -furA*. The signature GTAN₈TAC, usually flanked by A/T nucleotides, is strongly conserved in the promoters of NtcA-activated genes. However, the promoters of a number of nitrogen-

Fig. 1. (a, b) Northern blot analysis of RNA from cultures of (a) *Anabaena* sp. PCC 7120 (WT) and (b) CSE2 grown in the presence of ammonium and deprived of fixed nitrogen for 3, 6, 9 and 24 h. Total RNA (40 μ g) was hybridized with the *furA* gene, and the *rnpB* probe was used as loading and transfer control. Approximate sizes of the observed bands of RNA are indicated. (c) Western blot of FurA in *Anabaena* WT and CSE2 cells grown with ammonium or, for the time indicated in h, in the absence of fixed nitrogen.

assimilation genes have been reported that resemble, but do not match, the canonical consensus (Herrero *et al.*, 2001) shown in Fig. 2. Promoters of *furA* and of *alr1690- α -furA* have been defined previously by determination of their transcription start points (Hernández *et al.*, 2006a, b). Sequences present in the promoter regions of *furA* and *alr1690- α -furA* that are similar to the canonical targets of NtcA are shown in Fig. 2. P_{furA} exhibits three imperfect NtcA sites centred at bp -115.5 , -92.5 and -40.5 with respect to its *tsp*. A fourth sequence that matches the NtcA-binding consensus includes the translation start codon and part of the coding sequence. Similarly, the *alr1690- α -furA* promoter contains two sequences, at -146.5 and $+0.5$, that resemble NtcA-binding sites. The upstream sequences are similar to NtcA-activated NtcA-boxes, whereas the downstream sequence, which includes the *tsp*, could, depending on the nitrogen status of the cell, bind NtcA and act as a repressor. To verify whether NtcA is responsible for the increase in the expression of *furA* under conditions of nitrogen deprivation, we carried out Northern and Western analyses of cultures of the CSE2 strain subjected to nitrogen step-down. In all cases tested, Northern analysis of CSE2 cells grown in the presence of ammonium showed higher transcription of the *furA* message than that in the wild-type strain, in agreement with the amounts of FurA detected by Western analysis, which were around 1.3- to 1.5-fold higher in the CSE2 strain (lanes NH_4^+ from Fig. 1c). However, changes in the expression of FurA in CSE2 cultures in response to nitrogen step-down were much less than those in the wild-type strain. In the *ntcA* mutant, the 2.2 kb transcript dropped dramatically, while a 0.35 kb message appeared. Because non-specific hybridization of the *furA* probe with *furB* and *furC* homologues has been ruled out (Hernández *et al.*, 2006a), the 0.35 kb message may result from processing of the *alr1690- α -furA* transcript in CSE2 cells. Alternatively, transcription of *alr1690- α -furA* may be

repressed in the *ntcA* mutant, while a new 0.35 kb message is transcribed.

NtcA binds to P_{furA} and $P_{alr1690- α -furA}$

EMSA were carried out to test the ability of purified NtcA to bind to the regions upstream of *furA* and *alr1690- α -furA*. Since the region of P_{furA} that showed the highest homology to the canonical NtcA box contains the translation start point, binding assays were performed with two DNA fragments, P_{furA1} , which spans positions -331 to $+58$ with respect to the *tsp*, and a shorter fragment, P_{furA2} , which spans positions -331 to -25 and lacks the ATG. When NtcA was incubated with either P_{furA1} or P_{furA2} at least two NtcA-DNA complexes were detected (Fig. 3, lanes 3 and 6). Mobility-shift assays of $P_{alr1690- α -furA}$ showed that both FurA and NtcA bind to this DNA. FurA shifted only a small amount of the promoter fragment, whereas two NtcA- $P_{alr1690- α -furA}$ complexes were clearly observable.

As noted above, NtcA could act as an activator or as a repressor of transcription, depending on the location of the binding site. Because three putative NtcA boxes were identified in P_{furA} , as well as a putative site covering the translation start, DNase I footprinting assays were performed in order to clarify the role of NtcA in the modulation of expression of *furA*. Fig. 4(a) shows several NtcA-protected regions of nucleotides located in two main regions of P_{furA} , approximately from nt -122 to -100 and from nt -47 to nt $+57$. The protected nucleotides are identified in Fig. 4(b). All are located in the putative NtcA-binding sites depicted in Fig. 2. The protected G and C located at -47 and -34 bp, respectively, relative to the *tsp* are located in the imperfect NtcA binding site centred at -40.5 . This site forms a perfect palindrome and is

	Position of GT with respect to the <i>tsp</i>
P_{furA}	
AATAATAAGCATTGTTACTAGG	-122
TTTTGTAGGTGATTTTCACTAA	-99
ATAAGCAATGTTTGTTCGCAAT	$\text{N}_{16}\text{TAN}_3\text{T}$ -47
ATTAGTATGACTGTCATCAAA	+25
$P_{alr1690-\alpha-furA}$	
ATGTGTAGAAGACTATATGTAT	-153
AAATGTGAATATAGAAACACTT	-6
TTGCGTAATTTAGTACATCAAG	+23
Consensus (Herrero <i>et al.</i> , 2001)	
WWYWGTANCARNWRNTACA	WW

Fig. 2. Putative NtcA-binding motifs found in P_{furA} and $P_{alr1690- α -furA}$.

Fig. 3. Binding of FurA and NtcA to the P_{furA} and $P_{alr1690- α -furA}$ promoters. Mobility-shift assays were carried out using 500 nM recombinant His-tagged NtcA (Muro-Pastor *et al.*, 1999). All of the assays were performed in the presence of 100 μM Mn^{2+} and 1 mM DTT. Lanes: 1, free P_{furA1} (389 bp); 2, P_{furA1} with 500 nM FurA; 3, P_{furA1} (389 bp) with 500 nM NtcA; 4, free P_{furA2} (307 bp); 5, free P_{furA2} (307 bp) with 500 nM FurA; 6, free P_{furA2} (307 bp) with 500 nM NtcA; 7, free $P_{alr1690- α -furA}$; 8, $P_{alr1690- α -furA}$ with 500 nM FurA; 9, $P_{alr1690- α -furA}$ with 500 nM NtcA. Protein-DNA complexes are indicated with arrows.

Fig. 4. (a) DNase I footprinting of the *furA* promoter incubated with 1 μ M NtcA. The 389 bp fragment (0.05 pmol) containing the promoter region of *furA* was labelled in the non-coding strand, bound to NtcA and digested with DNase I. Black triangles show the NtcA-protected sites. (b) Sequence of *P_{furA}* showing the bases protected by NtcA (bold letters) and the sequences of the corresponding putative NtcA-binding sites (grey boxes). Iron boxes deduced by the DNase I footprinting assay with FurA (Hernández *et al.*, 2006b) are indicated with dotted lines. The transcription start point and the ATG codon are underlined. The positions of the oligonucleotides used to amplify both the 389 bp fragment of the *furA* promoter (Table 1, rows 3 and 4) and the 307 bp, truncated version (Table 1, rows 3 and 5) are indicated with arrows.

accompanied by the motif TAN₃T 20 nucleotides downstream of the NtcA signature, in good accord with the location of such a motif relative to canonical NtcA-activated promoters. This site is part of one of the two regions protected by FurA in DNase I footprinting experiments described by Hernández *et al.* (2006b). Perhaps this part of the promoter is occupied either by FurA or by NtcA depending on the relative abundance of the pools of iron and combined nitrogen. Binding of NtcA to nucleotides that span the translation start site would allow down-regulation of expression of *furA* under certain conditions. Such binding may account for the observation that in ammonium-containing media, where only vegetative cells are present, strain CSE2 produces more FurA than do cells of wild-type *Anabaena* sp. Our *in vitro* results show that NtcA can recognize and bind to NtcA-like signatures present in *P_{furA}*.

To test whether transcription of *furA* may be promoted by NtcA at a site other than the canonical *tsp* located at -26 nt from ATG (Hernández *et al.*, 2006b), primer extension studies were carried out using the RNAs isolated from cultures of both *Anabaena* sp. and the CSE2 strain grown both in the presence of ammonium and submitted to nitrogen step-down. In all cases tested, only the *tsp* previously reported was detected (not shown).

***furA* is strongly induced in proheterocysts and heterocysts**

To find out whether the increase in the expression of *furA* in the absence of fixed nitrogen was a general response produced throughout filaments of *Anabaena* sp. or was restricted to the heterocysts, expression from *P_{furA}* was investigated using Gfp as reporter (Fig. 5). Because nitrogen status does not affect transcription levels of *furB* and *furC* (as determined by Northern analysis; data not shown), we used, as controls, not only the plasmid pCZ20 which bears a 'non-promoted *gfp*', but also pCZ20-based vectors in which *P_{furB}* and *P_{furC}* were added to drive *gfp*. Recombinant *Anabaena* sp. bearing pCZS20, pCZS25, pCZS26 or pCZS27 was examined by fluorescence microscopy. The experiments were performed three times with independently grown cultures. Mature heterocysts were observed by light microscopy in recombinant *Anabaena* sp. strains 24 h after nitrogen step-down. We first examined the expression of *P_{furA}*. When cells were grown in the presence of ammonium, a faint fluorescence due to the expression of *furA* in vegetative cells was detected (Fig. 5a). A slight induction of Gfp from *P_{furA}* at regular intervals along the filament was observed after 15 h of nitrogen deprivation. Fluorescence reached its maximum by 24 h and remained approximately constant in mature heterocysts for at least 6 more days of nitrogen deprivation. Thus, *furA* is induced in proheterocysts and remains stably expressed in mature heterocysts. Fig. 5(c) shows that expression of Gfp from *P_{furC}* was nearly the same in heterocysts and vegetative cells. We were unable, at the same level of sensitivity, to detect expression of *P_{furB}* by fluorescence microscopy (Fig. 5b),

Fig. 5. Bright-field and fluorescence micrographs of *Anabaena* sp. (a) Cells bearing pCZS25 grown in BG11 supplemented with ammonium and deprived of fixed nitrogen for 15 h and 24 h. (b–d) Cells bearing (b) pCZS26, (c) pCZS27 or (d) pCZS20 deprived of fixed nitrogen for 4 days. Scale bars, 10 μm . White arrows, proheterocysts; black arrows, mature heterocysts. Images were photographed with the same settings to allow comparisons of intensities of fluorescence.

but by increasing the sensitivity, we could detect faint fluorescence that was also of nearly equal intensity in the two types of cells. Because no *gfp*-based fluorescence was observed with pCZS20 (which contains promoterless *gfp*; Fig. 5d), the observed fluorescence resulted from the activity of the *fur* promoters.

DISCUSSION

Regulation of Fur proteins has been studied extensively in several micro-organisms and in all of them the modulation of this global regulator is rather complex. In *Anabaena* sp., the expression and activity of FurA are controlled at every step of the flow of genetic information. At the transcriptional level, *furA* is auto-regulated and likely modulated by FurB and FurC. Post-transcriptionally, the *alr1690- α -furA* antisense RNA interferes with the *furA* transcript. *In vitro* analysis shows that binding of FurA to its DNA targets is

modulated by several parameters such as the redox potential, the availability of metal, and the presence of haem (Hernández *et al.*, 2006b, 2004b).

In this work, we show that nitrogen status also modulates the expression of *furA*. The use of a Gfp reporter fused to the promoters of the three *fur* homologues showed that P_{furA} is strongly activated in proheterocysts and remains over-expressed in the mature heterocyst. However, no changes were observed in the levels of expression of *furB* and *furC*. NtcA binds to P_{furA} and to the promoter of the dicistron *alr1690- α -furA*, whose transcription is severely affected in an *ntcA* mutant. A search for NtcA boxes in the promoter regions of both messages showed the presence of several putative NtcA-binding sites. Binding and footprinting assays showed that, *in vitro*, NtcA can bind to sites in both promoters. Similar observations have been made with the NtcA-dependent promoters of *nir* and *xisA* (Chastain *et al.*, 1990; Frías *et al.*, 2000). Like other regulators of the

CAP family, NtcA appears to act either as an activator or as a repressor depending on the metabolic status of the cells (Kolb *et al.*, 1993; Herrero *et al.*, 2001). In the case of *alr1690- α -furA*, NtcA would act as a repressor of the *alr1690- α -furA* message if it binds to the box that covers the *tsp* of this dicistron (Fig. 2). However, binding of NtcA to the site centred at -146.5 would activate transcription of that message. The structure of P_{furA} , which shows three imperfect NtcA-binding sites centred at -115.5 , -92.5 and -40.5 , is more complex. The last of these three NtcA signatures is located at the canonical distance observed in class II CAP-activated promoters (Busby & Ebright, 1997) and overlaps with the site II of FurA-protected regions (Hernández *et al.*, 2006b). The convergence of several transcriptional regulators in the promoter region of *fur* has been demonstrated in several micro-organisms (Zheng *et al.*, 1999; Fuangthong *et al.*, 2002). In *E. coli*, in addition to autoregulation by its product, transcription of *fur* is modulated by OxyR and SoxRS, the primary regulators of responses to oxidative stress (Zheng *et al.*, 1999). Moreover, transcription of *fur* is stimulated by the cAMP–CAP system (De Lorenzo *et al.*, 1988), and tight functional interactions between Fur and CRP have been confirmed using whole-transcriptome approaches (Zhang *et al.*, 2005).

In cyanobacteria, many iron-responsive genes, such as *nifH*, *nblA*, *petH*, and the putatively kinase-encoding genes *pkn41* and *pkn42*, are also modulated by NtcA (Valladares *et al.*, 1999; Luque *et al.*, 2001; Cheng *et al.*, 2006), suggesting that NtcA plays a key role in the coordination of iron acquisition and nitrogen metabolism. Interaction of FurA and NtcA at their overlapping binding sites in P_{furA} would result in integration of metabolic signals that involve C/N ratio, redox status, iron availability and/or ATP levels. We hypothesize that the enhanced expression of *furA* in the heterocysts of wild-type *Anabaena* sp., compared with the negligible increase in expression of the regulatory gene in the *ntcA* strain CSE2, in response to nitrogen step-down may be due to a synergistic effect of NtcA and other heterocyst-specific factor(s) or to the higher activity of NtcA exhibited in those differentiated cells (Ramasubramanian *et al.*, 1994). If, as we imagine, there is little free iron in the heterocyst, FurA would be released from its own promoter, thereby allowing binding of NtcA, whose activity in the heterocyst could be enhanced by increased levels of 2-oxoglutarate (Herrero *et al.*, 2001).

It has been shown that *Anabaena* sp. responds to nitrogen deprivation in several phases. First, if it was previously grown with ammonium, those of its genes repressed by ammonium ions are de-repressed (Cai & Wolk, 1997). Second, genes required for heterocyst differentiation, or related to the structure and function of heterocysts, are up-regulated in proheterocysts (Black *et al.*, 1993; Wolk *et al.*, 1993; Yoon & Golden, 2001). Finally, other genes related to heterocyst function are up-regulated, again not all at the same time (Ehira *et al.*, 2003; Ehira & Ohmori, 2006). FurA, which seems to belong to the second group of genes, could play diverse

roles in those differentiating cells. Although formation of the glycolipid layer of the heterocyst envelope presumably blocks the entry of ions directly from the medium, heterocysts continue to interact with vegetative cells, and so may have continued access to supplies of iron from vegetative cells. If developing heterocysts are deficient in free iron due to the large amount of iron required by the electron carriers that are involved in nitrogen fixation, it appears unlikely that FurA would serve only as a repressor in those cells.

In heterotrophic bacteria, under certain stress conditions, Fur proteins activate transcription of several genes involved in cell defence (Lee *et al.*, 2004). Accordingly, because the nitrogenase components are highly sensitive to oxygen, FurA may coordinate the availability of iron with responses to potential oxidative stress. Early activation of FurA in the proheterocyst when the heterocyst envelope is not yet completely formed may support this idea. Because heterocysts are not completely anoxic, we hypothesize that the low pO_2 in the heterocyst could activate the expression of enzymes that are involved in a concerted defence against oxidative stress. This hypothesis could receive support from the presence of catalase and superoxide dismutase in the heterocyst (Wolk *et al.*, 1994; Li *et al.*, 2002). Alternatively, FurA could exert a protective function in a heterocyst similar to that of DpsA (Peña & Bullerjahn, 1995). Although further studies have to be done to elucidate the role of FurA in the heterocyst, our results suggest that FurA is a principal node in the complex regulatory network that links iron and nitrogen-metabolic pathways and a key protein for the metabolism of the heterocyst.

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