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

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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 z-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
from inactivation by oxygen, the heterocyst lacks photosynthetic 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 GTAN₇TAC. 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 P₄₄furA in proheterocysts and heterocysts. In contrast, fusions of reporter genes to P₄₄furB and P₄₄furC 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-11 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 μM gfp 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, BG11C medium (i.e., BG11 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 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 pCZS5, 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 and 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 P₄₄furA 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 pRL2696 (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

<table>
<thead>
<tr>
<th>No.</th>
<th>Sequence (5’-3’*)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ATTACCATGGCTGTCTACAC</td>
<td>furA sense strand*</td>
</tr>
<tr>
<td>2</td>
<td>CTTGTAGCCTTACAGGGTC</td>
<td>furA antisense strand†</td>
</tr>
<tr>
<td>3</td>
<td>CTGCGCTAGAATTTACAAC</td>
<td>389 bp and 307 bp P₄₄furA coding strand</td>
</tr>
<tr>
<td>4</td>
<td>GCCTTGGAGAAGTTTGGT</td>
<td>389 bp P₄₄furA complementary strand</td>
</tr>
<tr>
<td>5</td>
<td>GAGTTGATTGCCAAACAAATTGCC</td>
<td>307 bp P₄₄furA complementary strand</td>
</tr>
<tr>
<td>6</td>
<td>GCGAGAGAGAATTTGGCCAG</td>
<td>P₄₄furA coding strand</td>
</tr>
<tr>
<td>7</td>
<td>TAGCGGAAAGCTGAGGGCA</td>
<td>P₄₄furA complementary strand</td>
</tr>
</tbody>
</table>

*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* 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 μl containing 10 mM Bistris/HCl (pH 7.5), 40 mM KCl, 1 mM dithiothreitol, 0.1 mM MgCl₂, 0.05 μg bovine serum albumin μl⁻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 (Friás 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 NcoI 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 with a volume of 20 μl, with incubations of 0.05 pmol labelled promoter with 1 μM purified NtcA in the presence of 1.5 μg 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 μg) 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*-x-*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 ~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*-x-*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*-x-*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-
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 NH₂ 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}

EMSAs 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

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**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} 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 Mn²⁺ and 1 mM DTT. Lanes: 1, free P_{furA1} (389 bp); 2, P_{furA1} (389 bp) 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.
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),
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*-x-*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<sub>furA</sub> 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<sub>furA</sub> and to the promoter of the dicistron *alr1690*-x-*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; Frias *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; Fungthong 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 nifJ, 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 at all 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 pO2 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.

ACKNOWLEDGEMENTS

The authors thank Dr Alicia Muro-Pastor and Dr Enrique Flores for NtcA and strain CSE2, as well as for helpful discussions, and Dr J. Naval and Dr J. Pardo for their help with fluorescence microscopy. S. L.-G. and J. A. H. were recipients of FPU fellowships (Ministerio de Educación y Ciencia, Spain). Work in the Wolk laboratory was supported by US Department of Energy grant DE-FG02-91ER20021.

REFERENCES


Fuangthong, M., Herbig, A. F., Bsat, N. & Helmann, J. D. (2002). Regulation of the Bacillus subtilis fur and perR genes by PerR: not all members of the PerR regulon are peroxide inducible. J Bacteriol 184, 3276–3286.


Edited by: K. Forchhammer