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Overexpression of FurA in *Anabaena* sp. PCC 7120 Reveals New Targets for This Regulator Involved in Photosynthesis, Iron Uptake and Cellular Morphology

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Previous genomic analyses of the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120 have identified three ferric uptake regulator (Fur) homologs with low sequence identities and probably different functions in the cell. FurA is a constitutive protein that shares the highest homology with Fur from heterotrophic bacteria and appears to be essential for in vitro growth. In this study, we have analysed the effects of FurA overexpression on the *Anabaena* sp. phenotype and investigated which of the observed alterations were directly operated by FurA. Overexpression of the regulator led to changes in cellular morphology, resulting in shorter filaments with rounded cells of different sizes. The *furA*-overexpressing strain showed a slower photoautotrophic growth and a marked decrease in the oxygen evolution rate. Overexpression of the regulator also decreased both catalase and superoxide dismutase activities, but did not lead to an increase in the levels of intracellular reactive oxygen species. By combining phenotypic studies, reverse transcription–PCR analyses and electrophoretic mobility shift assays, we identified three novel direct targets of FurA, including genes encoding a siderophore outer membrane transporter (*schT*), bacterial actins (*mreBCD*) and the PSII reaction center protein D1 (*psbA*). The affinity of FurA for these novel targets was markedly affected by the absence of divalent metal ions, confirming previous evidence of a critical role for the metal co-repressor in the function of the regulator in vivo. The results unravel new cellular processes modulated by FurA, supporting its role as a global transcriptional regulator in *Anabaena* sp. PCC 7120.

Keywords: *Anabaena* • Ferric uptake regulator • Growth • Morphology • Oxidative stress defenses • Photosynthesis.

Abbreviations: Amp, ampicillin; CAT, catalase; Cm, chloramphenicol; CM-H_2DCFDA, chloromethyl- 2,7-dichlorodihydrofluorescein diacetate; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; Fur, ferric uptake regulator; Km, kanamycin; NBT, nitro-blue tetrazolium; Nm, neomycin; PCV, packed cell volume; ROS, reactive oxygen species; RT–PCR, reverse transcription–PCR; SOD, superoxide dismutase; TCA, trichloroacetic acid.

Introduction

Iron is a scarce nutrient essential for almost all organisms. However, an excess of free iron in the cell is potentially toxic under aerobic conditions due to its ability to catalyze the formation of active species of oxygen by Fenton reactions (Andrews et al. 2003). Bacteria tightly regulate their iron metabolism by a predominant regulatory system orchestrated by the ferric uptake regulator (Fur), which controls the expression of iron uptake and storage machinery in response to iron availability (Escolar et al. 1999). The classical model of Fur regulation describes this protein as a sensor of the intracellular free iron concentration, that binds to Fe^{2+} under iron-rich conditions and acts as an active repressor by binding to 'iron boxes', located in the promoters of iron-responsive genes. Under iron-restricted conditions, the equilibrium is displaced to release Fe^{2+} and the repressor becomes inactive, allowing the transcription of target genes. Additionally, transcription of several genes has been shown to be directly or indirectly up-regulated by Fur, involving a variety of mechanisms (Masse and Gottesman 2002, Delany et al. 2004).

Fur proteins are considered global transcriptional regulators in prokaryotes. The Fur regulons so far identified include not only iron assimilation genes, but also a large number of genes and operons involved in many cellular processes such as respiration, redox stress resistance, glycolysis and the tricarboxylic acid cycle, methionine biosynthesis, purine metabolism, chemotaxis and synthesis of virulence factors, phage–DNA packaging, etc. Around 100 genes are regulated by Fur in *Escherichia coli* (McHugh et al. 2003), 59 genes in

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Helicobacter pylori (Danielli et al. 2006), 34 operons in *Yersinia pestis* (Gao et al. 2008) and 20 operons in *Bacillus subtilis* (Baichoo et al. 2002). Usually, more than one Fur homolog with different functions has been identified in the same microorganism (Bsat et al. 1998).

Cyanobacteria are widely distributed phototrophic prokaryotes that carry out oxygenic photosynthesis. As photosynthetic organisms, cyanobacteria particularly need iron for photosystem complexes and soluble proteins; but, on the other hand, they are exposed to the production of reactive oxygen species (ROS) concomitant with photosynthesis (Latifi et al. 2009). Therefore, regulation of iron metabolism in cyanobacteria must be particularly important, and Fur proteins could be involved in the control of new pathways not previously described in heterotrophic microorganisms.

The genomic analysis of the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120 led to the identification of three Fur homologs, named FurA, FurB and FurC, corresponding to the CyanoBase (http://genome.kazusa.or.jp/cyanobase) open reading frames *all1691*, *all2473* and *alr0957*, respectively (Hernández et al. 2004). The three proteins share typical Fur motifs, but are only distantly related. While FurA shares 40–45% homology with most well-known members of the Fur family, there have been few functional or structural characterizations of Fur members with close sequence homology to FurB or FurC. Western blot analyses of crude cellular extracts of *Anabaena* sp. have shown that FurA is a constitutive protein, whose expression slightly increases under iron deficiency (Hernández et al. 2002). Electrophoretical mobility shift assays (EMSAs) have shown that FurA binds the three *fur* homolog promoters, as well as those of genes involved in a variety of functions including photosynthesis and defense against oxidative stress (Bes et al. 2001, Hernández et al. 2004, Hernández et al. 2007, López-Gomollón et al. 2007). Divalent metal ions and thiol-reducing agents enhance the affinity of FurA for its target DNA sequences in vitro, suggesting the importance of the metal co-repressor and the redox status of the cysteines for the interaction with DNA in vivo (Hernández et al. 2006b). Attempts to inactivate *furA* have resulted in only a partial segregation of the mutated chromosomes, suggesting an essential role for this protein under standard culture conditions (Hernández et al. 2006a).

Overexpression of transcriptional regulators has been previously used in *Anabaena* sp. (Buikema and Haselkorn 2001, Liu and Golden 2002, Wu et al. 2004, Olmedo-Verd et al. 2005, Wu et al. 2007) and other bacteria (Marr et al. 2006, Ellermeier and Slauch 2008, Wang et al. 2008) as a tool to unravel the mechanisms of gene regulation and to identify direct DNA targets in complex regulatory networks. In order to gain new insights into the cellular functions of FurA, we have constructed a strain of *Anabaena* sp. that exhibits a high level of overexpression of this transcriptional regulator, and have analyzed some effects of such a level of FurA on various aspects of the cyanobacterial phenotype. We then investigated which of the observed phenotypic changes were the result of a direct FurA modulation of gene expression. This overexpression approach led us to identify three novel direct targets of FurA involved in different cellular processes in *Anabaena* sp.

Results

FurA overexpression from the copper-inducible *petE* promoter

A derivative strain of *Anabaena* sp. PCC 7120 showing a high level of expression of FurA was generated using the shuttle vector pAM2770 (Lee et al. 2003); it contained an extra copy of the wild-type *furA* gene located downstream of the copper-inducible *petE* promoter (Buikema and Haselkorn 2001). The strain obtained, named AG2770FurA, showed increased levels of the FurA protein both in standard BG-11 medium and in BG-11 supplemented with copper to 0.4 μM, according to Western blot analysis (**Fig. 1**). However, there was no appreciable increase in the amount of FurA after supplementation of BG11 with additional copper, suggesting a high induction of the *petE* promoter with the concentration of copper normally present in this medium. Consequently, standard BG-11 was used in further analyses. As previously found in cyanobacterial FurA (Pellicer et al. 2010), as well as in other Fur family proteins (Ortiz de Orue Lucana and Schrempf 2000, Lee and Helmann 2006), additional minor bands of FurA with different gel mobility appear as a result of oxidation of cysteines in the air and intrafragment disulfide bond formation.

Overexpression of FurA alters cyanobacterial morphology

Photoautotrophic growth of wild-type and *furA*-overexpressing strains in BG-11 medium was compared by following the cell yields during 24 d in terms of optical density at 750 nm (**Fig. 2**). The strain AG2770FurA showed a slower growth, with a doubling time of 4.07 d and a specific growth rate of 0.17 d^{-1} (in contrast to 3.15 d and 0.22 d^{-1} for the wild type), which determined minor cell yields in all growth phases. Values of packed cell volume (PCV) were in accordance with spectrophotometrical readings, showing a linear relationship. For this reason, the PCV was used as the parameter for cell mass in further determinations.

Microscopic examination of cultures in the exponential phase of growth revealed appreciable morphological changes in the strain AG2770FurA compared with its parental strain PCC 7120. Unlike the wild type, whose filaments formed large chains of elliptically shaped cells and highly uniform size (**Fig. 3A**), the *furA*-overexpressing strain showed an abundance of short filaments and even single cells, with rounded shape and loss of the characteristic uniformity in cell size, resulting in cells with both increased and diminished sizes compared with the wild type (**Fig. 3B**). In addition, the intrinsic fluorescence of cells appeared partially affected, since changes in the intensity of fluorescence of individual cells were observed along a single filament (**Fig. 3C, D**).

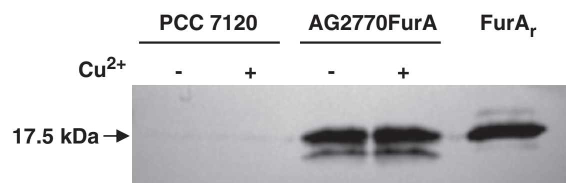

Fig. 1 Levels of FurA protein in wild-type *Anabaena* sp. strain PCC 7120 and the *furA*-overexpressing strain AG2770FurA revealed by Western blotting. Total cell extracts from filaments grown in BG-11 medium with (+) and without (−) additional supplementation with copper were separated by SDS–PAGE, electrotransferred and challenged with anti-FurA antiserum. Recombinant FurA overexpressed and purified from *E. coli* was included as control. The molecular weight is indicated.

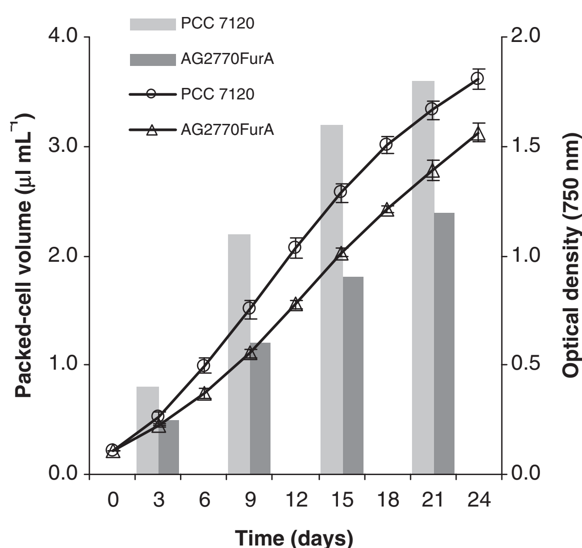

Fig. 2 Growth of *furA*-overexpressing strain AG2770FurA in comparison with the wild-type strain PCC 7120, expressed as both packed cell volume (columns) and optical density (lines). Values are the averages of three independent experiments; SDs are represented by vertical bars. Please note that in some instances the error is smaller than the symbols used.

Transmission electron microscopy analysis revealed differences in the ultrastructure, especially in the thylakoid arrangement. Whereas in the wild-type strain the thylakoids appeared in contorted arrays homogeneously distributed in the cytoplasm (**Fig. 3E**), in the *furA*-overexpressing strain the thylakoid membranes appeared evenly spaced and closely appressed to the periphery of cells (**Fig. 3F**).

Overexpression of FurA decreases photosynthetic activity

To determine whether overexpression of FurA affected the photosynthetic activity of *Anabaena* sp., we compared the oxygen evolution of wild-type and *furA*-overexpressing strains using a Clark-type oxygen electrode. Exponentially growing *Anabaena* cells overexpressing FurA evolved oxygen at about 50% of the rate of the wild type (**Table 1**), while dark respiration did not show significant variation between the strains.

Hence, the photoautotrophic growth data and measurements of oxygen evolution demonstrated that the *furA*-overexpressing strain was markedly compromised in photosynthetic function, but this decline could not be accounted for merely on the basis of differences in pigment contents. In fact, the strain AG2770FurA showed slightly higher amounts of Chl *a*, carotenoids and phycobiliproteins compared with the wild type (**Table 2**). Thus, the decrease in photosynthetic performance in a FurA overexpression background could be a consequence of changes in the expression pattern of photosynthesis genes, or could be a result of pleiotropic effects such as oxidative stress.

Overexpression of FurA decreases catalase and superoxide dismutase activities

Catalase (CAT) and superoxide dismutase (SOD) activities of cell-free extracts of wild-type and *furA*-overexpressing strains were measured to determine whether the enhanced expression of FurA affected the antioxidant defenses of *Anabaena* sp. Both enzymatic activities appeared clearly decreased in strain AG2770FurA compared with the wild type (**Fig. 4**), although CAT activity was particularly affected, displaying a >4-fold decrease. As expected, this appreciable reduction in the ability of cells to remove hydrogen peroxide (H_2O_2) led to a considerable decrease in the tolerance of the *furA*-overexpressing strain to oxidative stress resulting from exposure to exogenous H_2O_2 (**Fig. 5**). However, the AG2770FurA strain was still capable of effectively tolerating up to 300 µM of exogenous H_2O_2.

Levels of intracellular ROS in wild-type and *furA*-overexpressing strains were determined using two different methods. Fluorometric measurement of the oxidized form of the cell-permeant probe chloromethyl- 2,7-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) permits H_2O_2, hydroxyl radicals and peroxynitrite anions within the cell to be detected non-specifically (Latifi et al. 2005). However, since CAT activity was particularly affected in the AG2770FurA strain, the intracellular peroxide content was also specifically estimated by the ferrithiocyanate method (Thurman et al. 1972). Surprisingly, the reduction in the antioxidant capabilities observed in the *furA*-overexpressing strain did not lead to an increase in the levels of ROS accumulated by cells during the exponential phase of photoautotrophic growth. ROS production was similar and even slightly lower in the strain AG2770FurA compared with the wild type, perhaps as a result of a lower photosynthetic activity (**Table 3**). Similar results were observed when endogenously generated H_2O_2 was specifically measured by the ferrithiocyanate method. Taken together, these results suggested that FurA overexpression decreased oxidative stress defenses, but did not lead to induced oxidative stress in *Anabaena* sp.

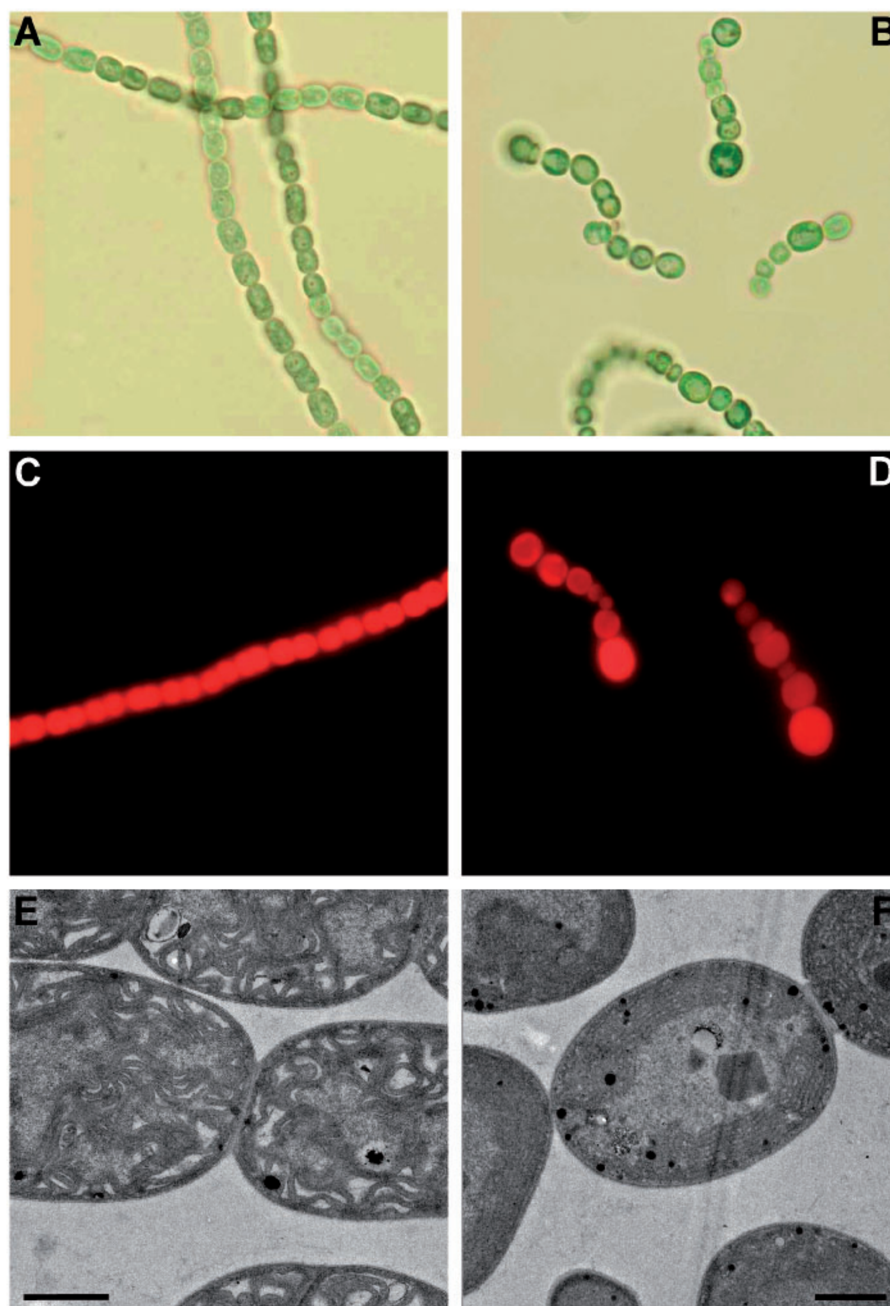

Fig. 3 Overexpression of FurA induced morphological and ultrastructural changes in *Anabaena* sp., as shown in photomicrographs of the wild-type strain PCC 7120 (A, C, E) and the *furA*-overexpressing strain AG2770FurA (B, D, F) taken at the exponential phase of growth. Bright-field (A, B) and fluorescence (C, D) microscopic examinations revealed alterations in filament length, cell shape/size and intrinsic fluorescence. Transmission electron microscope analysis (E, F) showed differences in thylakoid arrangement. The photomicrograph of each strain is representative of at least 10 different images. Bars = 1 µm.

Overexpression of FurA induces changes in the transcriptional pattern of a variety of genes

On the basis of the phenotypic changes observed in the AG2770FurA strain, we investigated the impact of FurA overexpression on the transcription of a variety of genes involved in several physiological processes such as photosynthesis, oxidative stress defense and control of cellular morphology. Semi-quantitative reverse transcription–PCR (RT–PCR) was used to compare the levels of transcripts of selected genes in wild-type and *furA*-overexpressing strains (**Fig. 6**). In order to obtain accurate determinations, each measure was performed at the early exponential phase of the PCR of each gene.

The *rnpB* housekeeping gene was included in all RT–PCR analyses to ensure that equivalent amounts of total RNA were being used in all reactions. The relative induction ratio observed for each analyzed gene is presented in **Supplementary Table S1**.

The analysis of the expression of some genes encoding subunits of PSI and PSII showed different effects of a FurA overexpression background on transcription of photosynthesis genes. Transcripts of the PSI genes *psaA* and *psaB*, encoding the reaction center proteins PsaA and PsaB, respectively, as well as those of the PSII gene *psbB* coding for the core antenna CP47 protein was clearly increased in the *furA*-overexpressing strain compared with the wild type, while transcripts of *psbA* encoding the PSII reaction center protein D1 were only slightly increased. However, at least one gene, *psbZ*, coding for an 11 kDa PSII protein, showed a reduced amount of transcripts. Thus, despite the increased pigment and phycobiliprotein contents found in the *furA*-overexpressing strain, the direct or indirect effect of FurA overexpression in down-regulation of other non-essential but important regulatory proteins, such as PsbZ (Bishop et al. 2007), could produce an imbalance among components of the photosystems in AG2770FurA cells.

The expression of several antioxidant enzymes described in *Anabaena* sp. PCC 7120 was also analyzed. Transcripts of the most abundant thiol peroxidases, namely GCT1 (*alr3183*) and GCT3 (*all2375*) (Cha et al. 2007), were reduced in the *furA*-overexpressing strain, while transcripts of glutathione reductase were increased. Interestingly, the transcriptional patterns of SODs observed in the *furA*-overexpressing strain resembled those previously described in wild-type strains of *Pseudomonas aeruginosa* (Hassett et al. 1996) and *E. coli* (Dubrac and Touati 2000) after iron deprivation. While transcripts of manganese-containing SOD (*sodA*) appeared increased, the amounts of the iron-containing SOD (*sodB*) were reduced. Additionally, some of the iron-responsive genes described in *Anabaena* sp. seemed to be up-regulated in the *furA*-overexpressing strain AG2770FurA, such as those encoding the siderophore outer membrane transporter SchT (Nicolaisen et al. 2008) and the IsiA protein.

The overexpression of FurA also led to an increase in the transcription of some genes involved in control of cellular

Table 1 Photosynthetic oxygen evolution and dark respiration of exponentially growing cells of *Anabaena* sp. strains PCC 7120 and AG2770FurA

Parameter	*Anabaena* sp. strain[a]	
	PCC 7120	AG2770FurA
Apparent O_2 evolution	45.6±2.1[b]	19.2±1.2[b]
	115.6±5.3[c]	46.5±2.8[c]
O_2 consumption	5.7±2.2[b]	7.7±3.5[b]
	14.6±5.6[c]	18.7±8.6[c]
Total O_2 evolution	51.4±4.3[b]	26.9±4.7[b]
	130.3±11.0[c]	65.4±11.4[c]

[a] *Anabaena* sp. strains were grown photoautotrophically in BG-11 medium to mid-log phase, and filaments were washed twice and resuspended to 10 μg ml^{-1} Chl *a*.
[b] Values are means of two independent determinations±SD, expressed as μmol O_2 mg Chl a^{-1} h^{-1}.
[c] Values are means of two independent determinations±SD, expressed as μmol O_2 ml PCV^{-1} h^{-1}.

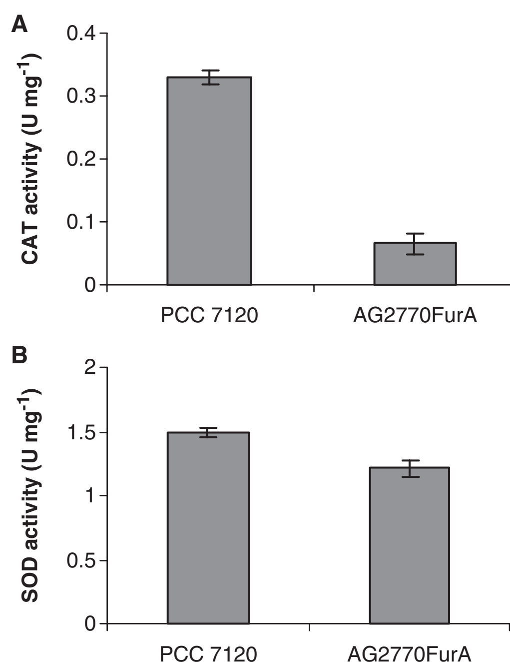

Fig. 4 Catalase (A) and superoxide dismutase (B) activities in wild-type *Anabaena* sp. strain PCC 7120 and *furA*-overexpressing strain AG2770FurA. Values are the averages of two independent experiments; SDs are represented by vertical bars.

Table 2 Pigments and protein contents of *Anabaena* sp. strains PCC 7120 and AG2770FurA at different stages of growth

Pigment/proteins[a]	Exponential phase		Stationary phase	
	PCC 7120	AG2770FurA	PCC 7120	AG2770FurA
Chl *a*	1.42±0.25	2.46±0.15	3.31±0.42	3.74±0.23
Carotenoids	0.19±0.03	0.31±0.02	0.58±0.06	0.61±0.01
Phycobiliproteins	6.15±0.46	7.75±0.35	22.5±0.8	21.5±0.6
Total soluble proteins	59.5±3.2	65±2.5	120±6.8	125±5.1

[a] Values are means of two independent determinations±SD, expressed as μg μl^{-1} PCV.

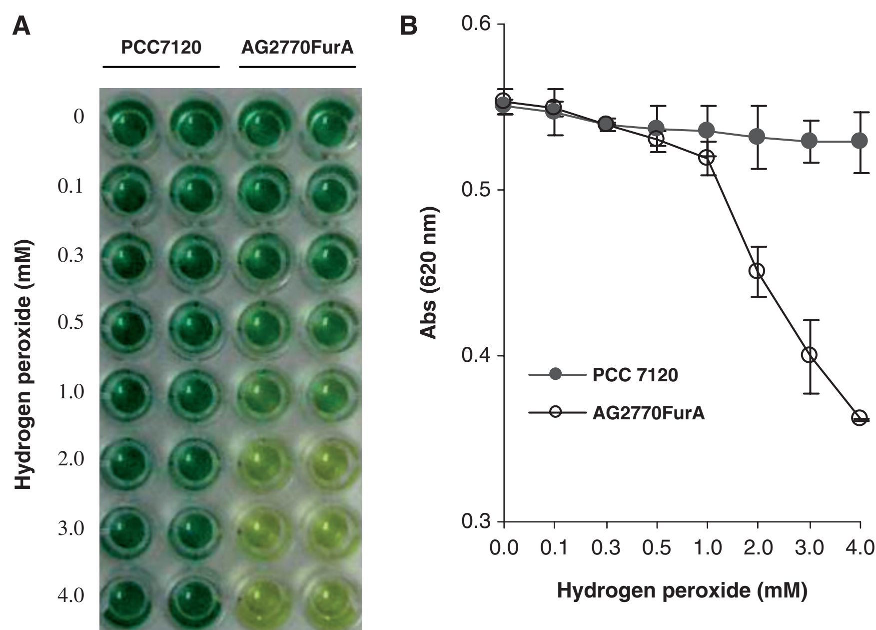

Fig. 5 Tolerance to oxidative stress induced by exogenous hydrogen peroxide exposure. (A) *Anabaena* sp. strains were grown in BG-11 medium to late log phase, washed and adjusted to the same cell density. Cell suspensions were exposed in duplicate to increasing concentrations of H_2O_2 up to 4 mM for 48 h in the dark, and results were documented by photography. (B) Estimation of chlorosis by reading the microtiter plate absorbance at 620 nm. Each experiment was done twice with similar results.

morphology, such as those for the bacterial actins MreB and MreC (Hu et al. 2007). Conversely, a second ferric uptake regulator described in *Anabaena*, FurB, with a possible role in oxidative stress protection (López-Gomollón et al. 2009), seemed to be down-regulated, while transcript levels of FurC decreased only slightly in the AG2770FurA strain.

Identification of novel FurA direct target genes

To determine whether the expression of genes previously analyzed by RT–PCR was directly regulated by FurA, we investigated the ability of FurA to bind in vitro the promoter regions of such genes by using EMSAs. To confirm the specificity of bindings, all assays included the promoter region of *nifJ* as non-specific competitor DNA, while binding of FurA to the promoter region of its own gene was used as a positive control. All gene promoters used in the analyses consisted of a 300–400 bp DNA fragment upstream of ATG. We assumed that genes giving a positive result in both RT–PCR analyses and EMSAs were direct targets of FurA.

Despite the high variation in transcript levels of photosynthesis genes under a FurA overexpression background, the EMSAs showed only a clear binding of FurA to the promoter region of *psbA*, a PSII gene coding for the reaction center protein D1 (**Fig. 7B**). Interestingly, *psbA* has been previously shown to respond to iron deprivation by increasing its transcriptional level (Singh and Sherman 2000), but this is the first evidence, to the best of our knowledge, of a direct regulation by a Fur. None of the other analyzed components of both photosystems showed specific binding to FurA according to EMSA (data not shown).

Table 3 Intracellular ROS levels of late-log growing cells of *Anabaena* sp. strains PCC 7120 and AG2770FurA

Strain	ROS level	
	CM-H_2DCFDA fluorescence[a]	Ferrithiocyanate[b]
PCC 7120	20.2 ± 0.4	0.073 ± 0.008
AG2770FurA	15.1 ± 0.1	0.055 ± 0.004
Control[c]	98.6 ± 0.6	0.322 ± 0.053

[a] Values are means of two independent determinations ± SD, presented in arbitrary units.
[b] Values are means of two independent determinations ± SD, expressed as µmol H_2O_2 mg FW^{-1}.
[c] Wild-type PCC 7120 strain treated with 1 mM H_2O_2 for 30 min.

Similarly, no apparent direct regulation by FurA of antioxidant enzyme expression was seen according to binding assays. Neither promoters of both SODs nor those of GCT1 and GCT3 thiol-peroxidases showed specific affinity of FurA. A similar result was also observed with the glutathione reductase promoter (data not shown).

It has been previously shown that the binding affinity of FurA for its target promoters is greatly affected by the redox status of the protein and the presence of divalent metal ions (Hernández et al. 2006b). Thus, the optimal binding conditions have been observed in the presence of both dithiothreitol (DTT) and Mn^{2+}, which are routinely used in the FurA EMSA experiments. Taking into account the tight connection between iron homeostasis and oxidative stress defenses, as well as previous reports of Fur direct regulation of SOD gene transcription

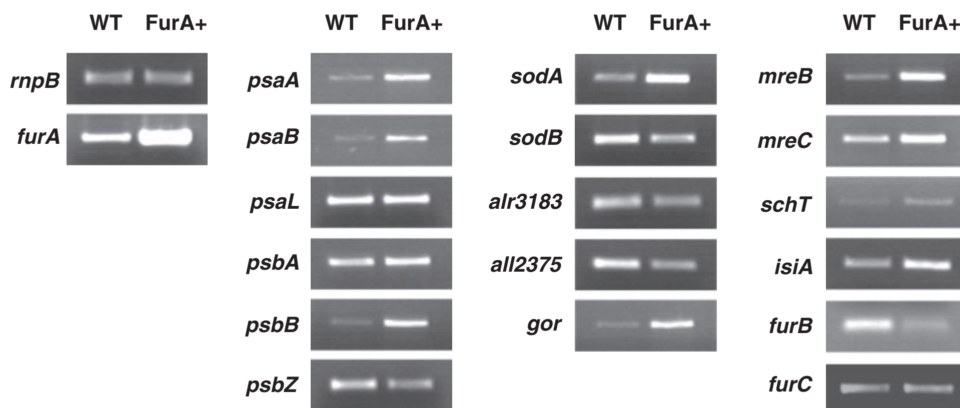

Fig. 6 Semi-quantitative RT–PCR analysis of the expression of several genes involved in a variety of physiological processes such as photosynthesis, oxidative stress defense, cellular morphology and iron stress response in wild-type strain PCC 7120 (WT) and the *furA*-overexpressing strain AG2770FurA (FurA+). Total RNA was isolated from cells grown in standard BG-11 medium during the exponential phase of growth. The housekeeping gene *rnpB* was used as control. Determinations for each gene were performed in the exponential phase of PCR. Experiments were repeated at least twice with independent RNA extractions, and the relevant portions of a representative gel are shown.

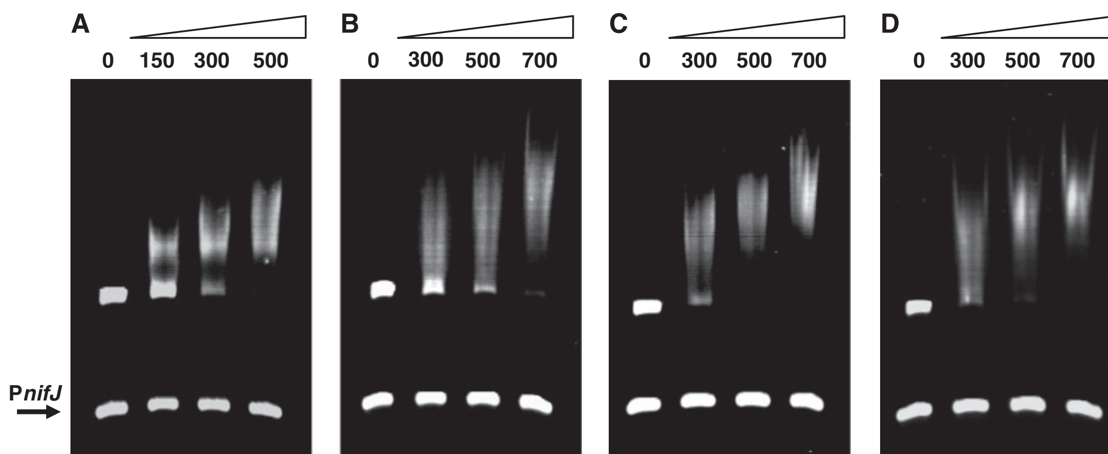

Fig. 7 Electrophoretic mobility shift assays showing the ability of FurA to bind in vitro the promoter regions of target genes *psbA* (B), *schT* (C) and *mreBCD* (D). Binding of FurA to its own promoter (A) was included as a positive control. DNA fragments free or mixed with increasing concentrations of recombinant FurA protein were separated by 4% PAGE. In gels, the protein concentration (indicated in nM) increases from left to right. The promoter region of the *nifJ* gene was used a as non-specific competitor DNA in all assays.

in other bacteria (Ernst et al. 2005), we further characterized the effect of divalent metal ion and redox conditions on the DNA-binding of FurA to SOD promoters, in order to determine whether pre-established binding conditions affected the affinity of FurA for these putative targets. Neither the absence of Mn^{2+} (+EDTA) nor non-reducing conditions improved the affinity of FurA for SOD promoters (data not shown).

As expected, the EMSA analysis showed that FurA bound with high affinity to the promoter region of *schT* (**Fig. 7C**), a gene encoding a membrane transporter of the siderophore schizokinen (Nicolaisen et al. 2008). Likewise, the *mreBCD* operon, encoding the bacterial actins MreB, MreC and MreD (Hu et al. 2007), seemed also to be directly regulated by FurA according to the binding assays (**Fig. 7D**). In agreement with the EMSA results, computational analysis of the promoter regions of those targets allowed the identification of potential AT-rich iron boxes (**Supplementary Fig. S1**).

Overall, the combination of RT–PCR analyses and EMSAs led us to identify three novel FurA direct targets: the photosynthesis gene *psbA*, the iron metabolism gene *schT* and the morphology-related operon *mreBCD*.

Metal co-repressor and reducing conditions are critical for the affinity of FurA for direct target genes

The overexpression of FurA showed two different effects on the transcriptional pattern of direct target genes. While most of those targets seemed to be up-regulated, at least one of them,

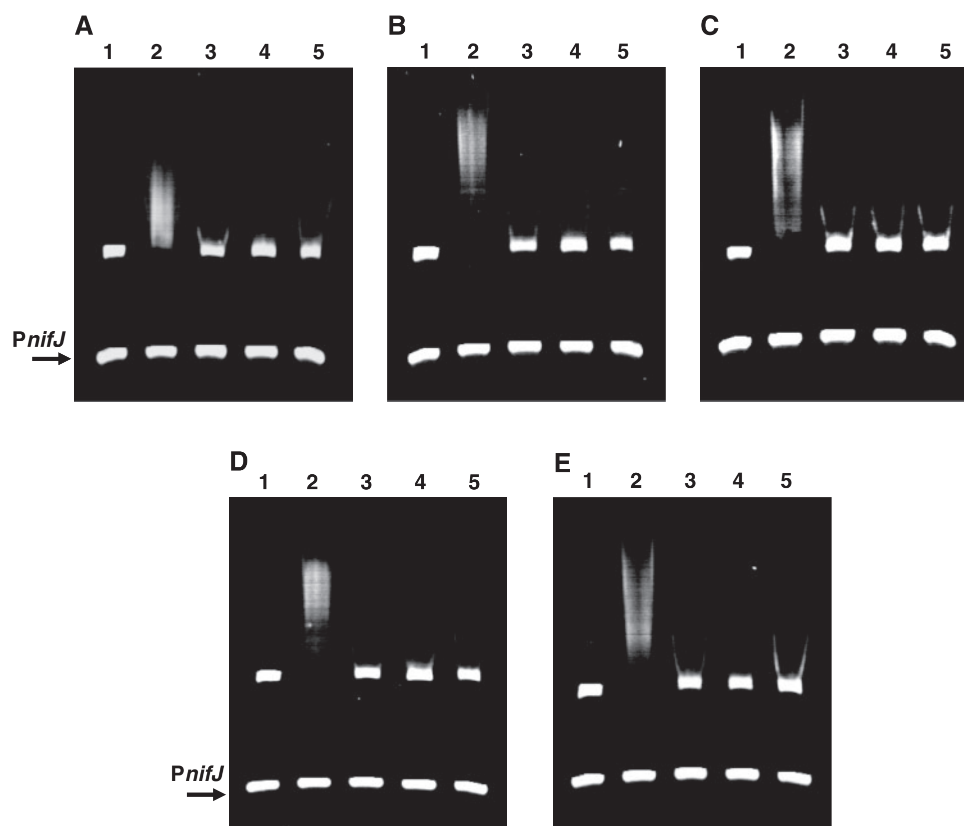

Fig. 8 Impact of a metal co-repressor and reducing conditions on the in vitro affinity of FurA for target genes *psbA* (A), *schT* (B), *mreBCD* (C), *furB* (D) and *isiA* (E). DNA fragments free (line 1) or mixed with recombinant FurA protein at a concentration of 500 nM (lines 2–5) in the presence of Mn^{2+} and DTT (2), EDTA and DTT (3), Mn^{2+} without DTT (4) and EDTA without DTT (5) were separated by 4% PAGE. The promoter region of the *nifJ* gene was used as a non-specific competitor DNA in all assays.

furB, decreased its transcript level. In order to determine whether these different effects were a consequence of differences in the mechanism of regulation, we further analyzed the impact of several binding conditions (by removing Mn^{2+} and/or DTT) on the affinity of FurA for the promoters of targets that had shown different transcriptional response in RT–PCR analyses. Notably, the affinity of FurA for all the target promoters analyzed was markedly affected in the absence of Mn^{2+} (and addition of EDTA) and/or DTT (**Fig. 8**). The results demonstrated that the presence of a metal co-repressor and reducing conditions are critical in the function of FurA, confirming previously obtained evidences (Hernández et al. 2006b). Thus, differences in the transcriptional patterns of target genes in a FurA overexpression background might reflect a coordinated action of more than one transcriptional regulator, rather than differences in the mechanism of regulation of FurA on its direct target genes.

Discussion

Although numerous studies have revealed insights into the structure, mechanism of action and functions of Fur proteins in heterotrophic bacteria (Escolar et al. 1999, Hantke 2001, Baichoo et al. 2002, Andrews et al. 2003, McHugh et al. 2003, Danielli et al. 2006, Lee and Helmann 2007, Gao et al. 2008), comparatively little is known about the role of this family of proteins in cyanobacteria. In the present study, we have constructed a strain of *Anabaena* sp. that overexpresses active FurA by using a shuttle plasmid with the copper-responsive *petE* promoter fused to a *furA* gene copy. Overexpression of FurA induced changes in the transcriptional pattern of a variety of genes, leading to alterations in photoautotrophic growth, filament integrity, cell morphology, ultrastructure, photosynthetic function and defense against oxidative stress. Although some of the effects observed in the FurA overexpression phenotype could result from an aberrant response unrelated to the normal function of the protein, the overexpression approach led us to identify novel direct targets of this apparently essential transcriptional regulator by combining the analysis of the phenotypic changes with both transcriptional profile variations and FurA–DNA interactions.

Perhaps the most evident effects of the overexpression of FurA on the *Anabaena* phenotype were the slowing down of photoautotrophic growth and the altered morphology of cells.

These effects appeared to be directly associated with the overexpression of the regulator, since the wild-type *Anabaena* sp. PCC 7120 and the *Anabaena* sp. strain harboring the empty vector pAM2770 showed identical phenotypes (data not shown).

Measurement of oxygen evolution of the strain AG2770FurA indicated a strong effect on the photosynthetic activity, while RT–PCR analyses revealed different effects of FurA overexpression on the transcriptional pattern of several genes involved in photosynthesis. At least one of those genes, *psbA*, encoding the PSII reaction center protein D1, was directly regulated by FurA according to EMSA. Although only a few genes were analyzed, at least one (*psbZ*) seemed to be down-regulated with the overexpression of FurA. The PsbZ subunit of PSII has been described in *Synechocystis* sp. as a modulator of the electron flow through the photosynthetic electron transfer chain (Bishop et al. 2007). Deletion mutants of the *pbsZ* gene showed a retarded photoautotrophic growth compared with the wild type. Since expression of photosynthesis genes is influenced by multiple factors and modulated by several transcriptional regulators (Li and Sherman 2000, Fujimori et al. 2005, Seino et al. 2009), our results could suggest not only a direct influence of FurA on photosynthesis regulation, but also an indirect effect, perhaps as a result of a coordinated action of different regulators, as observed with FurA and NtcA in nitrogen metabolism (López-Gomollón et al. 2007). The depletion in photosynthetic function undoubtedly had additional consequences on cellular physiology; many of these effects could be pleiotropic effects of FurA overexpression. The slightly increased amount of photosynthetic pigments and/or the rearrangement of thylakoid membranes could be adaptive responses of the microorganism in an attempt to improve the photosynthesis performance.

Our experiments led us to identify another direct target of FurA, the operon *mreBCD*. In particular, the bacterial actin MreB has been shown to play a critical role in determination of cell shape of *Anabaena* sp. PCC 7120 (Hu et al. 2007). Either *mreB* deletion or *mreBCD*-inducible overexpression caused drastic morphological changes in *Anabaena* cells. The increased level of *mreB*/*mreC* transcripts observed in a FurA overexpression background could explain the morphological alterations seen in the strain AG2770FurA. Likewise, although the increased fragility of filaments in the *furA*-overexpressing strain could be a result of different factors, the overexpression of FurA could directly or indirectly affect the expression of genes associated with filament integrity (Bauer et al. 1995, Nayar et al. 2007).

The intimate relationship between iron metabolism and oxidative stress has been extensively recognized in bacteria (Zheng et al. 1999, Andrews et al. 2003, Latifi et al. 2005). In *E. coli*, the expression of Fur is modulated by oxidative stress response regulators, and Fur directly or indirectly regulates the expression of antioxidant enzymes. *Anabaena* sp. PCC 7120 has a quorum of oxidative stress defenses, including Mn- and Fe-containing SODs (Li et al. 2002), peroxidases (Cha et al. 2007), glutathione reductase (Jiang et al. 1995) and several non-characterized CATs. In our experiments, the overexpression of FurA decreased both CAT and SOD activities, and had different effects of the transcriptional pattern of SODs, thiol-peroxidases and glutathione reductase. However, the results suggest that FurA does not directly regulate the expression of these antioxidant enzymes according to EMSA analyses. This fact could reflect a possible regulatory cascade or an indirect pathway of regulation of antioxidant enzyme gene expression by FurA, as previously described in other bacteria (Masse and Gottesman 2002). Likewise, the existence of two additional Fur homologs in *Anabaena* sp. (Hernández et al. 2004) makes it possible that there are other Fur-mediated regulation pathways, as described in microorganisms with more than one Fur protein (Horsburgh et al. 2001).

In cyanobacteria, siderophore-mediated iron uptake is thought to be a contributing factor in their ability to dominate eukaryotic algae. *Anabaena* sp. PCC 7120 synthesizes schizokinen as its major siderophore, although it also shows iron uptake from aerobactin and ferrioxamine B (Goldman et al. 1983). Recently, Nicolaisen and co-workers described an outer membrane transporter for schizokinen in *Anabaena*, named SchT, whose expression is induced under iron-limited conditions (Nicolaisen et al. 2008). Our results indicate that *schT* is a direct target of FurA.

Unexpectedly, most of the novel and previously described FurA direct targets seemed to be up-regulated rather than down-regulated in a transcriptional repressor overexpression background, including those recognized as iron-responsive genes. Additionally, the transcriptional patterns observed in SOD genes resembles those described in wild-type strains of *P. aeruginosa* (Hassett et al. 1996) and *E. coli* (Dubrac and Touati 2000) after iron deprivation. It has been previously hypothesized that Fur proteins might act as ferrous ion 'buffers' in the cell, and consequently have a dual action during redox stress, regulating the iron uptake and iron storage systems, but also themselves increasing the Fe^{2+}-binding capacity of the cytosol (Andrews et al. 2003). Since metal co-repressors seem to be essential for FurA DNA binding, we speculate that the high level of FurA overexpression achieved by the strain AG2770FurA using the P*petE*-pAM2770 system strongly reduces the free iron pool in the cytosol and leads to the release of the co-repressor from some FurA–Fe^{2+} complexes, allowing the transcription of target genes. A similar phenomenon has been observed in *Saccharomyces cerevisiae* when ferritins were overexpressed under iron-replete conditions (Kim et al. 2007).

Our data provide evidence that in *Anabaena* sp. PCC 7120, FurA acts directly or indirectly on the expression of genes involved in a variety of physiological processes, including iron metabolism, cellular morphology, photosynthesis and defense against oxidative stress. Taken together, the results strongly support the hypothesis that, as other Fur homologs described in heterotrophic bacteria, FurA is a global transcriptional regulator in cyanobacteria.

Materials and Methods

Strains and culture conditions

Bacterial strains and plasmids used in this study are described in **Table 4**. Wild-type *Anabaena* sp. strain PCC 7120 and its *furA*-overexpressing derivate strain AG2770FurA were grown photoautotrophically at 30°C in BG-11 medium (Rippka et al. 1979), supplemented with neomycin (Nm) 50 μg ml^{-1} in the case of strain AG2770FurA. For most experiments the strains were cultured in 250 ml Erlenmeyer flasks containing 60 ml of culture medium. The cultures were maintained in an incubator shaker at 120 r.p.m. under continuous illumination with white light at 20 μE m^{-2} s^{-1}.

For overexpression phenotype screening after conjugation, clones of exconjugant *Anabaena* sp. resistant to Nm were grown photoautotrophically in BG-11 medium supplemented with 50 μg ml^{-1} Nm to an optical density of 0.3 at 750 nm, then a filter-sterilized solution of $CuSO_4$ was added to BG-11 medium to a final concentration of 0.4 μM, in order to induce the *petE* promoter (Buikema and Haselkorn 2001), and cultures were further incubated for 72 h under standard growth conditions.

Escherichia coli strains carrying plasmids were grown in Luria broth supplemented, as appropriate, with 50 μg ml^{-1} kanamycin (Km), 50 μg ml^{-1} ampicillin (Amp) and/or 34 μg ml^{-1} chloramphenicol (Cm).

Nucleic acid manipulations

Total DNA and RNA from *Anabaena* sp. strains growing exponentially were isolated as described previously (Olmedo-Verd et al. 2005). Plasmid preparations were performed using the GenElute™ Plasmid Miniprep Kit (Sigma). DNA purification of PCR products was performed using the GFX™ PCR DNA and the Gel Band Purification Kit (GE Healthcare). Standard protocols were used for cloning, *E. coli* transformation and PCR (Sambrook and Russell 2001).

Plasmid pAM2770FurA, which contains a copy of the *furA* gene under the control of the *Anabaena* sp. PCC 7120 copper-inducible *petE* (plastocyanin) promoter, was constructed from the shuttle vector pAM2770 (Lee et al. 2003), a kind gift from Dr. James W. Golden (Texas A&M University, College Station, TX, USA). The entire coding region of *furA* was amplified by PCR using the primers 2770FurA_up and FurA_dw (**Table 5**), and genomic DNA from strain PCC 7120 as template. The PCR product was cloned into vector pGEM-T (Promega), digested with *Nde*I and *Bam*HI, and cloned into the same restriction sites of vector pAM2770. The construct was partially sequenced to ensure that no modifications in the nucleotide sequence occurred during amplification and cloning.

Construction of a *furA*-overexpressing strain

Plasmid pAM2770FurA was transferred to *Anabaena* sp. PCC 7120 by conjugation according to a previously described

Table 4 Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
E. coli		
DH5α	F$^-$ ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*argF*)U169 *recA1 endA1 hsdR17*(rK$^-$, mK$^+$) *phoA supE44 thi-1 gyrA96 relA1* λ$^-$, for cloning and maintaining plasmids	Invitrogen
ED8654	F$^-$ *e14*$^-$ (*mcrA*$^-$) *recA* 56 *lac-3 o lac*Y1 *gal*K2 *gal*T22 *gln*V44 *sup*F58 *met*B1 *hsd*R514 (rK$^-$ mK$^+$) *trp*R55, for triparental conjugation	Institute of Plant Biochemistry and Photosynthesis, Seville, Spain
CPB1893	*mcr*A$^-$ *mcr*B$^-$ M.*Eco*K$^+$ R.*Eco*K$^-$, for triparental conjugation	Institute of Plant Biochemistry and Photosynthesis, Seville, Spain
BL21(DE3)	*ompT hsdS$_B$* (r_B^- m_B^-) *gal dcm* (DE3), for expression and purification of recombinant FurA	EMD Biosciences
Anabaena sp.		
PCC 7120	Wild type	Pasteur Institute, Paris, France
AG2770FurA	PCC 7120 harboring pAM2770FurA, overexpresses FurA	This study
Plasmids		
pGEM-T	Cloning vector, Amp^r	Promega
pAM2770	Shuttle vector, Km^r/Nm^r	Lee et al. (2003)
pAM2770FurA	pAM2770 containing *furA* expressed from the *petE* promoter	This study
pRL443	Conjugal plasmid; Amp^r Tc^r; Km^s derivative of RP-4	Elhai et al. (1997)
pRL623	Conjugation helper plasmid; Cm^r; Mob$_{ColK}$, M·*Ava*II, M·*Eco*47II, M·*Eco*T221	Elhai et al. (1997)
pET28a	Expression vector, Km^r	EMD Biosciences

method (Elhai et al. 1997). Triparental mating was carried out using the *E. coli* conjugal donor strain ED8654, which contains the plasmid pRL443, and the *E. coli* conjugal helper strain CPB1893 carrying the plasmid pRL623. Clones resistant to Nm were screened by Western blotting in order to evaluate the copper-inducible overexpression of FurA. Finally, the clone selected as the *furA*-overexpressing strain was named AG2770FurA.

SDS–PAGE and Western blotting

Filaments of copper-induced exconjugants from 50 ml cultures were collected by centrifugation, washed with 50 mM Tris–HCl buffer (pH 8.0) and sonicated in an ice–water bath by five 30 s bursts with 30 s cooling intervals. The resulting crude extracts were centrifuged at 12,000×*g* for 5 min at 4°C to remove cell debris, and the protein concentration was determined using the BCA™ Protein Assay kit (Thermo Fisher Scientific). For each sample, 30 µg of total protein was loaded and separated by SDS–PAGE with a 17% polyacrylamide gel, and transferred to a polyvinylidine fluoride membrane (Millipore). Rabbit polyclonal antibodies raised against *Anabaena* sp. FurA recombinant protein were used (Hernández et al. 2002), and the blot was visualized with an Universal Hood Image Analyzer (Bio-Rad).

Measurements of growth and pigments

Growth of wild-type *Anabaena* sp. strain PCC 7120 and its derivative strain AG2770FurA was measured spectrophotometrically using a Cary 100 Bio UV-Visible spectrophotometer (Varian). The optical density of the cell suspensions was recorded at 750 nm every 72 h in triplicate with independent cultures. The specific growth rate and doubling time were calculated as previously described (Stein 1973). In order to confirm the spectrophotometric readings, the PCV was also determined at certain times during incubation using 5 ml graduated centrifuge tubes of 60 µl capacity. Readings of the PCV were taken after centrifugation for 5 min at 2,000×*g* using a swing-bucket rotor, and expressed as microliters of fresh cells per milliliter of culture.

Cultures were sampled at exponential and stationary phases of growth for measurement of pigments and total protein. Chl *a* (Nicolaisen et al. 2008), carotenoid (Davies 1976) and phycobiliprotein (Glazer 1976) contents were determined according to previously described methods. Total soluble protein present in cell extracts after sonication was determined using the BCA™ Protein Assay kit (Thermo Fisher Scientific). Pigments and protein contents were expressed in micrograms per microliter of PCV.

Microscopy

Bright-field and fluorescence microscopic examinations of *Anabaena* sp. filaments were carried out with a Nikon Eclipse 50i Epi-fluorescence microscope. Micrographs were taken with a Nikon DXM1200F camera coupled to the microscope. The images were processed with Adobe Photoshop version 6.0.

For electron microscopy, filaments were fixed in 2.5% (v/v) glutaraldehyde prepared in 66 mM phosphate buffer for 1 h at room temperature. After fixation, the samples were washed in phosphate buffer and then fixed again in 2% (w/v) osmium tetroxide for 1 h at room temperature. Dehydration was performed in a series of cold ethanol dilutions allowed to warm to room temperature at the higher concentrations, followed by infiltration with Spurr resin. Sections were cut on a Reichert Om U2 ultramicrotome, collected on nickel grids, stained with uranyl acetate and examined with a TECNAI G200 transmission electron microscope (FEI) operated at 120 V. The images were processed with Adobe Photoshop version 6.0.

Photosynthetic oxygen evolution

Oxygen evolution of exponentially growing cells was measured at 25°C with a Clark-type oxygen electrode model Chlorolab 2 (Hansatech). Cell suspensions (10 µg ml^{-1} Chl *a*) were illuminated with white light at saturating intensity (400 µE m^{-2} s^{-1}). Dark respiration was estimated from O_2 uptake by cells incubated in the dark. Total oxygen evolution was calculated as the sum of the apparent oxygen evolution in the light and dark respiration.

Antioxidant enzymes assays

Cyanobacterial cells were grown to late log phase in 50 ml of standard BG-11 medium, collected by centrifugation, and sonicated in 50 mM potassium phosphate buffer (pH 7). Supernatant obtained after centrifugation of the crude extracts was used for enzyme assays. Protein concentration was determined using the BCA™ Protein Assay kit (Thermo Fisher Scientific). CAT activity was determined according to the method of Beers and Sizer (1952). Dissociation of H_2O_2 was followed spectrophotometrically at 240 nm in a 3 ml reaction mixture containing 20 mM H_2O_2 and crude extract (600 µg ml^{-1} total proteins) at 25°C. One unit of enzyme was defined as the quantity that catalyzes the decomposition of 1 µmol of H_2O_2 in 1 min. SOD activity was determined according to the method of Winterbourn et al. (1975), based on the ability of SOD to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) by superoxide in a 3 ml reaction mixture containing 2 µM riboflavin, 50 µM NBT, 0.06 M EDTA, 20 µM sodium cyanide and crude extract (600 µg ml^{-1} total proteins). One unit of enzyme was defined as the amount causing half maximal inhibition of NBT reduction. Activities of enzymes were expressed as units per milligram of protein.

Tolerance to oxidative stress induced by H_2O_2

Tolerance of *Anabaena* sp. cells to the oxidative stress caused by H_2O_2 exposure was measured according to Shcolnick et al. (2009) with slight modifications. Basically, the strains were grown to late log phase in standard BG-11 medium, washed, and adjusted to an optical density at 750 nm of 1.0 in BG-11 medium. Aliquots of 200 µl of adjusted cultures were put on a microtiter plate and incubated for 48 h in the presence of 0–4 mM H_2O_2 in darkness. The results were documented by photography, and chlorosis was estimated by reading the

absorbance at 620 nm using a Multiskan EX microplate photometer (Thermo Fisher Scientific).

ROS determination

Levels of intracellular ROS in *Anabaena* sp. strains were quantified according to Latifi et al. (2005) by using the fluorescent probe CM-H_2DCFDA, which detects H_2O_2, hydroxyl radicals and peroxynitrite anions. Cyanobacterial cells were grown to late log phase in 50 ml of standard BG-11 medium, washed twice with 10 mM phosphate buffer and incubated with 25 µM CM-H_2DCFDA (Invitrogen) for 5 min at room temperature in darkness. Fluorescence was measured using an Aminco Bowman Series 2 spectrofluorometer (Thermo Fisher Scientific), with excitation and emission settings of 488 nm and 530 nm, respectively. Cells of the wild-type strain treated with 1 mM H_2O_2 for 30 min were used as the fluorescent-positive control.

Additionally, the amount of intracellular H_2O_2 was specifically determined by the ferrithiocyanate method (Thurman et al. 1972). Briefly, 95–100 mg of fresh cells were homogenized with 5% trichloroacetic acid (TCA), centrifugated at 12,000 × *g* for 5 min at 4°C, and the supernatant obtained was used for peroxide estimation. Reaction mixtures contained 800 µl of crude extract, 160 µl of 50% TCA, 160 µl of ferrous ammonium sulfate and 80 µl of potassium thiocyanate. The absorbance of the ferrithiocyanate complex was read at 480 nm using a Cary 100 Bio UV-Visible spectrophotometer (Varian), and compared with H_2O_2 standard. Peroxide content was expressed as micromoles per milligram of FW. Cells of the wild-type strain treated with 1 mM H_2O_2 for 30 min were used as the control.

Semi-quantitative RT–PCR

Total RNA (1 µg) was heated at 85°C for 10 min and used as template for the first-strand cDNA synthesis. Residual DNA in RNA preparations was eliminated by digestion with RNase-free DNase I (Roche, Basel, Switzerland). The absence of DNA was checked by PCR. Reverse transcription was carried out using SuperScript retrotranscriptase (Invitrogen) in a 20 µl reaction volume containing 150 ng of random primers (Invitrogen), 1 mM deoxyribonucleotide triphosphate mix (GE Healthcare) and 10 mM DTT. The sequences of the specific primers used for each RT–PCR analysis are given in **Table 5**. The housekeeping gene *rnpB* (Vioque 1992) was used as an internal control to compensate for variations in the input of RNA amounts and normalize the results. The exponential phase of PCR for each gene was determined by measuring the amount of PCR product at different numbers of cycles. For the final results, 20–23 cycles were used for the analysis at the early exponential phase of PCR in all genes tested. The PCR products were resolved by electrophoresis in 1% agarose gel, stained with ethidium bromide and analyzed using a Gel Doc 2000 Image Analyzer (Bio-Rad).

EMSAs

Recombinant *Anabaena* sp. FurA protein was produced in *E. coli* BL21(DE3) using the expression vector pET28a (EMD Biosciences), and purified according to a previously described

Table 5 List of oligonucleotides used in this study

Primer	Sequence (5′ to 3′)	Purpose
2770FurA_up	CATATGACTGTCTACACAAATACTTCG	Cloning of *furA* into pAM2770
FurA_dw	GGATCCCTAAAGTGGCATGAGCG	Cloning of *furA* into pAM2770
rnpB_up	AGGGAGAGAGTAGGCGTTG	RT–PCR
rnpB_dw	AAAAGAGGAGAGAGTTGGTGG	RT–PCR
FurA_up	CGGGATCCATGACTGTCTACACAAATAC	RT–PCR
FurA-5last_dw	ACGCGTCGACCTAACGTTGGCACTTGGG	RT–PCR
psaA_up	TGGCAGTTGTGACAATTTGG	RT–PCR
psaA_dw	GGGCGAACAATACACCTTTC	RT–PCR
psaB_up	CTTCGGTCACCTGGCAATC	RT–PCR
psaB_dw	GCCAGGGCCAATTGTTAAG	RT–PCR
psaL_up	ATGGCGCAAGCAGTAGACG	RT–PCR
psaL_dw	AGTAAGCGACTACTGCACCACC	RT–PCR
psbA_up	GCTAATTTATGGCATAGATTCGG	RT–PCR
psbA_dw	GAAAGAAATGCAAAGAACGGG	RT–PCR
psbB_up	GTAGTTCTGAATGACCCAGGGC	RT–PCR
psbB_dw	CGGCGTGACCAAAGGTAAAC	RT–PCR
psbZ_up	TTATGAAGCGCTATTGGTCACG	RT–PCR
psbZ_dw	ACCCCGTTTTAGTGCTGATTC	RT–PCR
sodA_up	CTCTGTGGCAACGGTTTATTG	RT–PCR
sodA_dw	CTTTGCCGTGAAGCTTGG	RT–PCR
sodB_up	CCACTACCCTACGACTTTAATGC	RT–PCR
sodB_dw	AAGCTGGGCGAGCATTTC	RT–PCR
GCT1_up	ATGCCAGTTAAAGTTGG	RT–PCR
GCT1_dw	CTATTTACTCGCCAACTG	RT–PCR
GCT3_up	ATGCCTCTAGCGGTTGGTAC	RT–PCR
GCT3_dw	TTACAGTCCCAAAGCCGC	RT–PCR
gor_up	GGCTGCTTCTAAACGAGCTGC	RT–PCR
gor_dw	GGAGGGATGAATGCCAACG	RT–PCR
mreB_up	GTGGGGCTTTTTAGGAACTTTC	RT–PCR
mreB_dw	CTACATATTTCGAGATCGTCCG	RT–PCR
mreC_up	ATGGTTACTGTACGTCGTTGGTG	RT–PCR
mreC_dw	CTAGTTGGACTTTTGTTGCTGTG	RT–PCR
alr0397_up	CATTAATCAGCCATCCTGGC	RT–PCR
alr0397_dw	GTTTCGCGGAGATAAAATGC	RT–PCR
FurB_up	ATGAGAGCCATACGCAC	RT–PCR
FurB_dw	CTAAATTTCACTCGCGTG	RT–PCR
FurC_up	ATGCAGCAACAGGCAATATC	RT–PCR
FurC_dw	GAGGATGCCGATATCCATAG	RT–PCR
IsiA_up	CTGCTCTGACAACCCTCTGG	RT–PCR
IsiA_dw	CAGCTAACTTGACTGTATCGGC	RT–PCR
PnifJ_up	GCCTACTCTGCGAGTTCTCCG	EMSA
PnifJ_dw	GGCCTGTGAGAGTTGCTGCAC	EMSA

(continued)

Table 5 Continued

Primer	Sequence (5′ to 3′)	Purpose
PfurA_up	CTCGCCTAGCAATTTAACAAC	EMSA
PfurA_dw	GCCTTGAGCGAAGTATTTGTC	EMSA
PpsaAB_up	GCAAACTCAGCAAATTTGTCG	EMSA
PpsaAB_dw	GAGGACTAATTGTCATTCGACC	EMSA
All0107-1	GCAGCTACTATCTGGCTGAC	EMSA
All0107-2	GATGCGTCTACTGCTTGCG	EMSA
Alr3742-1	CAGCATTGGCAACGGCTCC	EMSA
Alr3742-2	CATACATTCGATTTTCCGTACTG	EMSA
Ppsb_up	GTAACGGCGATCTGTGGGAAG	EMSA
Ppsb_dw	CATCGACTACGCTCCTCCTCG	EMSA
All1258-1	GCACATAAGCAGACTACACAAG	EMSA
All1258–2	GACAAGGGCAAGCAGACGTG	EMSA
PsodA_up	GATAGAATCAATGACTGCTC	EMSA
PsodA_dw	GTTGTTGCCTCTTATTTG	EMSA
PsodB_up	GCTTAGTAGTCCCTTTGC	EMSA
PsodB_dw	CATTTTGTGAAGTCCTCTC	EMSA
GCT1-Prom_up	GGTTTGGCTGTAATTTATCG	EMSA
GCT1-Prom_dw	CAGAGTCTCCAACTTTAACTG	EMSA
GCT3-Prom_up	GATCGCACCTACGGAAACG	EMSA
GCT3-Prom_dw	GGCATAATGAACCTTTCCTG	EMSA
PGor-1	CTCTCTGGACATCGCTTAGT	EMSA
PGor-2	CCAGAACCAGCACCAATTAC	EMSA
PmreBC_up	GCCTTATCCTTTAGTAC	EMSA
PmreBC_dw	GAAAGTTCCTAAAAAGC	EMSA
Palr0397_up	CATCCTACATGAAGATTAATG	EMSA
Palr0397_dw	CGCCTATTATTATTGACTTG	EMSA

method (Hernández et al. 2002). The promoter regions of each gene of interest were obtained by PCR using the primers listed in Table 5. EMSAs were performed as described previously (Hernández et al. 2006b). Briefly, 100–120 ng of each DNA fragment were mixed with recombinant FurA protein at a concentration of 150, 300, 500 and 700 nM in a 20 µl reaction volume containing 10 mM bis-Tris (pH 7.5), 40 mM KCl, 100 µg ml^{-1} bovine serum albumin, 1 mM DTT, 100 µM $MnCl_2$ and 5% (v/v) glycerol. In some experiments, EDTA was added to a final concentration of 200 µM. To insure the specificity of EMSA, the promoter region of the *Anabaena* sp. *nifJ* (*alr1911*) gene was included as non-specific competitor DNA in all assays (López-Gomollón et al. 2007). Binding assay of the promoter region of the gene *furA* was included as the EMSA positive control (Hernández et al. 2004, Hernández et al. 2006b). Mixtures were incubated at room temperature for 20 min and subsequently separated on a 4% non-denaturing polyacrylamide gel in running buffer (25 mM Tris, 190 mM glycine) at 90 V. The gel was stained with SYBR® Safe DNA gel stain (Invitrogen) and processed with a Gel Doc 2000 Image Analyzer (Bio-Rad).

Supplementary data

Supplementary data are available at PCP online.

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