FEBS Letters 581 (2007) 1351–1356

FurA modulates gene expression of *alr3808*, a DpsA homologue in *Nostoc* (*Anabaena*) sp. PCC7120

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Received 22 January 2007; revised 15 February 2007; accepted 18 February 2007

Available online 2 March 2007

Edited by Stuart Ferguson

Abstract The DNA-binding protein from stationary phase (Dps) protein family plays an important role in protecting microorganisms from oxidative and nutritional stresses. In silico analysis of the promoter region of *alr3808*, a *dpsA* homologue from the cyanobacterium *Nostoc* sp. PCC7120 shows putative iron-boxes with high homology with those recognized by FurA (ferric uptake regulator). Evidence for the modulation of *dpsA* by FurA was obtained using in vitro and in vivo approaches. SELEX linked to PCR was used to identify P_{dpsA} as a FurA target. Concurrently, EMSA assays showed high affinity of FurA for the *dpsA* promoter region. DpsA expression analysis in an insertional mutant of the *alr1690-xfurA* message (that exhibited an increased expression of FurA) showed a reduced synthesis of DpsA. These studies suggest that FurA plays a significant role in the regulation of the DpsA.

Keywords: Cyanobacteria; Fur; Ferric uptake regulator; DpsA; Iron stress; Oxidative stress; Nutritional stress

1. Introduction

Prokaryotes have developed efficient mechanisms to adapt rapidly and to survive a wide range of environmental conditions. Some members of the DNA-binding protein from stationary phase cells (Dps) family of proteins play an important role in protecting cells from oxidative and nutritional stresses. Dps are divergent members of the bacterioferritin/ferritin superfamily [1], and several homologues have been identified in different bacteria, playing roles in iron storage and DNA protection [2].

DpsA protein from the cyanobacterium *Synechococcus* sp. PCC7942 is a DNA-binding hemoprotein having heme-dependent catalytic activity [1,3] likely conferring resistance to peroxide damage during periods of oxidative stress and long term nutrient starvation [3]. Peña and Bullerjahn [1] suggested that DpsA could be playing a role related to protection against the oxidative stress associated with oxygenic photosynthesis. Indeed, DpsA null mutants exhibit growth inhibition under high light and the cells were hypersensitive to paraquat and exogenously added peroxide [4]. Expression of *dps* in *Escherichia coli* is dependent on integration host factor (IHF) and the alternative sigma factor RpoS in the stationary phase, and on OxyR in the exponential phase [5]. Sen et al. [3] stated that *dpsA* from *Synechococcus* sp. PCC7942 is under the control of an alternative sigma factor reported previously in *Synechococcus* sp. PCC7002 [6]. Because *dpsA* transcription increases about 12 times under low iron conditions, it was concluded that this protein is essential for growth under iron starvation. However, Michel et al. [7] found a fairly high transcription under regular growth conditions. Iron starvation caused a slight increase of the *dpsA* message, while the protein DpsA was clearly decreased in iron deficient conditions, suggesting a post-translational regulation. A *dpsA*-mutant shows interesting responses to iron deficiency in comparison with the wild-type strain, with an altered transcriptional/translational pattern, including a severe drop in the photosystem II activity in the mutant compared to the wild-type [7].

Using $P_{dpsA-lacZ}$ reporter gene constructs, Sen et al. [3] identified a region required for metal-dependent repression which sequence was apparently different from the site recognized by *E. coli* Fur (ferric uptake regulator). For that reason, they proposed that *dpsA* in cyanobacteria is under the control of another class of metal-dependent repressor. The ferric uptake regulator (Fur) is known to act as a Fe^{2+}-dependent transcriptional repressor of bacterial promoters. Fur also modulates responses to oxidative stress, and actually, the Dps homologue *mrgA* from *Bacillus subtilis* is under negative control of the repressor PerR [8,9], a Fur homologue responsive to oxidative stress. In *Nostoc (Anabaena)* sp. PCC7120 there are three Fur family members, FurA, FurB and FurC [10]. FurA is an essential auto-regulated protein whose in vitro activity is affected by the presence of Mn^{2+} and the redox status of its cysteines [11]. FurA binds heme in the micromolar range, and this interaction affects the in vitro ability of FurA to bind DNA [10]. Even though results from data in the surveyed literature contained uncertain interpretation, the tight link between iron and DpsA clearly pointed to a Fur-regulated protein. In this work we describe evidence indicating that in *Nostoc* sp. PCC7120, FurA participates in the regulation of the *dpsA* homologue *alr3808* expression.

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Abbreviations: Dps, DNA-binding protein from stationary phase; Fur, ferric uptake regulator; SELEX, systematic evolution of ligands by exponential enrichment; EMSA, electrophoretic mobility shift assays

0014-5793/$32.00
doi:10.1016/j.febslet.2007.02.053

2. Materials and methods

2.1. Strains and culture conditions

This study was carried out with the heterocyst-forming cyanobacterium *Nostoc* sp. strain PCC 7120 and the JAH3 mutant strain, which produces increased levels of FurA due to the disruption of the *alr1690-αfurA* message [12]. *Nostoc* sp. PCC7120 was obtained from the Pasteur Culture Collection [13]. Cells were photoautotrophically grown at 30 °C in $BG11_0C$ medium [13], supplemented with 0.84 g of $NaHCO_3$ per litre, in the presence of 8 mM NH_4Cl plus 16 mM TES [*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid]–NaOH buffer [pH 7.5] as a nitrogen source. Cultures were bubbled with a mixture of CO_2 (1% [vol/vol]) and air, and supplemented with 2 μg of streptomycin and 2 μg of spectinomycin ml^{-1} for mutant strains. Cells were harvested at the late exponential phase of growth (7–9 μg Chl/ml culture).

2.2. Cloning and overexpression of furA gene, and purification of the recombinant product

FurA was cloned and overexpressed as described previously [14]. The purification of the FurA protein for binding assays was performed according to [15].

2.3. SDS–PAGE and Western blotting

Crude extracts were prepared by sonication of *Nostoc* sp. PCC7120 cells followed by centrifugation to remove cell debris. Protein content was quantified using a bicinchoninic acid protein assay reagent (Pierce), and proteins were separated on 15% SDS–PAGE. For immunoblotting, proteins were electrophoretically transferred to a PVDF membrane (0.45 μm pore size transfer membrane from Waters), as described previously [16]. Rabbit polyclonal antibodies raised against *Nostoc* FurA protein were obtained as described in [15]. DpsA antibodies against the *Synechococcus elongatus* sp. PCC7942 protein were a kind gift from Prof. G.S. Bullerjahn.

2.4. RNA isolation and RT-PCR analysis

RNA was isolated according to [12]. Reverse transcription-PCR reactions were performed in a volume of 10 μl, using 1 μg RNA per reaction. The RT reaction was performed using the Superscript II kit (Invitrogen). *rnpB* transcript was used as internal control. Oligonucleotides dpsA-dir and dpsA-rev were used to analyze the levels of *dpsA* mRNA (Table 1). The products were amplified by PCR and then analyzed by electrophoresis on a 1% agarose gel. All the assays were repeated in the presence of DNase-free RNase as a negative control.

2.5. Electrophoretic mobility shift assays

A 245-bp fragment from nt −2 to −247 upstream the coding sequence of *dpsA* was amplified by PCR with the oligonucleotides PdpsA1 and PdpsA2 and was used in non-radioactive band-shift assays, as described previously [10]. Primers were designed according to the sequences available in the cyanobase (http://www.kazusa.or.jp/cyano/anabaena/). Binding assays were carried out in a final volume of 20 μl containing 10 mM Bis–Tris, pH 7.5, 40 mM KCl, 0.1 mM $MnCl_2$, 1 mM DTT, 0.05 mg/ml BSA, 75–100 ng of the DNA fragment to be tested, 75 ng of a control DNA fragment, and 5% glycerol. The DNA fragment used as non-related control DNA was a 224 bp non-specific competitor DNA (fourth exon of the human *apoE* gene). Assays were carried out with purified FurA protein. The binding was evaluated by estimating the remaining unbound DNA in each sample, compared to the band measured for free DNA, taken to be 100% (Gel Doc 2000 Image Analyser from BioRad).

2.6. In silico analysis of the promoter regions

Identification of putative iron boxes in the promoter region of *dpsA* was performed using ClustalW (http://www.ebi.ac.uk/clustalw). The DNA sequence containing 350-bp located upstream *dpsA* coding sequence was aligned with the iron boxes identified in P_{isiB} and P_{furA} in *Nostoc* sp. PCC7120 [12,17] and with the *E. coli* consensus GATAATGATAATCATTATC [18].

2.7. Cyclic selection procedure

The method was performed according to Valsangiacomo et al. [19], with some modifications. Chromosomal DNA from the *Nostoc* sp. strain PCC7120 isolated according to [20] was partially digested with *Nhe*I, *Spe*I, and *Xba*I. DNA fragments of approximately 200–900 bp were gel-purified using the GFX kit (Pharmacia). Two single-stranded oligonucleotides (Table 1) with complementary sequences and a compatible protruding end in oligonucleotide 1 were used in order to build an identical end in all DNA fragments (see Fig. 1). Oligonucleotide 1 was phosphorylated with T4 polynucleotide kinase (Takara) according to manufacturer instructions. For the annealing, a reaction mixture containing a total volume of 35 μl with 0.2 nmol of each oligonucleotide was maintained at 80 °C for 5 min, followed by slow cooling down to room temperature. Finally, the digested chromosomal DNA and the linker sequence formed by the annealed oligonucleotides were mixed in 15 μl, containing 250 ng of chromosomal DNA fragments, 2 μg of oligonucleotides and 2 U of T4-DNA ligase (Fermentas) in the buffer provided by the DNA-ligase manufacturer. The resulting DNA fragments were amplified by PCR using linker-specific primer C-3 (Table 1), designed to allow amplification. A single PCR primer was used in each reaction because the complementary sequence was present in both ends of the template DNA fragments. PCR products were purified using the GFX kit (Pharmacia).

In order to isolate DNA fragments capable of binding to FurA, 10–50 ng DNA of the purified PCR reaction were mixed in 20 μl final volume of binding buffer [10] with 700 nM purified FurA. After incubation of the reaction mixture for 30 min at room temperature, the sample was electrophoresed in 7% polyacrylamide gels. The retarded DNA fragments were isolated from the gel using the GFX kit (Pharmacia) and a new cycle of PCR was performed. The amplified DNA fragments were then subjected to five more rounds of FurA binding

Table 1
Oligodeoxyribonucleotides used in this study

#	Name	Sequence (5′–3′)	Description
1	proN	ctcgcctagcaatttaacaac	*furA* Promoter, direct
2	proC	gccttgagcgaagtatttgtg	*furA* Promoter, reverse
3	p2473-N	cttacttattgcccgatccc	*furB* Promoter, direct
4	p2473-C	gcgtatggctctcatagac	*furB* Promoter, reverse
5	p0957-N	cattggtcatcggtcattaga	*furC* Promoter, direct
6	p0957-C	gcctgttgctgcatatttatg	*furC* Promoter, reverse
7	fld-PN	gtcgcactcactttcgttac	*isiB* Promoter, direct
8	fld-PC	ccgtagaataaaccaattttc	*isiB* Promoter, reverse
9	pdpsA1	ctctactagcaaggcggagc	Promoter *dpsA* direct
10	pdpsA2	gttctcctcgcttttgggcag	Promoter *dpsA* reverse
11	dpsA-dir	tctagctagctttcaagcac	*dpsA* Sense strand
12	dpsA-rev	ctgagccgcttggcgac	*dpsA* Antisense strand
13	C-1	ctagggagtgactcttgacccgggcatgcgca	Cyclic selection procedure
14	C-2	tgcgcatgcccgggtcaagagtcactcc	Cyclic selection procedure
15	C-3	cccgggtcaagagtcactcc	Linker specific primer
16	rnpB-dir	aagccgggttctgttctctg	*rnpB* Direct
17	rnpB-rev	atagtgccacagaaaaataccg	*rnpB* Reverse

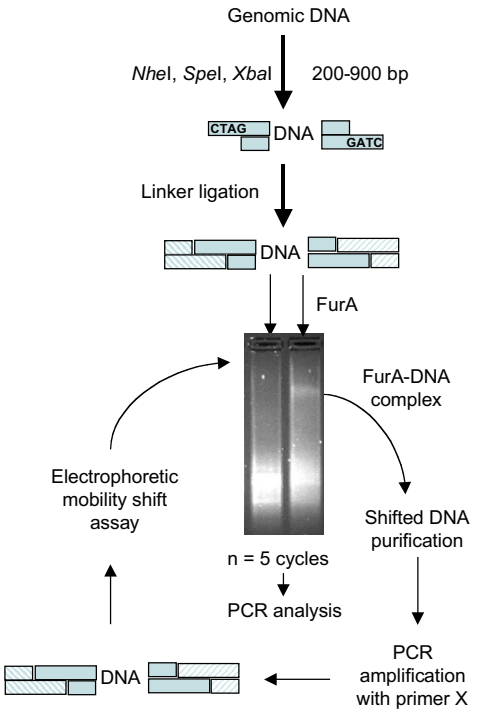

Fig. 1. Scheme of the SELEX procedure linked to PCR.

and PCR amplification. Presence of P_{furA}, P_{furB}, P_{furC}, P_{isiB}, and P_{dpsA} in the starting pool of DNAs and in the DNA fragments recovered after five selection cycles was directly assessed by PCR amplification using primers 1–10 listed in Table 1.

3. Results

3.1. Identification of a dpsA homologue in Nostoc sp. PCC 7120

Nostoc sp. PCC7120 genome does not include any ORF annotated as *dpsA;* however, *alr3808*, identified as a "nutrient-stress induced DNA binding protein", has high identity (68.8%) with *Synechoccocus* DpsA. The Alr3808 protein showed a conserved region of about 50 residues in their central region, characteristic of Dps proteins (Fig. 2). The presence of several histidine residues in conserved positions suggested that some of those amino acids could be involved in heme-binding by apo-Dps.

3.2. Analysis of the dpsA promoter region

Previous analysis of the *dpsA* promoter from *Synechococcus* sp. PCC7942 failed to reveal a Fur box sequence [3,21]. More recently, it has been shown that FurA from *Nostoc* binds to a series of A/T-rich sequences present in the promoters of *furA* and *isiB* [17]. While the GAT(A/T)AT "unit" of the Fur-binding motif proposed for *E. coli* is highly conserved in most promoters of Fur regulated genes from *Pseudomonas* sp. and *B. subtilis* [22,23], the number of matches with the cyanobacterial sequences recognised by FurA is lower. Alignment of the identified Fur binding sites with the canonical consensus from *E. coli* showed that the exact positions of A or T nucleotides is not critical [17]. Alignment of the *dpsA* promoter with the FurA-protected regions in P_{furA} and P_{isiB}, as well as with the *E. coli* consensus, shows several A/T rich, potential Fur-boxes highly homologous to the Fur-binding sites determined experimentally (Fig. 3A).

It has been reported that the alternative sigma factor *sigE* is involved in the switch of *dpsA* in *Synechococcus* sp. PCC7002 stationary phase gene expression [6]. Moreover, a potential operator involved in *dpsA* regulation has been identified by using different promoter constructs [3]. An A/T rich sequence showing high homology with the region upstream this putative repressor-operator is present in the *dpsA* promoter from *Nostoc* (Fig. 3), suggesting that in addition to *sigE*, FurA might act as a repressor of *dpsA* in cyanobacteria.

3.3. FurA binds specifically to dpsA promoter

A 245-bp fragment from the promoter region of *dpsA* from *Nostoc* sp. PCC7120, containing the putative iron-boxes was

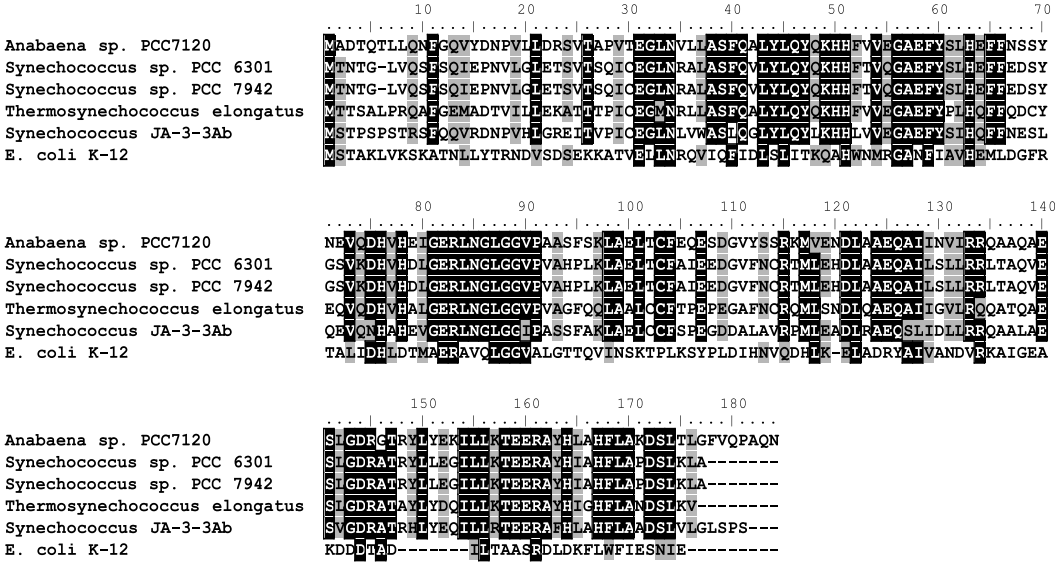

Fig. 2. Alignment of Alr3808 from *Nostoc* sp. PCC 7120 with Dps proteins from several cyanobacteria and *Escherichia coli*. Conserved amino acid residues are shadowed in black (identical residues) or grey (side chains belonging to the same group).

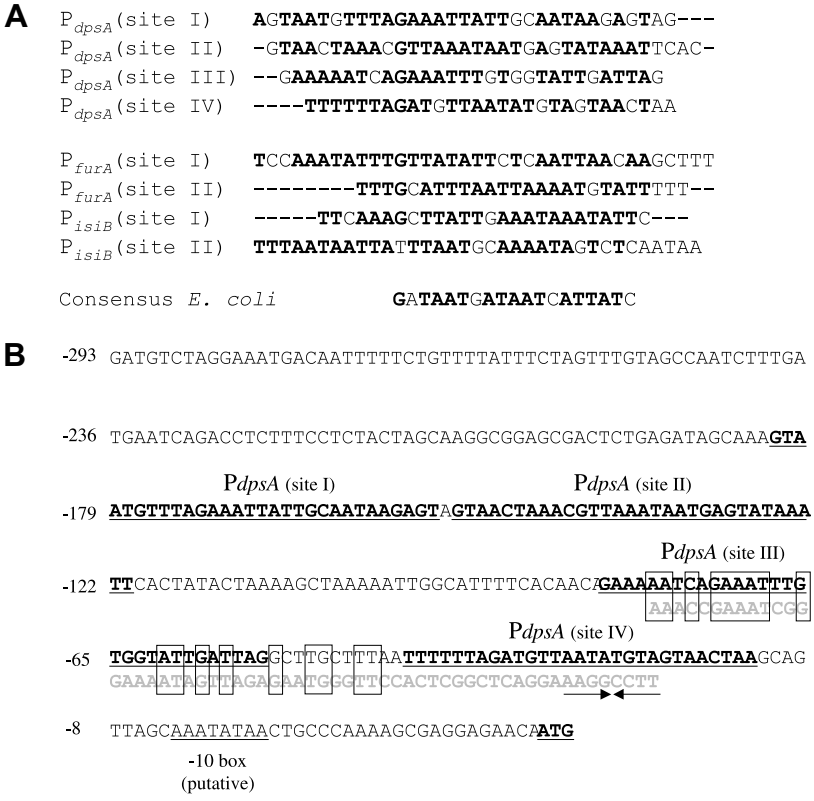

Fig. 3. Nucleotide sequence analysis of the upstream region of *dpsA* gene from *Nostoc* sp. PCC7120. (A) Alignment of the A–T rich regions from *PdpsA* with the Fur-protected regions found in *PfurA* and *PisiB* and the canonical consensus from *E. coli*. (B) Promoter region of *dpsA* from *Nostoc* showing the putative iron boxes, which are shown bold and underlined. The region in the promoter of *dpsA* from *Synechococcus* sp. PCC7942 showing high homology with the *Nostoc* sequence, in grey letter, is aligned with the *Nostoc alr3808* promoter. Identical bases are indicated by boxes, and the location of the repeat sequence studied in [3] is represented by arrows. The putative −10 box and the translation start site are underlined.

prepared by PCR and purified as described in the procedures section. Electrophoretic mobility shift assays (EMSA) was performed using purified FurA incubated with the amplified P_{dpsA} and a non-related competitor DNA. Fig. 4 shows that FurA binds specifically to P_{dpsA}.

3.4. Isolation of dpsA promoter from an enriched pool of Nostoc DNA fragments by a modified SELEX procedure linked to PCR

As part of an ongoing effort in our laboratory to identify and characterize some FurA-regulated genes in *Nostoc*, DNA fragments with potential FurA binding sites were isolated from *Nostoc* chromosomal DNA by a SELEX-like procedure. DNA fragments present in an enriched chromosomal DNA library (see Section 2), were incubated with purified FurA. The resulting FurA-DNA complexes were enriched exponentially using EMSA followed by purification of the retarded FurA-DNA and further PCR. This procedure was performed repeating the cycle five times (Fig. 1).

To identify whether P_{dpsA} was among the DNA fragments recognized by FurA, the FurA–DNA complexes were collected after five cycles of this SELEX-like procedure in several independent experiments. The selected DNA pool was used as a template in a PCR reaction, in comparison with the starting pool of DNA fragments. The promoter of the *dpsA* gene was identified as one of the FurA targets, like the promoters of other FurA-regulated genes, such as P_{isiB} and P_{furA}, which were also amplified by PCR from the retarded DNA fragments after five cycles of enrichment. In contrast, the promoter regions of other non-regulated genes that were clearly present

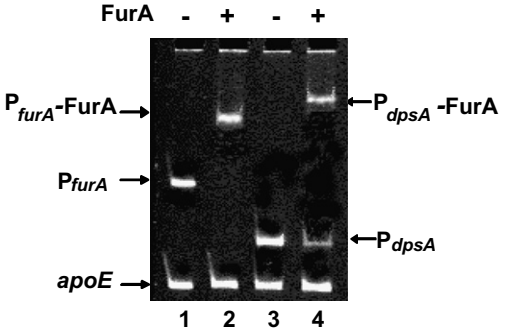

Fig. 4. Gel mobility shift assays of the promoter region of *alr3808* in the presence of purified FurA. A 245-bp fragment containing the putative iron-boxes was incubated with 500 nM FurA in the presence of an equimolar amount of unrelated DNA and analyzed for reduced mobility in a native gel. Binding of FurA to its own promoter was used as positive control. Lane 1: *furA* promoter region. Lane 2: *furA* promoter region in presence of FurA. Lane 3: *alr3808* promoter region. Lane 4: *alr3808* promoter region in the presence of FurA. Assays were performed in the presence of 100 μM $MnCl_2$ and 1 mM DTT. A fragment of exon IV from the human *apoE* gene was used as non-specific competitor DNA.

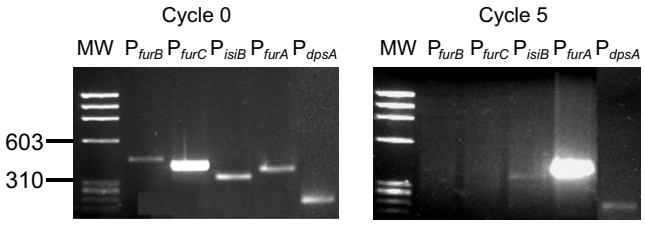

Fig. 5. PCR analysis showing the presence of P_{furA}, P_{furB}, P_{furC}, P_{isiB}, and P_{dpsA} in the starting pool of DNAs (left panel) and the recovery of P_{furA}, P_{isiB}, and P_{dpsA} after five selection cycles (right).

in the starting library, such as P_{furB} or P_{furC}, could not be amplified after the selection cycles (Fig. 5).

3.5. *dpsA* is down-regulated in Nostoc mutants with increased FurA expression

Previous analysis of the *furA* expression in wild-type cells in comparison to the JAH3 mutant showed that the absence of the antisense *furA* RNA resulted in a marked increase of the FurA synthesis [12]. Since Δ*furA* mutants in *Nostoc* sp. PCC7120 could not be isolated [12], the JAH3 strain was a valuable tool to investigate deregulation of Fur regulated genes. To test whether the level of FurA affected transcription and translation of *dpsA*, RT-PCR and Western analysis were performed in the *Nostoc* WT and the JAH3 mutant. The Western analysis showed a single, high molecular weight signal

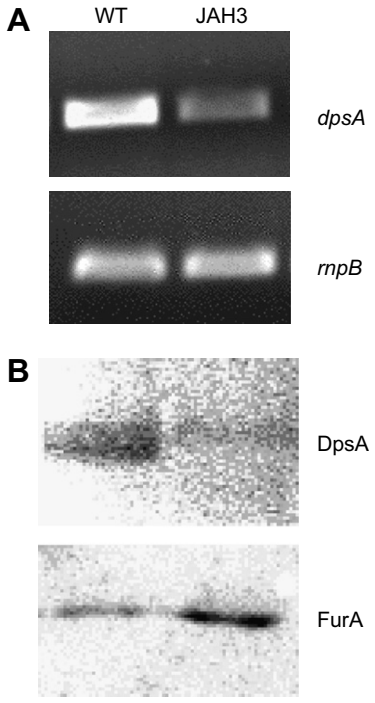

Fig. 6. (A) RT-PCR analysis of the *dpsA* transcript in *Nostoc* sp. PCC 7120 and the JAH3 strain. Cells were grown in BG11 in the presence of ammonium (please, see Section 2) and harvested in the late exponential phase. RT-PCR of *rnpB* was performed as internal control. (B) Western blot analysis of DpsA expression. 25 μg of total protein from WT and JAH3 strains were separated on 15% PAGE, transferred to PVDF and analyzed using polyclonal antibodies anti-DpsA from *S. elongatus* sp. PCC7942 (kind gift of Prof G.S. Bullerjahn). Lane 1: Crude extract of wild-type *Nostoc* sp. PCC7120. Lane 2: Crude extract of JAH3 mutant.

probably corresponding to a hexameric aggregate of DpsA, as has been observed in *Synechococcus* sp. PCC7942 [1]. Fig. 6 shows that in the JAH3 mutant, transcription of *dpsA* notably decreased, according to the amount of immunodetected DpsA that was around three times lower than in the wild-type strain. Therefore, in vivo *dpsA* expression correlates well with the expression of FurA.

4. Discussion

There is strong evidence supporting the link between Dps proteins and iron metabolism in many prokaryotes. In *E. coli*, the interaction of DNA with the negatively charged surface of Dps is finely tuned by the intracellular concentration of divalent cations [24]. Depending on the nutritional status of the cells and the pressure of environmental conditions, Dps proteins can act as ferritins and as a defense against oxidative stress via either the ability in non-specific DNA binding or the function in protection of DNA from Fenton mediated damage. Dps homologue *mrgA* from *B. subtilis* is under negative control of the repressor PerR [8,9], a Fur homologue belonging to the Fur family. DpsA of *Synechococcus* sp. PCC7942 is a DNA-binding hemoprotein possessing a weak catalase activity, with no ferroxidase activities described [1]. There is no data about the DpsA from *Nostoc*, but the high identity with the protein from *Synechococcus* indicates that its characteristics are very similar between each other.

While the *dps* homologue *mrgA* from *B. subtilis* is under negative control of the repressor PerR [8,9], transcription in *Synechococus* is not sensitive to peroxide treatment [4]. This fact, together with the low homology with Fur boxes of the operator region identified in *Synechoccocus*, suggests that *dpsA* may be under the control of a divergent Fur-like repressor, in addition to the sigma switching control previously described [3,6]. In our opinion, the presence of Fur-binding sequences in P_{dpsA} from *Synechococcus* should also be considered since homology of cyanobacterial iron-boxes with the canonical consensus described in *E. coli* is relatively low.

Systematic Evolution of Ligands by Exponential Enrichment (SELEX) is a powerful tool for isolation of target nucleic acid sequences when a purified sequence specific binding protein is available. To obtain relevant DNA fragments, we used a SELEX-like procedure followed by PCR in order to isolate FurA-binding fragments. As a result, the fragment with *dpsA* upstream sequences was found among others, after five cycles of selection. This was a very good indication of *dpsA* as a FurA-regulated gene, which was reinforced by our *in vivo* results (Fig. 6).

In good concordance with the results observed in *Synechococcus*, transcription of *dpsA* in *Nostoc* does not increase after peroxide or paraquat treatment (RT-PCR analysis, data not shown). The crosstalk between iron metabolism, oxidative stress, and redox regulation is even tighter in photosynthetic organisms. However, it does not mean that synthesis of DpsA in cyanobacteria should respond to the different stresses in identical fashion than the protein from heterotrophic bacteria. Cyanobacteria present a battery of iron-responsive genes, such as *idiA*, *idiB*, *isiA*, or *isiB*, which are also induced by oxidative stress. Some of those genes are directly involved in the protection of the photosynthetic machinery against photo-oxidative stress. The protective function exerted by DpsA in *E. coli*

and other heterotrophic bacteria could be carried out by a different mechanism in cyanobacteria.

Michel et al. [7] found already high levels of *dpsA* transcription in iron replete conditions. The level of *dpsA* mRNA decreases in mild iron starvation, and it rises slightly under severe iron deficiency. On the other hand, Sen et al. [3] described that *dpsA* was essential for growth under iron-deficient conditions and *dpsA* mRNA levels were 12-fold increased when iron was limiting, more in accordance with a Fur derepression. These results were obtained with cells in the stationary growth phase; however, the Northern blot analysis performed by Michel et al. [7], was carried out using cells apparently in exponential growth phase, where *dpsA* transcription is much lower [4].

It is noticeable that the raise of *dpsA* mRNA under iron limitation is not accompanied by increasing protein concentrations [7]. Since the ability of the Dps protein complex to bind DNA is regulated by the presence of bound metal ions [24], these results could be explained assuming that in the diverse conditions tested, the distribution of the total Dps protein between the soluble pool and the DNA-bound fraction could be different, being this later hardly detected by antisera [25].

In our case, the role of DpsA as an iron-storage protein would be in good concordance with the lower amount of DpsA in the FurA overexpressing strain whose endogenous content of iron is lower than in the wild-type *Nostoc* but still enough to prevent starvation (our unpublished results).

In this work we show for the first time that a *dps* homologue gene is Fur-regulated. Evidence includes (a) EMSA showed that FurA bound specifically to the *dpsA* promoter region, (b) P_{dpsA} was recovered after five cycles of SELEX, (c) transcription and translation of *dpsA* were down-regulated in a FurA overexpressing mutant, and (d) several putative iron boxes were identified using cyanobacterial consensus. All these data indicate that *Nostoc* FurA modulates *dpsA* expression.

Acknowledgments: We thank Dr. Bullerjahn for his kind gift of anti-DpsA antibodies. This work was supported partially by Grants CTM2005-03751 and BFU2006-03454 from Ministerio de Educación y Ciencia (MEC) of Spain. J.A.H. was recipient of a FPU (MEC) fellowship and S.P. was granted by Diputacion General de Aragon.

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