2-oxoglutarate modulates the affinity of FurA for the *ntcA* promoter in *Anabaena* sp. PCC 7120

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2-oxoglutarate (2-OG) is a central metabolite that acts as a signaling molecule informing about the status of the carbon/nitrogen balance of the cell. In recent years, some transcriptional regulators and even two-component systems have been described as 2-OG sensors. In the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120, two master regulators, NtcA and FurA, are deeply involved in the regulation of nitrogen metabolism. Both of them show a complex intertwined regulatory circuit to achieve a suitable regulation of nitrogen fixation. In this work, 2-OG is found to bind FurA, modulating the specific binding of FurA to the *ntcA* promoter. This study provides evidence of a new additional control point in the complex network controlled by the NtcA and FurA proteins.

Keywords: 2-oxoglutarate; *Anabaena* sp. PCC 7120; FurA; NtcA

Cyanobacteria frequently use nitrate, nitrite, and ammonium as nitrogen sources in natural environments. However, some species are able to fix atmospheric N_2 in conditions in which combined nitrogen availability is insufficient. Nitrogen fixation is performed by the nitrogenase complex, whose activity is greatly affected by the presence of oxygen. In order to avoid enzyme inactivation by molecular oxygen, multicellular nitrogen-fixing cyanobacteria spatially separate photosynthesis and nitrogen fixation. Such fixation is restricted to differentiated cells called heterocysts [1,2]. Heterocyst differentiation is a complex process that requires the actuation of different regulatory and structural genes at specific stages during nitrogen deficiency [3,4]. NtcA and HetR are two transcriptional

regulators involved in the hierarchical activation of different proteins that finally trigger heterocyst formation. NtcA is a transcriptional regulator belonging to the CRP (cAMP receptor protein) family whose regulon comprises genes involved in nitrogen scavenging and assimilation but also genes included in other diverse categories [5]. Upon nitrogen decline, an increase in 2-oxoglutarate (2-OG) levels activates NtcA that is present at low levels under nitrogen-replete conditions. Then, NtcA binds its own promoter increasing the levels of the NtcA protein and in turn triggering the transcriptional response of this global regulator [6]. Most promoters of genes that are activated by NtcA comprise the consensus binding site TGTA-(N_8)-TACA centered at -41.5 nucleotides upstream of the

Abbreviations
2-OG, 2-oxoglutarate; EMSA, electrophoretic mobility shift assay; ITC, isothermal titration calorimetry.

transcription start site [7]. After nitrogen deprivation, NtcA activates in the first place the expression of nitrate assimilation proteins (*nir* genes) and ammonia translocators (*amt* genes) in order to obtain combined nitrogen sources [7]. Afterward, the process of heterocyst differentiation starts with the expression of the *nrrA* and *hetC* genes, followed by activation of *xisA* and *devBCA* and finally *pipX*, *cox*, and *nifHDK* [3]. On the other hand, NtcA is able to activate the expression of HetR through NrrA [8].

2-oxoglutarate is a central metabolite playing an important role in the Krebs cycle and in NH_4^+ assimilation through the GS/GOGAT cycle. Apart from being a metabolite involved in the central metabolism, 2-OG seems to be a signal molecule in prokaryotes and eukaryotes [9]. In bacteria and archaea, some proteins act as 2-OG sensors such as the PII protein, NifA, NrpR, PTS ^{Ntr}, GltC, and the two-component system KguS/KguR, most of them being involved in the regulation of nitrogen and carbohydrate metabolism but showing different action mechanisms [9].

In cyanobacteria, NtcA seems to sense the cellular carbon/nitrogen balance by binding to 2-OG since its accumulation indicates an increase in the carbon/nitrogen ratio and, therefore, a symptom of nitrogen deficiency [10]. NtcA is not the only protein in *Anabaena* PCC 7120 able to bind 2-OG. The PII protein also binds this molecule and the regulatory activity of PipX depends on the presence of 2-OG, both of them being deeply involved in the regulation of nitrogen metabolism [11]. The crystal structure of NtcA in complex with 2-OG has already been solved [12,13]. NtcA comprises a C-terminal helix–turn–helix DNA-binding domain and an N-terminal effector domain that contains a pocket in which oxoglutarate binds. This binding triggers conformational changes that are transmitted to the DNA-binding domain, possibly resulting in a better joining to DNA [13]. A tighter coiled-coil conformation of two C helices promotes a suitable distance between two F helices that is crucial for DNA recognition [13].

FurA from *Anabaena* PCC 7120 is a master regulator that senses iron availability and the redox state of the cell, regulating the expression of many genes involved in iron homeostasis but also in many other important cellular processes [14,15]. *In vitro* and *in silico* analysis has shown a significant overlap between targets regulated by FurA and NtcA proteins from *Anabaena* PCC 7120 [14]. Moreover, the expression of FurA is notably upregulated after nitrogen deprivation, mainly in proheterocyst and heterocyst cells [16]. This response is modulated by NtcA since this induction was not observed in a *ntcA* deletion mutant [16].

Finally, it has been found that FurA was able to bind *in vitro* the *ntcA* and *hetR* promoters, which was consistent with the altered expression of these genes in a *furA*-overexpressing strain [17]. Since it seems clear that FurA has an important role in the regulation of nitrogen metabolism, we speculated whether FurA could sense the nitrogen status of the cell through its interaction with 2-OG. In the present study, we demonstrate stoichiometric binding of 2-OG to FurA using isothermal titration calorimetry (ITC). We also show the effect of this interaction on the ability of FurA to bind its target promoters.

Experimental procedures

Purification of FurA wild-type and mutant variants

Recombinant *Anabaena* sp. FurA protein and R24R63FurA and R70FurA variants were produced in *Escherichia coli* BL21 (DE3) using the expression vector pET28a (EMD Biosciences) and purified following a previously described method [18]. A R24R63FurA_-pET-28a plasmid containing a double mutation in two arginines located in positions 24 and 73 and a R70Fur-A_pET-28a plasmid holding a single mutation in arginine located in position 70 were obtained by site-directed mutagenesis (synthesized by GenScript). In both FurA variants, the arginines were replaced by alanines.

The FurA, R24R63FurA, and R70FurA proteins were purified as follows. Briefly, 10 g of *E. coli* cell paste overexpressing recombinant Fur variants was resuspended in 50 mL of buffer A (0.1 M NaH_2PO_4, 0.01 M Tris, 2 M guanidine/HCl, pH 8) containing 1 mM PMSF and disrupted by sonication. Cell lysates were clarified by centrifugation at 18.000 *g* for 30 min, filtered through an 0.45-μm-pore-size filter (Merck Millipore), and applied to a previously equilibrated 10 mL Zn-iminodiacetate column (Chelating Sepharose Fast Flow; GE Healthcare). The column was first washed with 5 vol. of 0.5 M $(NH_4)_2SO_4$ in buffer A and then with 35 mM glycine in buffer A until A_{280nm} was lower than 0.1. FurA was eluted with a 3 vol. linear gradient of 0–1 M imidazole in buffer A. Selected fractions were pooled and dialyzed against 10 mM acetic acid/acetate buffer at pH 4.

Similarly, 10 g of *E. coli* cells overexpressing FurA was resuspended in Tris 50 mM pH 8 containing 200 U of DNase, 4 mM of $MgCl_2$, and 1 mM PMSF and sonicated. Cell lysate was clarified by centrifugation at 18.000 *g* for 30 min, filtered through an 0.45-μm-pore-size filter (Merck Millipore), and applied to a

previously equilibrated heparin column. The column was washed with 10 vol. of Tris 50 mM at pH 8 and then eluted with a 5 vol. linear gradient of 0–1 M of Tris 50 mM, pH 8 NaCl. Selected fractions were pooled and dialyzed against Tris 50 mM, pH 8, 150 mM NaCl.

Prediction of a three-dimensional model for the structure of FurA from Anabaena sp. PCC 7120

The structural model for FurA from *Anabaena* sp. PCC 7120 was constructed using the SWISS-MODEL server (https://swissmodel.expasy.org/) that creates automated protein structure homology models. The template was selected by sequence comparison using the BLAST program and PDB structures, considering statistical significance higher than a total score of 50. The structures of Fur from different microorganisms, namely *Francisella tularensis* (PDB codes: 5NBC and 5NHK), *Magnetospirillum gryphiswaldense* MSR-1 v2 (PDB codes: 4RAZ and 4RAY), *Vibrio cholera* (PDB code: 2W57), *Campylobacter jejuni* subsp. *Jejuni* (PDB codes: 4ETS and 6D57), *Helicobacter pylori* 26695 (PDB code: 2XIG), and *E. coli* BL21 (PDB code: 2FU4), were suitable as templates. The ModFOLD Model Quality Assessment Server was used to obtain a single score and *P*-value relating to the predicted quality of each 3D model of the FurA structure and to rank the different 3D models of FurA according to the predicted model quality (Table S1). The structural model with the best quality score was finally evaluated using the PROCHECK validation program (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/), the Pro-SA web (https://prosa.services.came.sbg.ac.at/prosa.php), ProQ (https://proq.bioinfo.se/ProQ/ProQ.html; Table S2), and MolProbity (http://molprobity.biochem.duke.edu/; Table S3).

A model of FurA complexed with DNA was constructed using the TFmodeller web server (http://maya.ccg.unam.mx/˜tfmodell/). For this purpose, the sequence of FurA *Anabaena* sp. PCC 7120 was introduced in this 3D footprint database of DNA-binding protein structures and was used to find a crystallized protein in complex with DNA that was homologous to FurA from *Anabaena* sp. PCC 7120. The template selected was the Fur-DNA complex of *M. gryphiswaldense* MSR-1 v2 (PDB code: 4RB3).

Prediction of 2-oxoglutarate-binding sites

The prediction of putative 2-OG-binding sites of FurA from *Anabaena* sp. PCC 7120 was carried out using the online docking web server SwissDock (http://www.swissdock.ch/) and the previously obtained structural models of FurA, both free and in complex with DNA. PYMOL software was used for the structure visualization and production of figures.

Isothermal titration calorimetry

FurA, R24R63FurA, and R70FurA interaction with 2-OG was analyzed with a MicroCal Auto-iTC200 calorimeter (Malvern Panalytical) at a constant temperature of 25 °C. Protein solution was used at a final concentration of 20 μM in freshly prepared 10 mM acetic acid/acetate buffer pH 4 to avoid protein precipitation or in Tris 50 mM, pH 8, 150 mM NaCl. Similarly, ligand solution containing 200 μM 2-OG or 200 μM glutamate was prepared in 10 mM acetic acid/acetate buffer pH 4 or in Tris 50 mM, pH 8, 150 mM NaCl. A sequence of 2 μL injections ($0.5\ \mu L \cdot s^{-1}$ injection rate) spaced 150 s apart and a stirring speed of 750 r.p.m. was programmed. The dissociation constant was obtained through nonlinear least squares regression analysis of the experimental data to a model for a single ligand-binding site for 2-OG in FurA. When necessary, 1 mM of a reducing agent (DTT) and/or 100 μM Mn^{2+} was added to the solutions in order to assess their influence on the FurA-2-OG interaction. Appropriate controls were performed: calcium-EDTA titrations to calibrate/test the calorimeter, and 2-OG dilution in buffer in order to evaluate the effect of heat associated with the process.

Electrophoretic mobility shift assay

Gene promoters used in the analyses consisted of a 150–350 bp DNA fragment upstream of ATG. These were obtained by PCR, using the *Anabaena* sp. PCC 7120 genome as a template. Primers used in the PCR amplifications were $P_{ntcA\text{-}for}$ (5′-GCAATCTA-TACCCCTATACCC-3′) and $P_{ntcA\text{-}rev}$ (5′-CTTATCTTGTGTCACGATCATC-3′) for the *ntcA* promoter, P_{isiB_up} (5′-CTTCTCTACGTTTGCGC-3′) and P_{isiB_dw} (5′-CATTATGACACCTGATCTTTAG-3′) for the *isiB* promoter, and if*pkn*_up (5′-AAAGATGAATTA-CACTGGCG-3′) and if*pkn*_dw (5′-CTGCAAACTGTGGCAGAATA-3′) for nonspecific competitor DNA. Binding assays were carried out incubating purified FurA wild-type or FurA variants in 10 mM Bis/Tris, pH 7.5, 40 mM KCl, 2 mM $MgCl_2$, 0.05 mg·mL^{-1} BSA, 1 mM DTT, 1 mM $MnCl_2$, and 5% glycerol with 50 ng of DNA promoters in standard Electrophoretic mobility shift assays (EMSAs) and with 0.1 pmol of each promoter in competition assays. When required, concentrations between 0.3 and 1 mM of 2-OG were

tested. The mixture was incubated for 30 min at room temperature and loaded into nondenaturing 6% polyacrylamide gels. The gels were stained with SYBR ® Safe (Invitrogen) and visualized in a Gel Doc 2000 device (Bio-Rad). Specificity of FurA binding to the studied promoters was tested including an internal fragment of the *pkn22* gene (if*pkn22*) as a nonspecific competitor DNA in all reactions. Densitometric analysis of the bands appearing in the EMSAs was performed with Quantity One Analysis Software (Bio-Rad), and the results were expressed as percentages of unbound DNA.

Multiple sequence alignments

Protein sequences of FurA orthologs in different cyanobacteria were retrieved from Cyanobase (http://genome.microbedb.jp/cyanobase/). Regarding heterotrophic bacteria, the FurA protein sequence of *Anabaena* sp. PCC 7120 was compared with the proteins encoded in the whole genome of each heterotrophic bacterium by using BlastP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE = Proteins). Those proteins showing the highest score in the comparison were selected as potential FurA orthologs. Multiple sequence alignments between sequences retrieved from cyanobacteria as well as from heterotrophic bacteria were performed with ClustalW (https://www.genome.jp/tools-bin/clustalw).

Results

Identification of two potential 2-oxoglutarate-binding sites in FurA from Anabaena sp. PCC 7120

Since the crystal structure of *Anabaena* sp. PCC 7120 FurA is lacking, a model of the tridimensional structure of the FurA chain was built in order to search for possible binding sites for 2-OG. The best model was the one based on Fur from *H. pylori* (PDB code: 2XIG; ModFOLD global model quality score 0.6076; Table S1). This was a good and reliable model according to quality control checks with multiple programs (Table S2 and Table S3). The overall fold closely resembled that of other Fur homologues [19]. It consisted of two domains, an N-terminal helix–wing–helix domain and a C-terminal domain containing three antiparallel β-strands and one α-helix (Fig. 1A). This FurA model was docked with 2-OG using SwissDock. The lowest energy conformation of the most populated cluster was selected. The resulting complex was visualized using PYMOL and showed that 2-OG was able to bind to FurA (Fig. 1B). The position and orientation

of 2-OG in the complex were well suited to establish contacts with two arginine residues, namely Arg 24 and Arg 63. In addition, a potential second 2-OG-binding site involving an arginine in position 70 was predicted (Fig. 1B). Figure 1C shows the electrostatic surface representation of the protein model. In both cases, 2-OG is positioned in two binding pockets (Fig. 1C).

As FurA is a DNA-binding protein, a new model was constructed in complex with DNA using the TFmodeller server (Fig. 1D). The model was built using as a template the Fur-DNA complex of *M. gryphiswaldense* MSR-1 v2 (PDB code: 4RB3). Since the *M. gryphiswaldense* MSR-1 v2 structure complexed with DNA contains two Fur molecules bound to DNA, in the model obtained for FurA from *Anabaena* sp. PCC7120, FurA is shown as a dimer in complex with DNA. In this model, the two arginines (arginine 24 and arginine 63) of the first 2-OG-binding site were in fact interacting with DNA. Curiously, docking simulations with the FurA-DNA complex model again predicted the second binding pocket involving arginine 70 (Fig. 1E). Electrostatic surface representation of the FurA-DNA complex showed that the binding site involving arginines 24 and 63 was occupied by the DNA fragment, so that 2-OG could only bind to the binding pocket corresponding to arginine 70 (Fig. 1F). In order to determine the involvement of these residues in the binding of 2-OG, two FurA variants were constructed and purified, one double mutant in which arginine 24 and arginine 63 were replaced by alanine (called R24R63FurA) and a single mutant with the same substitution at arginine 70 (called R70FurA).

2-oxoglutarate is able to bind FurA *in vitro* in the presence of manganese and reducing conditions

In view of the *in silico* predictions in which a possible interaction between FurA and 2-OG could be inferred, ITC experiments were performed with purified FurA and 2-OG under different conditions. The potential interaction was examined under reducing and nonreducing conditions and in the presence and the absence of manganese. The analysis of the experimental data through nonlinear regression [20] led to the conclusion that indeed 2-OG could bind to FurA and that for an effective binding both the reducing agent and the presence of manganese were necessary (Fig. 2A,B). In these conditions, FurA was capable of interacting with 2-OG showing a dissociation constant K_d of 2.8 μM (Fig. 2B). Nevertheless, in the absence of manganese
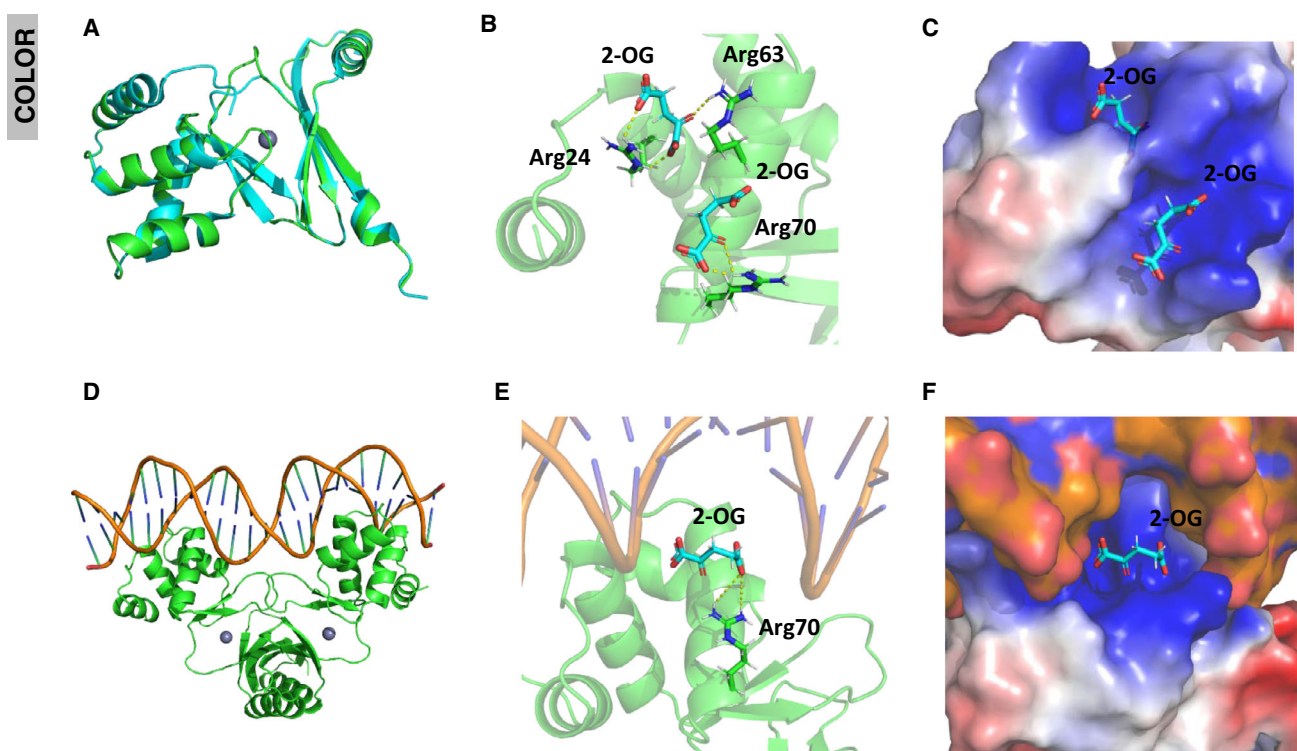
Fig. 1. Prediction of the two potential 2-OG-binding sites in FurA from *Anabaena* sp. PCC7120. (A) Superimposition of the FurA structural model (green) based on the structure of Fur from *H. pylori* (PDB ID: 2IXG cyan). (B) Cartoon representation of the two putative 2-OG-binding sites in the FurA structural model, one involving arginines 24 and 63 and another involving arginine 70. (C) Surface representation of the two putative 2-OG-binding sites in the FurA structural model. (D) FurA structural model (green) in complex with DNA (orange). (E) Cartoon representation of the putative 2-OG-binding site (involving arginine 70) in the FurA structural model in complex with DNA. (F) Surface representation of the putative 2-OG-binding site in FurA structural model in complex with DNA. 2-OG is colored in cyan, and residues which were predicted to interact with 2-OG are represented as sticks.

and/or DTT, FurA was unable to interact with 2-OG (data not shown). Therefore, in view of the results, the binding of FurA to 2-OG depends on the presence of both DTT and Mn^{2+}. It is noticeable that FurA also requires the presence of its co-repressor metal (Mn^{2+}) and reducing conditions to bind to Fur-regulated promoters [21]. In spite of the fact that the apparent reaction stoichiometry indicated an *n* value of 0.55, the binding of one molecule of 2-OG to one molecule of FurA protein is proposed. The low *n* value obtained in the ITC experiments could indicate that only the 55% of FurA molecules are in fact binding-competent and active, which could be attributed to partial aggregation of the regulator or the formation of incorrect disulfide bridges [15].

The interaction of R24R63FurA and R70FurA variants with 2-OG was also analyzed by ITC in the presence of DTT and Mn^{2+}, conditions in which FurA was able to bind this ligand. The results indicated that the R24R63FurA mutant interacted slightly with 2-OG (dissociation constant K_d of 12 mM), whereas no

interaction was observed in the R70FurA mutant (Fig 2C,D).

As a specificity control of the FurA-2-OG binding, ITC experiments were carried out adding glutamate as a ligand to FurA. These experiments, performed again in the presence of DTT and Mn^{2+}, revealed no interaction between glutamate and FurA (Fig. 2E), which suggests that the binding between 2-OG and FurA is specific.

In previous experiments, FurA was dissolved in 10 mM acetic acid/acetate buffer at pH 4 since in this buffer the FurA protein is stable. However, ITC experiments were also performed with FurA dissolved in Tris/HCl pH 8 150 mM NaCl and 2-OG in the presence of both DTT and Mn^{2+}. This pH value was selected in order to compare the ITC results with those obtained from EMSAs performed at pH 7.5 (see below) and because this pH value is closer to physiological pH. The results showed again a clear interaction between FurA and 2-OG at pH 8. Indeed, no significant changes were observed in the K_d values between FurA at pH 4 and FurA at pH 8 and 2-OG (Fig. 2F).
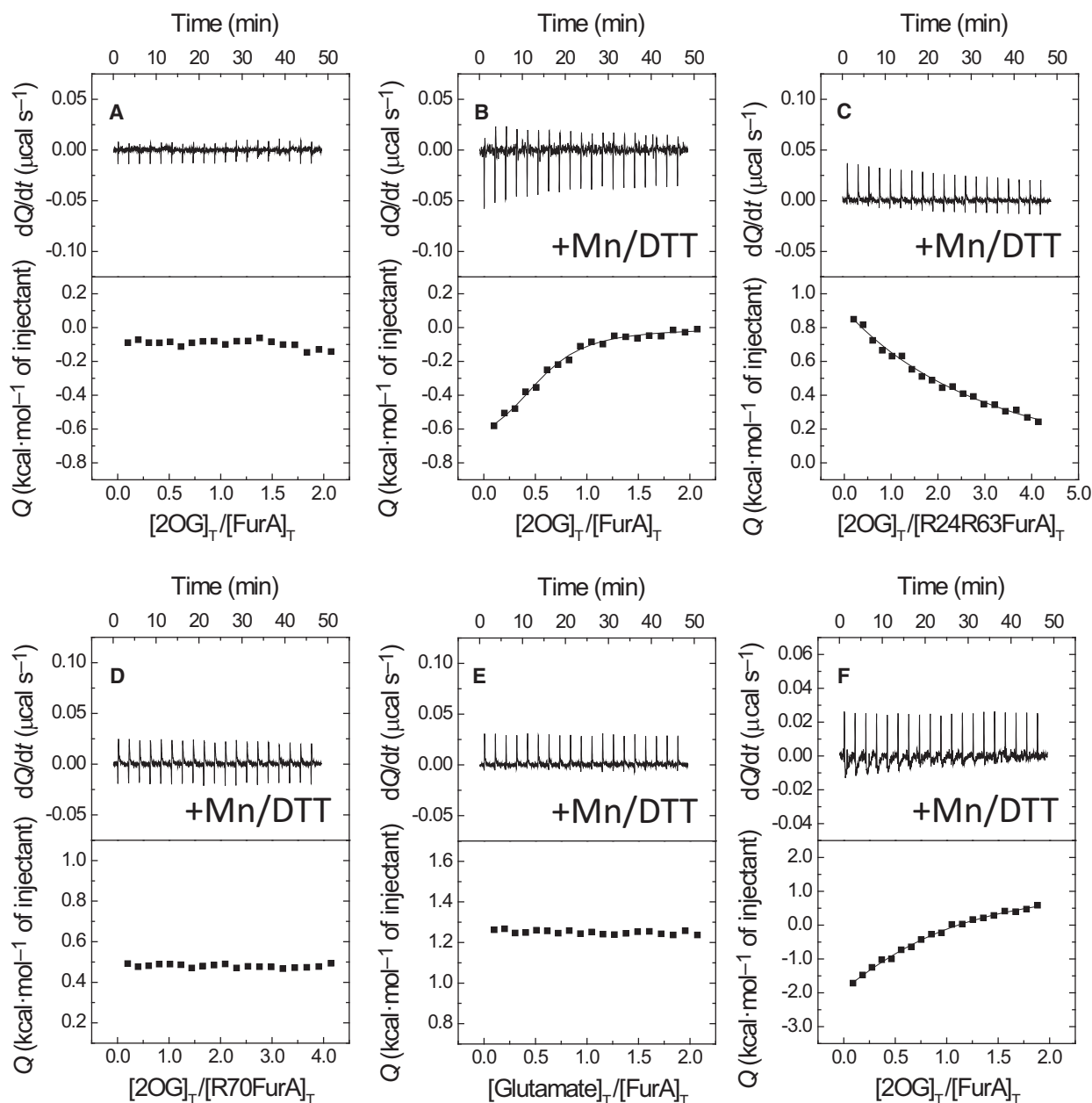
Fig. 2. Analysis of the interaction between FurA and 2-OG by ITC under different experimental conditions. (A) Titration of FurA in the presence of 2-OG at pH 4; (B) titration of FurA and 2-OG in the presence of 100 μM manganese and 1 mM DTT at pH 4; (C) titration of R24R63FurA variant and 2-OG in the presence of 100 μM manganese and 1 mM DTT; (D) titration of R70FurA variant and 2-OG in the presence of 100 μM manganese and 1 mM DTT; (E) titration of FurA in the presence of glutamate; (F) titration of FurA and 2-OG in the presence of 100 μM manganese and 1 mM DTT at pH 8.

2-oxoglutarate modulates the binding affinity of FurA to the ntcA promoter

FurA usually acts as a transcriptional repressor of many important genes involved in different cellular processes, such as the adaptation to nitrogen starvation or the regulation of iron metabolism. In order to study whether the binding of 2-OG had functional consequences on the binding of FurA to its target promoters, EMSAs were performed in the presence of 2-OG. Two target genes were selected as representative of nitrogen and iron availability adaptation mediated by Fur, the gene encoding the

A   ***ntcA* promoter**

FurA-binding site

TTATAC**ATGGTTAGCAAAAATGATGATTATTAAGG**GCGTTTTTTAGAATAAAATGTTTGTTATTTCT

                                                                                                +1
TAAGATATTTTCAAGATTACTTAATTTTTTCTGCAAAAAACCTGATGGTTAGAGAAAGTAGAAG**T**AT

        +1   NtcA-binding site
CTATCA**A**GAACTGCTAGCTAAAAATAGCAAAA**GTTGGGTATCATTATGAACAAATAAC**CATAAAGTT

                                                                              −10
GTACGTTTGAGTATAGGAAAGTACAGAAAGGTTAACGGTGCTTTATTGATTTTTCAGGTG**TATTCT**T

        +1
AGGT**A**ATTAGTTTTTAGTTTTTTGTTATTCCGGCATTGGGTAGGGGAAGGGAG**ATG**ATCGTGACACA

Fig. 3. Scheme of the *ntcA* and *isiB* promoters. The FurA- and NtcA-binding sites are located in each promoter and represented in bold. The −10 and −35 sequences as well as transcriptional start points represented as angled arrows previously reported for these genes are also included. Note that in both promoters three transcriptional start points were described. Start codons of both genes are also shown in bold. (A) is adapted from [23], and (B) is adapted from [21].

B   ***isiB* promoter**

FurA-binding site   +1

CAAAA**TTTAATAATTATTTAAT**GCAAAATAGTCTCAATAAATCT**GATACTATA**TTT**TCAAAGCTTAT**
FurA-binding site   −35   −10
TGAAATAAATATTCAATAAGTTAACGCCCTTCTCTACGTTTGCGCCTGCACGAAGGCATTTTTATCT

ACACAGACTAAAAAACTTATTTTTCGTAGTAAGTGTAGTATCACACTTCAGGTTTCGGTGAGTTGAG

                        −35   −10   +1
TTTTCCAACTCACTCTAATCTGTG**CTGAGG**AAAAATCCCGGTTCCATTG**CCTAGT**GCATCACGAATA
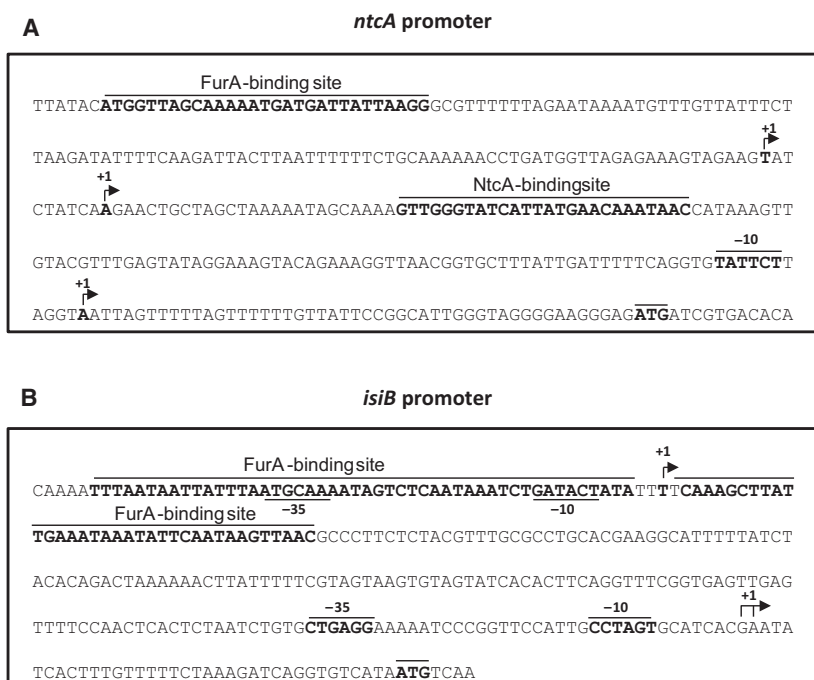
TCACTTTGTTTTTCTAAAGATCAGGTGTCATA**ATG**TCAA

master regulator of the nitrogen metabolism NtcA and the gene encoding flavodoxin (*isiB*). These two genes had been previously described as direct targets of FurA and both of them contained Fur-binding sites in their promoters [21–24], as shown in Fig. 3. First, EMSAs were performed with different concentrations of the FurA protein (350, 400, 500, and 600 nM) and the *ntcA* promoter in the presence and absence of 2-OG. These assays were performed in the presence of manganese and under reducing conditions. Densitometric analysis of the bands obtained in the EMSAs shown in Fig. 4A indicated that 2-OG was able to mildly enhance the binding of FurA to the *ntcA* promoter under each of the tested FurA concentrations. This increase in FurA affinity would produce a higher repression of *ntcA* expression. However, when similar experiments were performed with the *isiB* promoter, no effect of 2-OG was observed in the FurA-binding activity (Fig. 4B).

A

[2-OG] 0.8 mM   −   −   −   −   −   +   +   +   +   +

[FurA] nM   0   350 400 500 600   0   350 400 500 600

P*ntcA* ▶

ifpkn22 ▶

            100   87   76   76   74 100   61   63   51   50

% unbound DNA

B

[2-OG] 0.8 mM   −   −   −   −   −   +   +   +   +   +

[FurA] nM   0   250 350 500 600   0   250 350 500 600

P*isiB* ▶

ifpkn22 ▶

            100 100   97   92   84 100 100   96   90   76
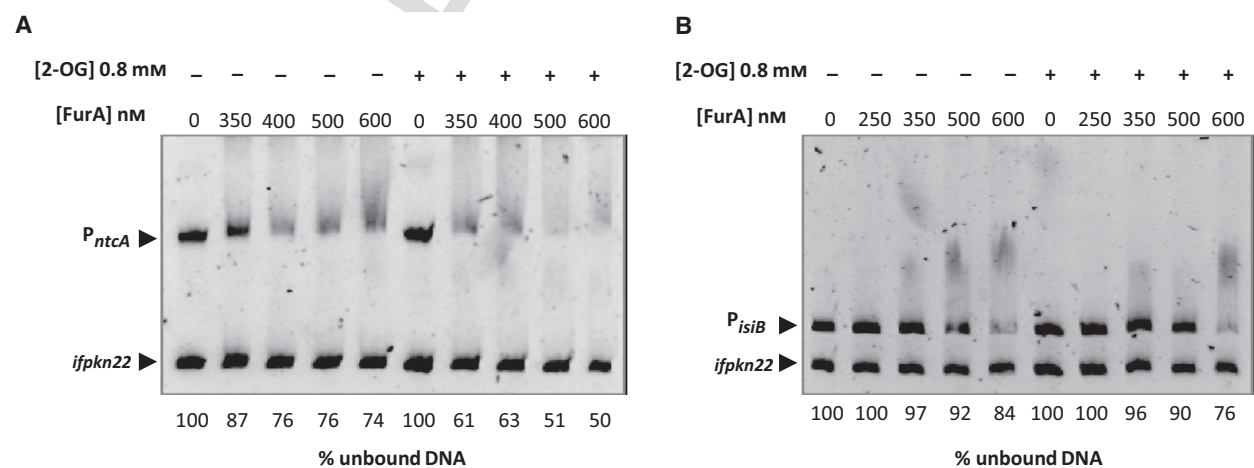
% unbound DNA

Fig. 4. Influence of 2-OG in the interaction of FurA with the *ntcA* and *isiB* promoters. (A) EMSAs showing the binding of different concentrations of FurA to the *ntcA* promoter, (B) Gel retardation of the *isiB* promoter. The internal fragment of *pkn22* gene was used as control for unspecific binding. Percentage of unbound DNA was included.

A

[FurA] 400 nM   −   +   +   +   +   +   +
[2-OG] mM   0   0   0.3 0.4 0.6 0.8 1.0

P$_{ntcA}$ ▶

ifpkn22 ▶

100 84   77   75   72   71   65

% unbound DNA

B

[FurA] 600 nM   −   +   +   +   +   +   +
[2-OG] mM   0   0   0.3 0.4 0.6 0.8 1.0

P$_{isiB}$ ▶
ifpkn22 ▶

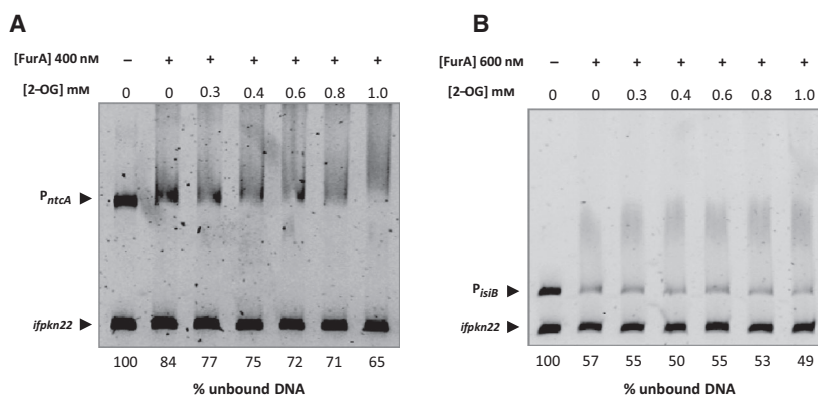100 57   55   50   55   53   49

% unbound DNA

Fig. 5. Effect of concentration of 2-OG in the binding of FurA to the *ntcA* and *isiB* promoters. (A) EMSAs showing the effect of increasing concentrations of 2-OG in the interaction of FurA with the *ntcA* promoter, (B) and with the *isiB* promoter. The internal fragment of *pkn22* gene was used as control for unspecific binding. Percentage of unbound DNA was included.

Taking into account that the levels of 2-OG change in the cell as a consequence of various situations, such as nitrogen deprivation, different concentrations of 2-OG (0.3, 0.4, 0.6, 0.8, and 1 mM) were used to treat the FurA protein prior to the incubation with the *ntcA* promoter. As observed in Fig. 5A, increasing concentrations of 2-OG enhanced the binding of FurA to the *ntcA* promoter. In contrast, the same concentrations of 2-OG did not yield any difference in the complex formed by FurA and the *isiB* promoter (Fig. 5B).

The interacting behavior of the R24R63FurA and R70FurA variants with the *ntcA* promoter was also analyzed by EMSA. The experiments revealed that the R24R63FurA mutant was not able to bind the *ntcA* promoter, supporting the view that indeed these two arginine residues are involved in DNA binding. However, the R70FurA variant was able to slow down the electrophoretic migration of the *ntcA* promoter but

was insensitive to 2-OG, which reinforced the idea of its involvement in 2-OG binding (Fig. 6A,B).

Discussion

Fur proteins are widely distributed in bacteria and FurA orthologs are present in environmental bacteria such as *E. coli* or *Pseudomonas* spp., and in important pathogens such as *Salmonella* or *Clostridium* spp. [25]. In these heterotrophic microorganisms, FurA has a well-conserved role in the regulation of iron metabolism, sometimes linked to pathogenesis [25]. However, in *Anabaena* sp. PCC 7120, FurA displays a more comprehensive role controlling several important processes different from iron metabolism, such as heterocyst differentiation in nitrogen fixation [14,17,24]. 2-OG is a key metabolite that signals the carbon/nitrogen status of the cell. In the present study, FurA was predicted to

A

FurA (800 nM)   R24R63FurA (800 nM)
−   +   +   −   +   +

P$_{ntcA}$ ▶

ifpkn22 ▶

[2-OG] −   −   +   −   −   +
(0.8 mM)

B

[2-OG] 0.8 mM   −   −   −   −   −   +   +   +   +   +
[R70FurA] nM   0 600 800 1000 1200 0 600 800 1000 1200

P$_{ntcA}$ ▶

ifpkn22 ▶

100 84   75   70   68 100 92   76   77   69

% unbound DNA
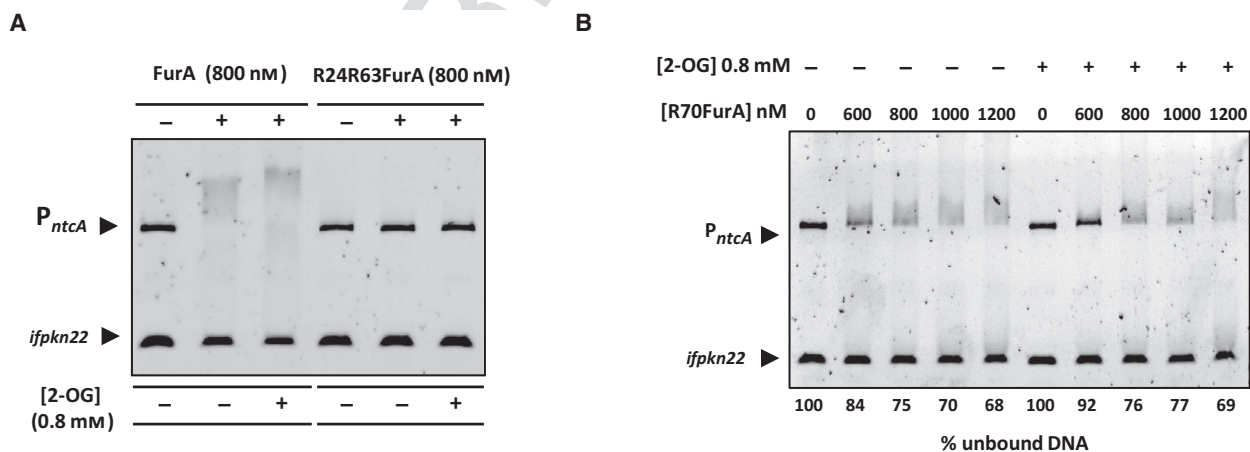
Fig. 6. Study of the interaction between R24R63FurA and R70FurA variants with the *ntcA* promoter by EMSAs. (A) EMSAs showing the effect of R24R63FurA variant in the *ntcA* promoter retardation in the presence and absence of 2-OG. A FurA-positive control of DNA binding is included. (B) Evaluation of the binding of different concentrations of R70FurA variant to the *ntcA* promoter in the presence and the absence of 2-OG. The internal fragment of *pkn22* gene was used as control for unspecific binding. Percentage of unbound DNA was included.

LOW RESOLUTION FIG

A

```
A. 7120    --------------------MTVY--TNTSLKAELNERGWRLTPQRETILHIFQELPQG    37
S. 7002    ------------------MLVPSY--DVSALKTELNAKGWRLTPQREKILRTFLNLPKG    39
C. ra      --------------------MTVY--TTGSLKAELNDRGWRLTPQRETILHIFQELPQG    37
F. JSC-11  --------------------MTVY--TAASLKAELNDRGWRLTPQRETILHIFQDLPQG    37
C. 336/3   --------------------MTAY--TASSLKAELNERGWRLTPQREIILHIFQELPQG    37
N. pu      --------------------MTVY--TTTSLKAELNERGWRLTPQRETILHIFQELPQG    37
N. sp      --------------------MTVY--TSTSLKAELNDRGWRLTPQRETILHIFQELPQG    37
S. 6803    -------------------MSY--TADSLKAELNARGWRLTPQREKILTIFQNLPEG    36
M. ae      -------------------MSAY--TASSLKAELNARGWRLTPQREKILHVFQNLPKG    37
P. ma      MLQPLSLAIIIENNVLADMTLNVGYGSRSSPLELGLHKNGRRLTPQRKKILDLFQRIGSG    60
                         *        *:  *: .* ******: ** *  : .*

A. 7120    EHLSAEDLYHRLEADGEGISLSTIYRTLKLMARMGILRELELGEGHKHYEINQPYPHHHH    97
S. 7002    EHLSAEDLHNELLEQGEKISLSTVYRSVKLMTRMGILRELELAEGHKHYELNQSQAH-HH    98
C. ra      EHLSAEDLHYRLENGGESISLSTVYRTLKLMARLGILRELELGEGHKHYELNQPYPHHHH    97
F. JSC-11  EHLSAEDLYHRLESNGEGISLSTIYRTLKLMARMGILRELELGEGHKHYELNQPYPYHHH    97
C. 336/3   EHLSAEDLYHRLETEGEGISLSTIYRTLKLMARMGILRELELGEGHKHYELNQPHPHHHH    97
N. pu      EHLSAEDLYHRLETDGEGISLSTIYRTLKLMARMGILRELELGEGHKHYEINQPYPHHHH    97
N. sp      EHLSAEDLYHRLESDGEGISLSTIYRTLKLMARMGILRELELGEGHKHYEINQPYPHHHH    97
S. 6803    EHLSAEELHHRLEEEREKISLSTVYRSVKLMSRMGILRELELAEGHKHYELQQASPHHHH    96
M. ae      NHLSAEELQELLDKRGEGISLSTVYRSVKLMSRMGILRELELAEGHKHYELNQPYPHHHH    97
P. ma      IHLSAEEVHAHLLQNKTRVSLATIYRTLRLLVRMGFLHELELSEGGHRFEILSKDNPDHH    120
           *****::    *    :**:*:**:::*: *:*:*:****.** :::*: .      **
```

B

```
A. 7120    -----------------------MTVYTNTSLKAELNERGWRLTPQRETILHIFQELP    35
H. py      --------------------MKRLETLESILERLRMSIKKNGLKNSKQREEVVSVLYR--    38
C. di      --------------------MANTMDLLKDKLKETGFKITPQRRAIVEILLK-H       33
L. mo      --------------------MEGRIGRIKAQLHDASYKLTPQREATVRVLLE-N       33
M. tu      --------------------------MSAAGVRSTRQRAAISTLLET-L          22
S. en      -------------------------MTDNNTALKKAGLKVTLBRLKILEVLQE-P       29
P. ae      ------------------------MVENSELRKAGLKVTLERVKILQMLDS-A      28
P. pu      ------------------------MVENSELRKAGLKVTLPRVKILQMLDS-T      28
E. co      MFFLEHGKVRTFLTPTLRCPMEKT-TTQELLAQAEKICAQRNVRLTPQRLEVLRLMSL--    57
                                       . : :    *        ::

A. 7120    QGEHLSAEDLYHRLEADGEGISLSTIYRTLKLMARMGILRELELGEGHKHYEINQP--YP    93
H. py      SGTHLSPEEITHSIRQKDKNTSISSVYRILNFLEKENFICVLETSKSGRRYEIAAK--E-    95
C. di      DHSHLSSEEIYDLVRVDCPEIGLATVYRTMQLLDEIGLISKLNLDDGCIRYEISLHKEDC    93
L. mo      EKDHLSAEEVFLRVKDIAPDTGLATVYRTLELLTELRVVDKINFGDGVSRYDLRQE-GAK    92
M. tu      D-DFRSAQELHDELRRRGENIGLTTVYRTLQSMASSGLVDTLHTDTGESVYRRCSE--H-    78
S. en      DNNHVSAEDLYKRLIDMGEEIGLATVYRVLNQFDDAGIVTRHNFEGGKSVFELTQQ--H-    86
P. ae      EQRHMSAEDVYKALMEAGEDVGLATVYRVLTQFEAAGLVVRHNFDGGHAVFELADS--G-    85
P. pu      EQRHMSAEDVYKALMEAGEDVGLATVYRVLTQFEAAGLVVRHNFDGGHAVFELADG--G-    85
E. co      QDGAISAYDLLDLLREAEPQAKPPTVRALDFLLEQGFVHKVESTNSYVLCHLFDQ--PT    115
           .   *  ::  :          ::** :  :    .:   .   .
```
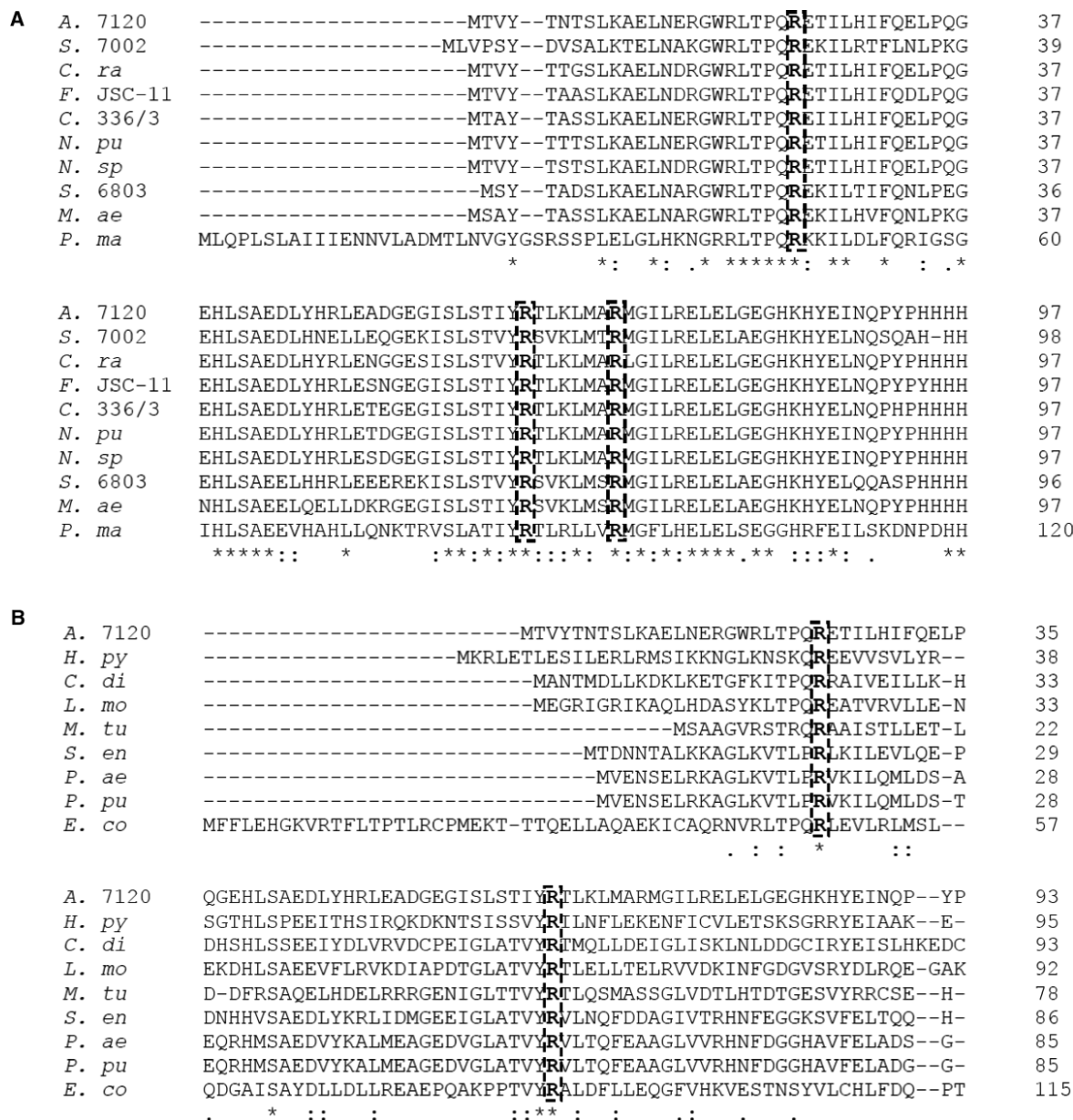
Fig. 7. Multiple sequence alignments comparing the FurA sequence from *Anabaena* sp. PCC7120 with other FurA sequences present in cyanobacteria or heterotrophic bacteria. (A) Cyanobacteria. (B) Heterotrophic bacteria. Abbreviation for each sequence is as follows: *Anabaena* sp. PCC 7120 (*A. 7120*), *Synechococcus* sp. PCC 7002 (*S. 7002*), *Cylindrospermopsis raciborskii* CS-505 (*C. ra*), *Fischerella* sp. JSC-11 (*F. JSC-11*), *Calothrix* sp. 336/3 (*C. 336/3*), *Nostoc punctiforme* PCC 73102 (*N. pu*), *Nodularia spumigena* CCY9414 (*N. sp*), *Synechocystis* sp. PCC 6803 (*S. 6803*), *Helicobacter pylori* NCTC 11637 (*H. py*), *Clostridium difficile* 630 (*C. di*), *Listeria monocytogenes* serotype 1/2a (*L. mo*), *Mycobacterium tuberculosis* H37Rv (*M. tu*), *Salmonella enterica* subsp. enterica serovar Typhimurium str. LT2 (*S. en*), *Pseudomonas aeruginosa* str. PAO1 (*P. ae*), *Pseudomonas putida* KT2440 (*P. pu*), and *Escherichia coli* str. K-12 substr. MG1655 (*E. co*). A colon (:) indicates strongly conserved amino acid, a dot (·) weakly conserved amino acid, and an asterisk (*) indicates identical amino acid. The three conserved arginines (Arg 24, Arg 63, and Arg 70) are indicated in bold and located inside squares.

contain two potential binding sites for 2-OG, and furthermore, this signal molecule was found to bind *in vitro* to FurA in the presence of metal and reducing conditions. These results provide the first evidence of the binding of 2-OG to Fur proteins. In this work, we have found that a FurA variant having arginine 70

replaced by alanine was not able to interact with 2-OG in ITC experiments (Fig. 2D), and moreover, the positive effect of 2-OG in the FurA binding to the *ntcA* promoter was not observed when the R70FurA variant was used in EMSAs (Fig. 6B). Thus, the results shown in this work strongly suggest that arginine 70 is deeply involved in the binding of 2-OG. Regarding the other potential binding site involving arginines 24 and 63, it has been proved that these two arginines are necessary for the binding of FurA to DNA (Fig. 6A). However, when the interaction of the R24R63FurA variant with 2-OG was analyzed by ITC, only a slight interaction was observed so that participation of arginine 24 and/ or arginine 63 residues in the FurA interaction with 2-OG cannot be entirely ruled out (Figs 2E and 6A). If the model of FurA is observed, arginines located in positions 24 and 63 are very close in the structure to arginine 70 (Fig. 1B) so that alterations in these residues may modify the arginine 70 binding site, abolishing the binding of 2-OG. Another possible explanation could be that as FurA was only able to bind 2-OG in the optimal configuration for binding DNA, that is, in the presence of manganese and reducing conditions (Fig. 2B), and since the R24R63FurA variant was not able to bind DNA, this variant was not in the suitable configuration and was not able to bind 2-OG. Multiple sequence alignments carried out comparing the protein sequence of FurA from *Anabaena* sp. PCC 7120 and other Fur protein sequences from different bacteria and cyanobacteria revealed that the two arginine residues located in positions 24 and 63 in the *Anabaena* sp. PCC 7120 FurA sequence are well conserved in Fur proteins from most of these microorganisms (Fig. 7A,B). In contrast, arginine 70 is only present in cyanobacteria and not in heterotrophic bacteria (Fig. 7A,B). This could be evidence that only FurA proteins from cyanobacteria can bind 2-OG, although more investigation should be done in this field. One piece of evidence that strengthens this hypothesis is that when ITC experiments were carried out with two FurA proteins from heterotrophic bacteria, those of *Pseudomonas aeruginosa* PAO1 and of *Clostridium difficile*, no interaction with 2-OG was observed (Fig. S1).

FurA is a transcriptional repressor of the *ntcA* and *isiB* promoters, among many others. If the *ntcA* and the *isiB* promoters are analyzed in detail, it can be seen that the *isiB* promoter holds two FurA-binding sites, whereas the *ntcA* promoter contains only one (Fig. 3A,B). Indeed, when competitive EMSAs were performed with FurA and both promoters mixed in equimolar concentrations, the results showed a higher FurA affinity for the *isiB* promoter (Fig. 8). This could indicate that FurA can fully repress the expression of

isiB in the presence of the co-repressor metal and reducing conditions, but in contrast, FurA may need some additional signal to fully repress the expression of the *ntcA* promoter. Taken together, these results suggest that this signal could be 2-OG. As occurs in the complex with NtcA, 2-OG could trigger conformational changes in FurA resulting in a tighter interaction with DNA [13]. Moreover, in agreement with the results obtained in this work, 2-OG exerts a positive effect on the binding of NtcA to NtcA-regulated promoters, but the magnitude varies depending on the promoter [26].

A clear intertwining between FurA and NtcA in *Anabaena* sp. PCC 7120 has been previously demonstrated. These two important regulators are involved in a complex cross-talk for the regulation of the response to nitrogen step-down, both of them being key players in the process of heterocyst differentiation [7,17]. First, the NtcA response is activated by nitrogen deficiency and then this global transcriptional activator elicits the heterocyst differentiation response [7]. During the early phase of differentiation, NtcA upregulates the expression of FurA in the proheterocyst, so that the increased levels of FurA may negatively regulate the *ntcA* expression in order to arrest the differentiation once it has been triggered. Maybe the presence of 2-OG is another control point in the timing of the transient NtcA induction. Thus, when the two conditions are achieved simultaneously, on the one hand an
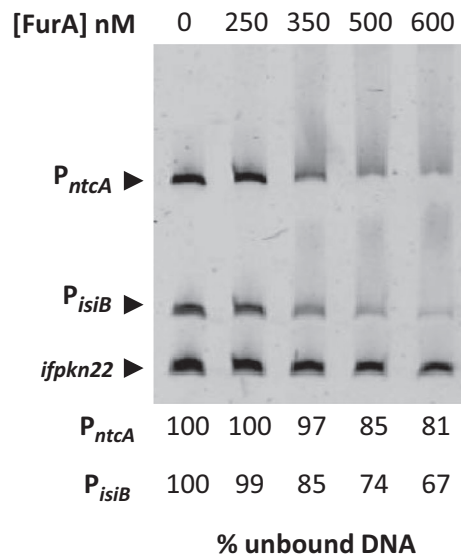

[FurA] nM   0   250   350   500   600

P*ntcA* ▶

P*isiB* ▶

ifpkn22 ▶

| P*ntcA* | 100 | 100 | 97 | 85 | 81 |
| P*isiB* | 100 | 99 | 85 | 74 | 67 |

% unbound DNA

Fig. 8. Competitive EMSA analysis of FurA binding to the *ntcA* and *isiB* promoters. Equimolar concentrations of each promoter were used. The internal fragment of *pkn22* gene was used as control for unspecific binding. Percentage of unbound DNA was included.

increase in FurA and on the other the presence of high levels of 2-OG, FurA is able to bind tightly to the *ntcA* promoter, hampering its transcription and slowing down the differentiation process. In cyanobacteria, the control of heterocyst differentiation requires fine-tune regulation, and it seems clear that FurA and NtcA proteins are involved in this process, providing a link between carbon, nitrogen, and iron metabolism. The results presented here point to 2-OG as a novel player allowing another key point in the regulation displayed by FurA. Nevertheless, further studies need to be carried out to define the degree of importance of 2-OG in the modulation of the expression of FurA-regulated genes.

Finally, the present work provides further evidence that FurA from *Anabaena* PCC 7120 is a versatile regulator deeply involved in the maintenance of cell homeostasis, integrating various environmental and nutritional signals. Indeed, FurA appears to be a multisensory protein able to modulate its transcriptional activity depending not only on iron availability and the redox state of the cell but it could be feasible to propose that the interaction of FurA with 2-OG is a way of sensing the carbon/nitrogen ratio in *Anabaena* sp. PCC 7120.

Acknowledgements

Authors' contribution

Xxxx.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Analysis of the interaction between FurA from A. *Pseudomonas aeruginosa* PAO1 and B. *Clostridium difficile* with 2-oxogutarate performed by ITC.

Table S1. P-value and global model quality score of all models of FurA structure.

Table S2. Quality check results for the FurA model and FurA model in complex with DNA.

Table S3. MolProbity analysis of FurA model.