FurC (PerR) from *Anabaena* sp. PCC7120: a versatile transcriptional regulator engaged in the regulatory network of heterocyst development and nitrogen fixation

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Summary

FurC (PerR) from *Anabaena* sp. PCC7120 was previously described as a key transcriptional regulator involved in setting off the oxidative stress response. In the last years, the cross-talk between oxidative stress, iron homeostasis and nitrogen metabolism is becoming more and more evident. In this work, the transcriptome of a *furC*-overexpressing strain was compared with that of a wild-type strain under both standard and nitrogen-deficiency conditions. The results showed that the overexpression of *furC* deregulates genes involved in several categories standing out photosynthesis, iron transport and nitrogen metabolism. The novel FurC-direct targets included some regulatory elements that control heterocyst development (*hetZ* and *asr1734*), genes directly involved in the heterocyst envelope formation (*devBCA* and *hepC*) and genes which participate in the nitrogen fixation process (*nifHDK* and *nifH2*, *rbrA* rubrerythrin and *xisHI* excisionase). Likewise, *furC* overexpression notably impacts the mRNA levels of *patA* encoding a key protein in the heterocyst pattern formation. The relevance of FurC in these processes is bringing out by the fact that the overexpression of *furC* impairs heterocyst

development and cell growth under nitrogen step-down conditions. In summary, this work reveals a new player in the complex regulatory network of heterocyst formation and nitrogen fixation.

Introduction

Among the three FUR (Ferric Uptake Regulator) paralogues (FurA, FurB and FurC) present in *Anabaena* sp. PCC7120 (also known as *Nostoc* sp. PCC7120), FurC (PerR) (hereafter FurC) is the most puzzling member. This protein was previously proposed to work as a PerR orthologue since it directly regulates the transcriptional regulation of key genes involved in the oxidative stress response such as *prxA* (peroxiredoxin A), *srxA* (sulfiredoxin A) or *ahpC* (alkyl hydroperoxide reductase) and because such transcriptional regulation is dependent on metal catalysed oxidation, a distinctive feature of PerR proteins (Yingping *et al*., 2014; Sevilla *et al*., 2019). Afterwards, *prxA*, *srxA* and *ahpC* genes were found to be derepressed in the presence of hydrogen peroxide in a *furC*-overexpressing variant (EB2770FurC) of *Anabaena* sp. PCC7120, which was in agreement with the canonical action mode of PerR proteins (Sevilla *et al*., 2019). However, while the phenotype of EB2770FurC variant was being characterized, it was observed that FurC displayed an important role in the management of the photosynthetic apparatus linking the oxidative stress response of the cyanobacterium with photosynthetic metabolism. Significant alterations in pigment and photosystem composition were found in the EB2770FurC strain and new unexpected direct targets of FurC such as the major thylakoid membrane protease *ftsH* involved in D1 recycling processes were identified (Sevilla *et al*., 2019). These results strongly suggest that unlike what was observed in heterotrophic bacteria, FurC role was not only restricted to the maintenance of redox homeostasis but this transcriptional regulator could display a more global function in *Anabaena* sp. PCC7120.

Because of its ability to fix atmospheric nitrogen, the control of nitrogen metabolism in *Anabaena*

sp. PCC7120 engages an intricate regulatory network that selectively responds to the source of nitrogen available (Herrero and Flores, 2019). After nitrogen step-down, the master regulator NtcA activates the expression of nitrate assimilation proteins (*nir* genes) and ammonia translocators (*amt* genes) (Herrero *et al*., 2004). On the other hand, morphological and metabolic changes lead to heterocyst formation and nitrogen fixation. NtcA early induces the expression of the *nrrA* and *hetC* genes, followed by activation of *xisA* and *devBCA* and finally *pipX*, *cox* and *nifHDK* (Herrero *et al*., 2013). In addition, HetR, a key transcriptional regulator required for heterocyst differentiation, binds the promoters of important genes involved in heterocyst development such as *hetP*, *hepA*, *hetZ* and *patS* (Huang *et al*., 2004; Higa and Callahan, 2010; Feldmann *et al*., 2011; Du *et al*., 2012). Nevertheless, the promoter of *hetR* does not present NtcA binding sites so that the effect of NtcA on *hetR* expression seems to be mediated by NrrA (Ehira and Ohmori, 2006; Muro-Pastor *et al*., 2006). The oligomeric state of HetR may be functionally relevant and could be regulated by phosphorylation (Valladares *et al*., 2016). Indeed, HetR was described as being susceptible to phosphorylation by the Pkn22 kinase in *Anabaena* sp. PCC7120 (Roumezi *et al*., 2019). FurA, the master regulator of iron homeostasis in *Anabaena* sp. PCC7120, also influences heterocyst development. FurA directly binds the promoters of *ntcA* and *hetR* (Gonzalez *et al*., 2013) and conversely, NtcA controls the expression of *furA* under nitrogen-step down leading to a notable increasing of *furA* expression in heterocysts (Lopez-Gomollon *et al*., 2007). Furthermore, FurA is able to bind 2-oxoglutarate (2-OG), a signal molecule that informs about the C/N balance in the cell (Guio *et al*., 2020). In the last years, some articles point out a strong cross-talk between iron deficiency, oxidative stress response and nitrogen metabolism (Pernil and Schleiff, 2019). It is well known that in *Anabaena* sp. PCC7120 under nitrogen-deprived conditions, the demand for iron increases (Saxena *et al*., 2006; Kaushik and Mishra, 2019) and subsequently, iron deficiency can lead to oxidative stress (Latifi *et al*., 2005; Kaushik *et al*., 2015; Kaushik *et al*., 2017). Likewise, it has been demonstrated that iron deficiency influences the regulation exerted by NtcA on genes involved in fatty acid desaturation and heterocyst envelope formation in *Anabaena* sp. PCC7120 (Kaushik and Mishra, 2019). Recently, Robles-Rengel *et al*. (2019), described that hydrogen peroxide alters the expression of some genes related to nitrogen metabolism and interestingly revealed that H_2O_2 presence diminishes 2-OG levels in the cell.

In *Anabaena* sp. PCC7120, FurC promoter region contains an NtcA box suggesting a potential involvement of FurC in nitrogen metabolism (Lopez-Gomollon *et al*., 2007). Likewise, it was observed that the *furC*-overexpressing strain (EB2770FurC) was unable to develop heterocysts under nitrogen-starvation conditions (see below) so that we wondered whether FurC was involved in this process. Therefore, with the aim to better understand the role of FurC in *Anabaena* sp. PCC 7120, transcriptomic analysis was performed to compare the mRNA levels of EB2770FurC with those of the wild-type strain both grown under standard culture conditions and under nitrogen deficiency. EB2770FurC was used instead of a *furC* deletion mutant since previous attempts to inactivate the *furC* gene in *Anabaena* sp. PCC 7120 were unsuccessful (Yingping *et al*., 2014; Sevilla *et al*., 2019). In this strain, the levels of *furC* mRNA were upregulated 44-fold relative to those in *Anabaena* sp. PCC7120 (Sevilla *et al*., 2019). The results shown in the present work revealed that FurC directly and indirectly regulates the expression of key genes involved in the adaptive processes to nitrogen starvation among others suggesting that FurC plays a critical role in the regulation of heterocysts formation and patterning as well as in nitrogen fixation.

Materials and methods

Cultures and RNA extraction

Total RNA was prepared from *Anabaena* sp. PCC7120 and EB2770FurC cultures. The EB2770FurC strain is a *furC*-overexpressing strain that contains the pAM2770FurC plasmid harbouring the *furC* gene downstream the copper-inducible *petE* (plastocyanin) promoter (Sevilla *et al*., 2019). Three independent cultures of each strain were set up by diluting a pre-inoculum from the late exponential phase until an OD750 of 0.4 in a final volume of 100 ml in BG11 or BG11$_0$ (BG11 medium without nitrate). Cultures were grown in Erlenmeyer flask at 28°C on an orbital shaker at 120 rpm under a continuous light regime of 30 μmol photons m^{-2} s^{-1}. For nitrate-deprived samples, cells were collected by centrifugation and washed three times in BG-11$_0$ before resuspension in BG11$_0$ to the final optical density 0.4. RNA was extracted from 25 ml of each culture after 48 h of nitrogen deficiency following a method adapted from Olmedo-Verd *et al*. (2005). Briefly, cyanobacterial cells were rapidly harvested by centrifugation at 4000*g* for 5 min at 4°C and transferred to a 2 ml Eppendorf and collected by centrifugation at 16 500*g* for 5 min at 4°C. The supernatant was carefully removed and the cell pellet was resuspended in 600 μl of 50 mM Tris–HCl pH 8, 100 mM EDTA and 130 μl of chloroform and mixed thoroughly by inversion. The suspension was centrifuged at 16 500*g* for 5 min and the cell phase was collected and frozen in liquid nitrogen for storage at −80°C. Afterwards, frozen pellets were resuspended in 300 μl resuspension buffer (0.3 M

sucrose, 10 mM sodium acetate, pH 4.5) and 100 μl of 250 mM Na$_2$-EDTA (pH 8), 400 μl of lysis buffer (2% SDS, 10 mM sodium acetate, pH 4.5) and 1 ml of acid phenol at 65°C. Samples were vortexed for 30 s and incubated at 65°C for 2.5 min (this step was repeated three times). The suspension was centrifuged at 16 500g for 5 min and the aqueous phase was collected and sequentially extracted with Trizol, Trizol-Chloroform and Chloroform. Finally, two volumes of precooled absolute ethanol were added and RNA was precipitated O/N at −80°C. For the DNAse I treatment, the pellet was washed with precooled 70% ethanol, dissolved in H$_2$O and treated with RNase-free DNase I (Roche). The absence of DNA in the RNA samples was checked by Real-Time PCR, using oligonucleotides for the housekeeping gene *rnpB* (Vioque, 1992). RNA was quantified spectrophotometrically using a SPECORD® PLUS Analytik Jena spectrophotometer.

RNA seq

RNA seq was performed by Era7 bioinformatics company (Spain) from RNAs extracted and processed from two RNA biological replicates of *Anabaena* sp. PCC7120 and EB2770FurC strains cultured in standard conditions (BG11) and after 48 h of nitrogen deprivation (BG11$_0$). Ribosomal RNA (rRNA) was removed from the samples using 'Ribo-Zero rRNA magnetic Kit Bacteria' (Illumina) and RNA libraries were prepared according to standard procedures using the TruSeq RNA kit (Illumina). The pool of libraries was sequenced using a NextSeq 500 Mid Output Kit (Illumina) in a 1 × 75 single-read sequencing run on a NextSeq 500 sequencer (Illumina). The RNA sequencing data have been deposited in ArrayExpress with the accession number E-MTAB-8996.

Bioinformatics analyses

Bioinformatics analysis of differential gene expression for the RNAseq data was performed by Era7 bioinformatics (Spain) following the protocol described by Trapnell *et al*., 2013 (Trapnell *et al*., 2013). Quality control of raw readings was performed with the FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Alignment to the reference genome *Anabaena* sp. PCC 7120 (assembly ASM97v1) was performed using the tool Bowtie (Langmead *et al*., 2009), integrated into the Tophat suite software (Trapnell *et al*., 2009). Transcripts assembly was performed with the Cufflinks tool and transcripts merged with the Cuffmerge tool. Analysis of differences in gene expression was performed with the Cuffdiff tool. Statistical significance was considered for *P*-value <0.05 (corrected by the multiple testing Benjamini-Hochberg method). Only genes with a log2 (fold change) being ±1.5 were considered for further analyses.

Differentially expressed genes were subjected to search in the Cyanobase (http://genome.microbedb.jp/cyanobase) and KEGG (https://www.genome.jp/kegg) to discard pseudogenes and describe gene symbol and protein function. In some exceptional cases in which the number of reads associated with one of both strains was 0, the fold changes were established as −650-fold or +650-fold in order to avoid the nomenclature +infinite or −infinite.

Identification of a putative FurC-DNA binding motif was performed using MEME motif discovery tool on the MEME Suite server (http://meme-suite.org/tools/meme) (Bailey and Elkan, 1994) using as an input the FASTA sequences of the promoter regions whose respective genes were found to be directly regulated by FurC in this and previous works (Yingping *et al*., 2014; Sevilla *et al*., 2019). This MEME search was performed requesting a motif between 15 and 19 bp long using the ZOOPS model (zero or one occurrence per sequence) and the other default parameters (Bailey *et al*., 2006). Subsequently, the MEME output was scanned in all promoter regions by Find Individual Motif Occurrences (FIMO) analyses (Grant *et al*., 2011). The identified FurC motif was compared with known transcriptional factor motifs from bacteria using TomTom (Gupta *et al*., 2007) using the default parameters.

In order to locate the putative FurC box in the selected promoters of the genes directly regulated by FurC, the sequences of their promoters were retrieved from Cyanobase and included in Supplementary Fig. S1. Finally, the transcriptional start points of each gene, as well as −10 sequences (TATA boxes) that previously reported (Flaherty *et al*., 2011; Mitschke *et al*., 2011), were included in the sequences (Supplementary Fig. S1).

Real-time RT-PCR

The pool of cDNA was synthesized by reverse-transcription of 2 μg of total RNA using SuperScript retrotranscriptase (Invitrogen) following the manufacturer's conditions. Real-time PCR was performed using the ViiA™ 7 Real-Time PCR System (Applied Biosystems). Each reaction was set up by mixing 12.5 μl of SYBR Green PCR Master Mix with 0.4 μl of 25 μM primer mixture and 10 ng of cDNA template in a final volume of 30 μl. Amplification was performed at 60°C. Negative controls with no cDNA were included. The sequences of specific primers of selected genes are defined in Table S1. Transcript levels of target genes were normalized to those of the housekeeping gene *rnpB* measured with the same samples (Vioque, 1992). Relative quantification was performed according to the comparative Ct method (ΔΔCt Method). The minimum fold-change threshold was set up to ±1.5 fold.

4 *C. Sarasa-Buisan* et al.

Electrophoretic mobility shift assays

Promoter regions used in the analyses consisted of 150–350 bp DNA fragments and were obtained by PCR, using the *Anabaena* sp. PCC 7120 genome as template and the primers included in Table S1. Electrophoretic mobility shift assays (EMSA) analyses were performed with a FurC protein purified by using a protocol modified from previously described (Sevilla *et al.*, 2019). Briefly, the purification involved a first step of a Heparin-Sepharose affinity chromatography in 50 mM Tris–HCl pH 7.5 in presence of 10 mM EDTA to avoid metal-catalysed oxidation and a second step of an anion exchange chromatography on DEAE Cellulose in 50 mM Tris–HCl pH 7.5. Moreover, all washing steps were carried out with a buffer with higher ionic strength (0.1 M NaCl) to obtain a better degree of purity in each step. Finally the protein was dialyzed against 50 mM Tris–HCl pH 7.5, 150 mM NaCl. Reactions for EMSA analyses were performed mixing purified FurC in a final volume of 20 μl with 50 ng of DNA promoters in a binding buffer containing 10 mM Bis Tris–HCl, pH 7.5, 40 mM KCl, 0.1 mg ml^{-1} BSA, 1 mM DTT (1,4-dithiothreitol), 100 μM MnCl$_2$ and 5% (vol./vol.) glycerol. The resulting mixture was incubated for 30 min at room temperature and loaded into a non-denaturing 6% polyacrylamide gel. Both gel and running buffer included 100 μM MnCl$_2$. Gels were stained with SYBR® Safe (Invitrogen) and visualized in a GelDoc 2000 device (Bio-Rad).

Viability tests

Viability tests of *Anabaena* and EB2770FurC strains were carried out as a spot assay on BG11 agar plates with or without NaNO$_3$ as a nitrogen source (BG11$_0$). An aliquot was retrieved from cultures grown until late exponential phase and diluted to an OD$_{750}$ of 0.4 in BG11 or BG11$_0$. For nitrogen-deprived samples, cells were washed three times in the same volume of BG11$_0$ before dilution. Hereafter a 5 μl volume of liquid cultures was applied per spot. These agar plates were exposed to continuous light of 30 μmol photons m^{-2} s^{-1} for 7 days. Two biological replicates were tested separately.

Microscopy

Bright-field examinations of *Anabaena* sp. PCC 7120 and EB2770FurC filaments were carried out after 72 h of nitrogen deficiency. 10–40 μl of cyanobacterial cultures were immobilized in agar-solidified BG11 medium for nitrogen-sufficient conditions and in agar-solidified BG11$_0$ medium for nitrogen-deficient conditions. Immediately, small blocks were cut and placed on an inverted sample holder to visualize the cells using an Olympus IX81 microscope. Images were collected using a CCD camera (Model XC50; Olympus) under a 40× objective (lens specification LUCPLFLN 40× NA 0.6). The images were processed with ImageJ and Adobe Photoshop CC 2017. For staining of heterocyst polysaccharide layers, cell suspensions were mixed (5:1) with a 1% (wt./vol.) Alcian Blue 8 GX (Panreac) solution in water before its immobilization in agar plates.

Results

Transcriptional profile of the EB2770FurC strain under standard culture conditions

RNA from EB2770FurC and *Anabaena* sp. PCC7120 strains cultured until mid-exponential growth phase was obtained and analysed by RNA-seq. *furC* overexpression modified the mRNA levels of 197 genes more than ±1.5-log2 (fold change) that correspond with ±2.8-fold (*P*-value <0.05). Among the selected genes, 75 were annotated belonging to different functional categories (Table S2 and Fig. 1A). Genes annotated as hypothetical proteins are included in Supplementary Table S4. The expression of some remarkable genes involved in photosynthesis was deregulated in the EB2770FurC strain such as *psaI*, *psbK*, *psbX* encoding PSI and PSII structural subunits and two *psbA* genes (*psbAIV* and *psbAII*) encoding two isoforms of the PSII reaction center protein D1. These isoforms are related with the recycling of D1 protein in photoxidative processes (Mulo *et al.*, 2009). Likewise, *wcaG* encoding a GDP-fucose synthase which takes part in the pathway of carotenoid biosynthesis (Mochimaru *et al.*, 2008) was downregulated in the EB2770FurC strain. In addition, genes included in iron metabolism category were upregulated in the EB2770FurC variant. They were genes that encode proteins involved in uptake and transport of Fe^{3+}-citrate called FecB, FecD3 and FecE3 (Stevanovic *et al.*, 2012) and genes encoding proteins taking part in iron transport named IacT and SchE (Nicolaisen *et al.*, 2010). Among nitrogen metabolism category, the mRNA levels of the RNA polymerase sigma G factor and two proteins similar to nitrile hydratases (All2027 and All2026) were deregulated in the EB2770FurC strain. Furthermore, some genes encoding proteins involved in oxidative stress and detoxification processes such as the peroxiredoxin A, a glutathione S-transferase, a cytochrome P450 and an oxidoreductase showed altered expression in the EB2770FurC strain. In the energy metabolism category, the mRNA levels of a succinyl-CoA synthetase and a 6-phosphofructokinase were strongly upregulated and on the other hand, six genes described as glycosyltransferases also increased their transcription in the EB2770FurC strain. Several genes displaying
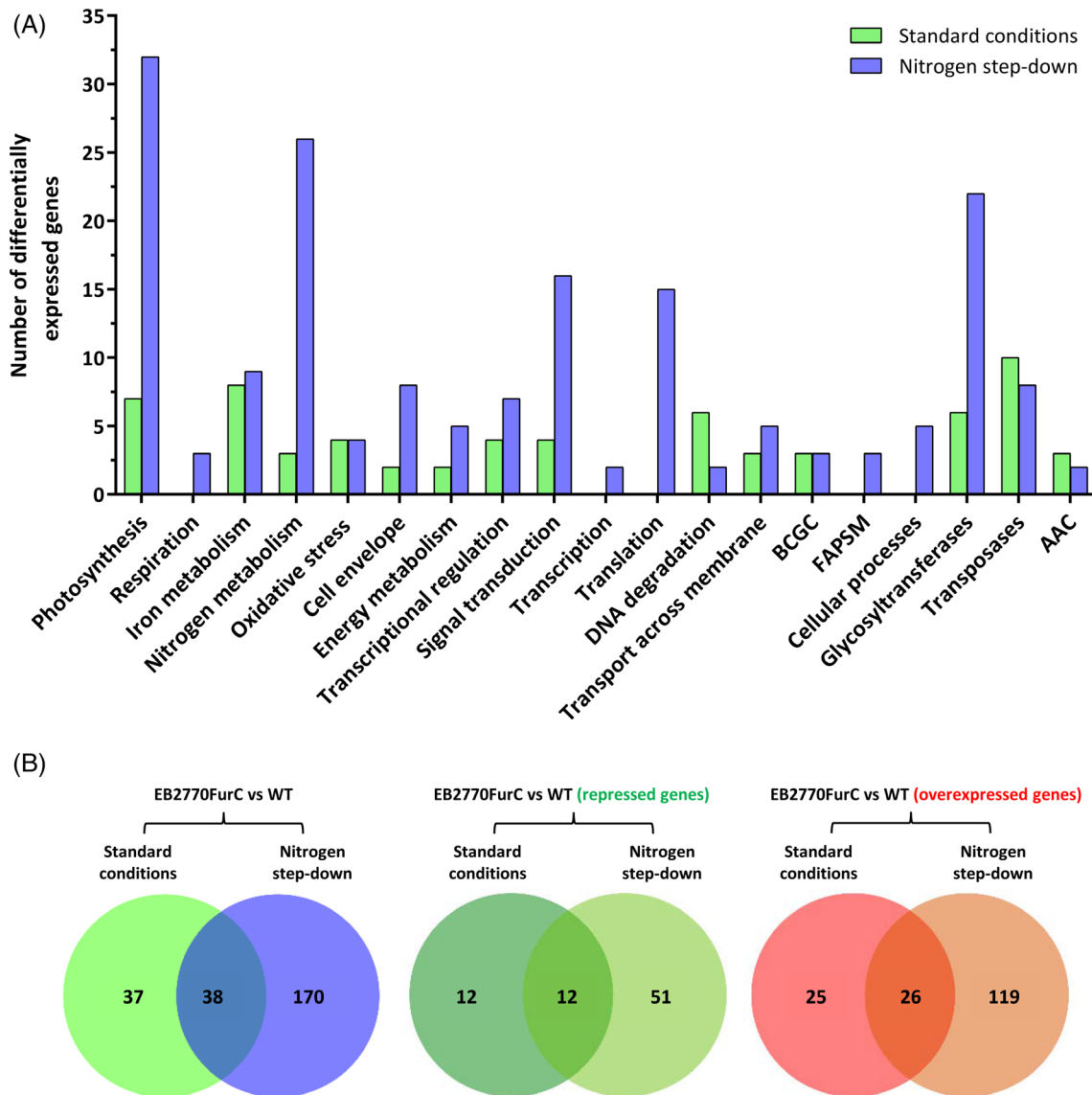
Fig 1. A. Functional annotation of the total number of differentially expressed genes in EB2770FurC strain relative to *Anabaena* sp. PCC7120 under standard conditions (green) and after 48 h of nitrogen step-down (blue). Functional categories were described according to the CyanoBase (http://genome.microbedb.jp/cyanobase/) and KEGG classification (www.genome.jp/kegg/). BCGC: Biosynthesis of cofactors, prosthetic groups and carriers; FAPSM: Fatty acid, phospholipid and sterol metabolism; AAC: Adaptations and atypical conditions.
B. Overlap between differentially expressed genes with known functions in EB2770FurC strain versus wild-type strain grown under standard and nitrogen step-down conditions. In the Venn-type diagrams, all the differentially expressed genes and among them those upregulated and down-regulated are represented. [Color figure can be viewed at wileyonlinelibrary.com]

regulatory functions as transcriptional regulators or genes encoding proteins conforming two component systems and serine/threonine kinases were deregulated in the EB2770FurC strain. Finally, the mRNA levels of the non-specific endonuclease NucA and its inhibitor NuiA (Muro-Pastor *et al*., 1997; Meiss *et al*., 2000) were down-regulated in the EB2770FurC strain. Some differentially expressed genes in EB2770FurC strain such as *nucA* and *nuiA* are harboured in plasmids. These transcriptional changes should be taken with caution because it has recently revealed that plasmid genes may suffer genome rearrangements in mutant strains of *Anabaena* sp. PCC7120 (Camargo *et al*., 2021). The expression of seven selected genes belonging to different functional categories was analysed in the EB2770FurC strain related to *Anabaena* sp. PCC7120 by Real-Time RT-PCR in order to validate the results obtained in RNA-seq analysis. Three biological and three technical replicates were assayed for each gene. These genes were *asl3849*, *fecB*, *iacT*, *all1648*, *alr7354*, *nucA* and *sigG*.
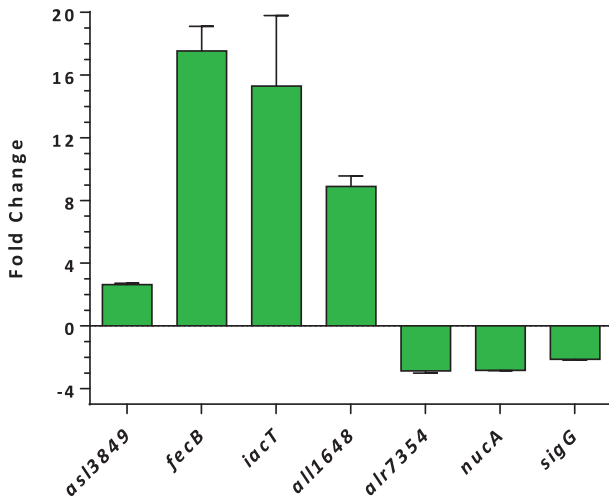
Fig 2. Validation of RNA-seq results obtained in EB2770FurC and *Anabaena* sp. PCC7120 strains grown under standard conditions. Relative transcription of selected genes determined by Real-Time RT-PCR in EB2770FurC cells with respect to *Anabaena* sp. PCC7120. Values are expressed as fold change and correspond to the average of three independent assays; the standard deviation is indicated. [Color figure can be viewed at wileyonlinelibrary.com]

The transcriptional profile of these genes was in agreement with RNA-seq data in all the cases (Fig. 2). It should be noted that in most cases the transcriptional changes are substantially higher when RNA expression levels were analysed by Real-Time RT-PCR as it is the case of *fecB* (17.5-fold), *iacT* (15.3-fold) or *all1648* (8.9-fold) levels.

FurC directly controls the expression of genes involved in photosynthesis, iron homeostasis and energy metabolism

EMSA assays were carried out by using FurC and 28 selected promoters whose genes showed altered expression under standard conditions in the EB2770FurC strain. Results are indicated as positive for EMSA assays (genes directly regulated by FurC) or negative for EMSA assays (genes indirectly regulated by FurC) in Table S2. Gels obtained from EMSA assays that yielded positive band-shift retardation are included in Fig. 3. Results showed that FurC was able to bind to the promoter of *psbAIV* encoding an isoform of the PSII reaction center protein D1. Likewise, FurC bound to the promoter of the *alr4028-alr4033* operon which includes the components of the Fec system involved in uptake and transport of Fe^{3+}-citrate. Interestingly, FurC also displayed band shift retardation with the promoter of the operon comprising *all3913* and *all3914* genes which encode the succinyl-CoA synthetase alpha and beta chains respectively. These subunits form the succinyl-CoA synthetase of *Anabaena* sp. PCC7120 that catalyses the conversion of

succinyl-CoA into succinate in the Krebs cycle. Regarding to regulatory functions, FurC bound to the promoters of the transcriptional regulator *all7016* and *alr9013* two-component response regulator, both of them displaying unknown functions.

Nitrogen starvation deeply impacts the transcriptional profile of the EB2770FurC strain

The RNA from EB2770FurC and *Anabaena* sp. PCC7120 strains, both cultured under nitrogen step-down conditions for 48 h, were purified and analysed by RNA-seq. Comparative analyses revealed that 504 genes showed altered expression in the EB2770FurC strain related to *Anabaena* sp. PCC7120 using a log2 (fold change) with a cut-off value of ±1.5 that correspond with ±2.8-fold and a *P*-value <0.05 (Table S3; Fig. 1A). Among them, 296 genes annotated as hypothetical proteins were included in Supplementary Table S5. It is important to note that a high percentage of the genes showing altered expression in the EB2770FurC strain under nitrogen-deficient conditions do not change under normal conditions suggesting that FurC could be strongly involved in the adaptive response to nitrogen deficiency (Fig. 1A). In fact, the overlap between differentially expressed genes under both conditions yielded only 38 genes (Fig. 1B). Interestingly, most of the deregulated genes are upregulated in the EB2770FurC strain in both transcriptomic analyses. As it can be seen in Table S3, photosynthesis-related genes are markedly affected in the EB2770FurC strain under nitrogen step-down conditions. Several genes encoding both photosystem I (PsaL, PsaJ, PsaX, PsaC) and photosystem II proteins (PsbAIV, PsbAII, PsbO, PsbAIII, PsbL) change their expression in the EB2770FurC strain. Furthermore, 18 genes encoding proteins that constitute phycobilisomes and three genes involved in carotenoid and chlorophyll biosynthesis were deregulated. The mRNA levels of some proteins related to iron uptake showed altered expression in nitrogen step-down conditions as happened in normal conditions. These proteins are those composing the Fec system and SchE, the putative transporter involved in siderophore secretion. However, under nitrogen step-down conditions, some new genes involved in iron transport such as *futA* and *futC* and the two *all1100* and *all2609* genes encoding iron (III) dicitrate-binding periplasmic proteins were deregulated in the EB2270FurC strain. In the other categories, it should be noted the high number of genes showing regulatory functions whose expression is modified, comprising genes of transcriptional regulators as well as proteins composing two component systems and serine/treonine kinases. Interestingly, 22 glycosyltransferases exhibited altered transcription levels in the EB2770FurC strain, being all of them upregulated.
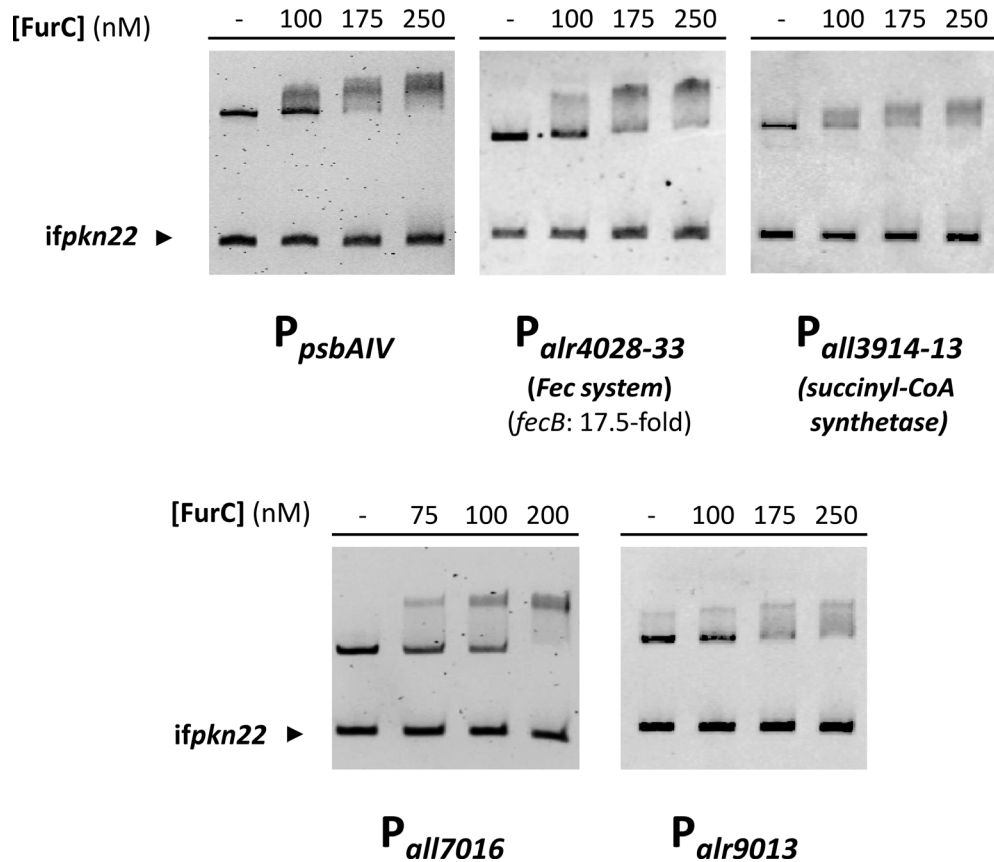
Fig 3. Electrophoretic mobility shift assays testing the ability of FurC to bind *in vitro* the promoter regions of selected genes. DNA fragments free or mixed with increasing concentrations of recombinant FurC (nM) were separated by 6% PAGE. An internal fragment of the gene *pkn22* was used as non-specific competitor DNA. The fold changes of genes whose transcription was analysed by Real-time RT-PCR in EB2770FurC strain relative to wild-type strain are indicated.

FurC overexpression deregulates the expression of several genes involved in heterocyst development and nitrogen fixation

Some genes that show altered expression in the EB2770FurC strain grown under nitrogen starvation are those related to nitrogen metabolism, heterocyst differentiation and nitrogen fixation (Table S3). Among nitrogen metabolism, changes in the transcription of *nirA-nrtABCD* operon responsible for nitrate import and of *all2026* and *all2027* encoding proteins similar to nitrile hydratases were found. Regarding to nitrogen fixation, *nifH2* (a copy of the *nifH* gene), two genes called *xisH* and *xisI* involved in DNA excision and *rbrA* encoding a rubrerythrin were also affected. Finally, mRNA levels of several genes/operons that take part in heterocyst differentiation were found deregulated in the EB2770FurC strain. They were *hepC* (similar to UDP-galactose-lipid carrier transferase), two *devBCA* operons (*alr4973-75* and *alr3647-49*) related to heterocyst glycolipid transport, *hglE2* (potential heterocyst glycolipid synthase), *henR* (response regulator that regulates polysaccharide deposition), *hetZ*

(transcriptional regulator in heterocyst development), *sigC* (sigma factor) and finally *asr1734* (heterocyst development negative regulator). In order to corroborate RNA-seq data, the expression of some selected genes showing key functions in the nitrogen metabolism (*hetZ*, *henR*, *hepC*, *alr3646*, *alr4973*, *hglE2*, *xisH*, *nifH2*, *asr1734* and *nrtA*) was analysed by Real-time RT-PCR. Three biological and three technical replicates were analysed for each gene. Results shown in Fig. 4 indicated that all the transcriptional changes observed in the Real-Time PCR assays were in good concordance with those retrieved from RNA-seq data. Indeed, in general, the fold changes observed in Real-time PCR analyses were sensibly higher (Fig. 4; Table S3). Most of these nitrogen-related genes showed an increase in their expression in the EB2770FurC mutant under nitrogen starvation conditions. For instance, *hetZ* increases 7.8-fold, *henR* 3.6-fold, *hepC* 5.8-fold, *alr3646* 3.7-fold, *alr4973* 4.2-fold, *hglE2* 28.4-fold *xisH* 5.1-fold, *asr1734* 4.6-fold and *nrtA* 12.6-fold. In contrast, *nifH2* was downregulated -threefold in the EB2770FurC strain. In order to analyse if these genes were direct targets of FurC, EMSA assays were
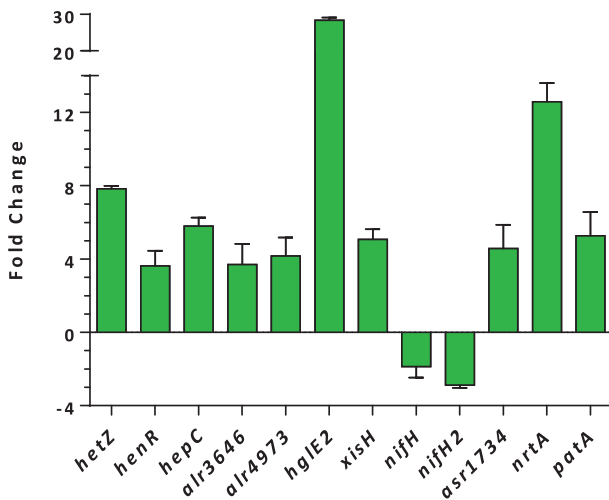
Fig 4. Influence of *furC* overexpression on the mRNA levels of selected genes related to the heterocyst differentiation and nitrogen fixation upon 48 h of nitrogen step-down. A relative quantification procedure was used. Values are expressed as fold change and correspond to the average of three independent assays; the standard deviation is indicated. [Color figure can be viewed at wileyonlinelibrary.com]

performed using 15 promoter regions from genes related to nitrogen metabolism and heterocyst development. The results retrieved from EMSA assays are summarized in Table S3. Genes whose promoters were positive in EMSA assays such as *hetZ*, *hepC*, *alr4973-75* (*devBCA*), *nifH2*, *rbrA*, *xisHI*, *asr1734* and *alr2165* were identified as direct targets of FurC protein (Fig. 5). Since the *nifH2* gene was a direct target of FurC, we decided to investigate if the canonical dinitrogenase reductase encoded in *nifH* (which forms part of the *nifHDK* operon) was a direct target of FurC. Interestingly, the expression of *nifH* was found to be slightly downregulated in the EB2770FurC strain (−1.8-fold). Afterwards EMSA assays corroborated that FurC was able to bind to the promoter region of the *nifHDK* operon (Fig. 5). The promoter region used in EMSA assays spans −350 bp and +10 relative to the translation initiation codon of *nifH*.

Inference of a putative FurC box

In order to find a putative FurC box, the sequences of the promoter regions whose genes were found to be directly regulated by FurC in the present and previous works (Yingping *et al*., 2014; Sevilla *et al*., 2019) were used as an input for analysis with the MEME software. From a total input of 22 promoter regions, MEME algorithm automatically selected the motifs found in the promoter regions of 18 genes (*prxA*, *ftsH*, *srxA*, *furC*, *nifH2*, *alr4404*, *xisH*, *ahpC*, *CGT3*, *hepC*, *hetZ*, *devBCA2*, *asr1734*, *all7016*, *all2165*, *psbAIV*, *alr9013* and *alr1174*)

to build a nearly palindromic 19-bp FurC-box (Fig. 6A). The FurC DNA-binding consensus sequence 5′-CAAAATCATAACGACTTTG-3′ was inferred selecting the most conserved nucleotides located in each position within FurC-box. The predicted consensus suggests that FurC from *Anabaena* sp. PCC 7120 presumably associates with DNA at an imperfect 9-1-9 inverted motif which shares 58% homology to the classical 9-1-9 inverted consensus sequence from *E. coli* Fur: 5′-GATAATGATAATCATTATC-3′ (de Lorenzo *et al*., 1987). Moreover, by using the motif comparison tool 'TOMTOM', the predicted FurC-box also showed similarities with other FUR motifs from other bacteria. The best three matches were obtained with the Fur boxes from *Listeria monocytogenes*, *Salmonella enterica* and *Yersinia pestis* (Fig. S2) (Ledala *et al*., 2007; Gao *et al*., 2008; Teixido *et al*., 2011). Finally, FIMO analyses were carried out to scan the putative FurC-box within the 22 promoter regions used as an input in MEME studies. The putative FurC-binding sequences located in the promoter region of FurC direct targets reported in the present and previous works are shown in Table 1.

The EB2770FurC strain is unable to develop heterocysts

The EB2770FurC and the wild-type *Anabaena* strains were grown under nitrogen sufficiency and nitrogen step-down conditions and the cells compared by using bright-field microscopy. As it can be seen in Fig. 7B and D, no heterocyst differentiation was observed in EB2770FurC cells after 72 h under nitrogen deficiency, whereas several heterocysts were observed in the wild-type strain. EB2770FurC cells did not differentiate heterocysts neither after 96 h nor after 120 h of nitrogen deprivation. In order to confirm these results, the filaments of both strains were stained with Alcian blue after 72 h of step-down and no cell staining was observed in the EB2770FurC strain (Fig. 7E and F). As we previously reported, nitrate-grown EB2770FurC cells exhibited altered morphology compared with *Anabaena* sp. PCC7120 (Fig. 7A and B) (Sevilla *et al*., 2019). However, nitrogen step-down caused even more drastic modifications of the EB2770FurC phenotype, resulting in abnormal cell pattern among the filament (Fig. 7C and D). In view of these results, we wondered if HetR and PatA expression could be deregulated in the EB2770FurC strain. Results revealed that the expression of *hetR* was not affected in EB2770FurC strain, whereas *patA* expression was notably upregulated (5.3-fold) in the EB2770FurC strain (Fig. 4) although FurC was unable to bind the *patA* promoter in EMSA assays (Data not shown). Finally, viability tests in agar plates were carried
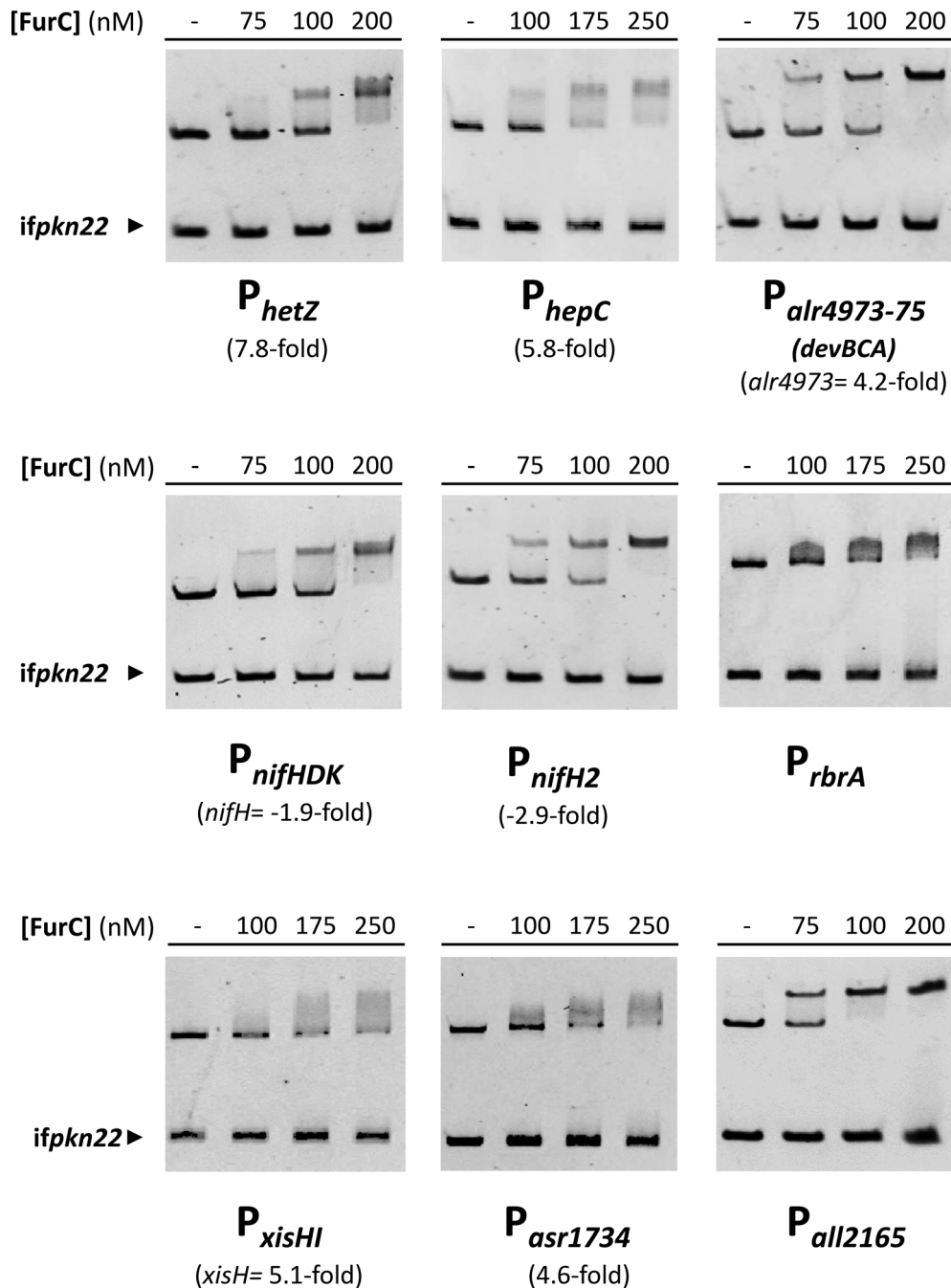
Fig 5. Electrophoretic mobility shift assays showing the binding of FurC to the promoter regions of genes related to the heterocyst differentiation, nitrogen fixation and signal transduction. DNA fragments free or mixed with increasing concentrations of recombinant FurC were separated by 6% PAGE. An internal fragment of the gene *pkn22* was used as non-specific competitor DNA. The fold changes of genes whose transcription was analysed by Real-time RT-PCR in EB2770FurC strain relative to wild-type strain are indicated.

out with *Anabaena* sp. PCC7120 and EB2770FurC strains in nitrogen-sufficient and -deficient conditions. The results showed in Fig. 8 revealed that the growth of the EB2770FurC strain was substantially impaired in nitrogen-starved cultures likely as a consequence of its inability to fix nitrogen due to the lack of hetero-cysts (Fig. 8).

Discussion

PerR is a master regulator that coordinates the oxidative stress response integrating the metal availability and the presence of hydrogen peroxide. Among PerR proteins, PerR of *Bacillus subtilis* is by far the best-characterized orthologue. In this heterotrophic bacterium, the PerR
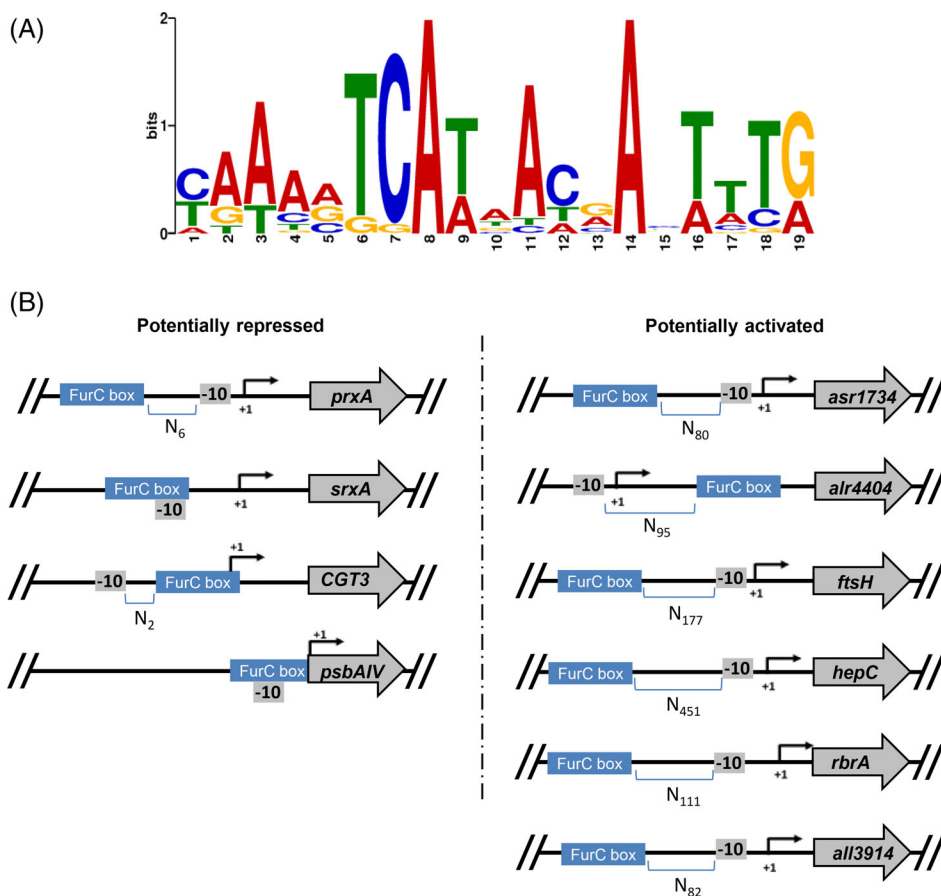
(A)

(B)

Fig 6. A. Predicted 19-bp FurC-box from *Anabaena* sp. PCC7120 identified by MEME using as input the promoter regions of the FurC direct targets found in the present and previous works.
B. Graphical representation of the Fur box location in the promoter region of selected genes. On the left, genes potentially repressed by FurC are shown. On the right, genes potentially activated by FurC are shown. Transcriptional start points are indicated as +1 and TATA boxes are represented as −10. N_x is the distance in nucleotides between the FurC box and the TATA box. [Color figure can be viewed at wileyonlinelibrary.com]

Table 1. Predicted FurC binding sequences found in the promoter regions of genes directly regulated by FurC.

Gene[a]	*P*-value	Putative FurC binding sequence[b]
prxA_alr4641	2.40×10^{-9}	CATAGTCATAACGATTTTG
ftsH_all4776	1.05×10^{-8}	CGAAGTCATTACGAATTTG
furC_alr0957	1.25×10^{-8}	CAAACTCATTACAACTTTA
srxA_asl4146	1.98×10^{-8}	CGAAGTCATAATGACTATG
nifH2_alr0874	2.78×10^{-8}	AAAAATCATAACGATATTG
ahpC_all1541	3.65×10^{-7}	TATAATCATAATGACTACG
hepC_alr2834	5.23×10^{-7}	CTTAATCATGACAACTTTA
alr4404	7.36×10^{-7}	TAAAGGCAAAACAACATCG
CGT3_all2375	7.64×10^{-7}	CAAAGTCATCAAAAATCTG
xisH_alr1461	1.05×10^{-6}	TGACCTCAAAACCAGATTG
hetZ_alr0099	1.56×10^{-6}	CTACATCATGACAATTCTG
asr1734	1.93×10^{-6}	TAAAGTCAACAATAGTTTG
devBCA2_alr4973-75	2.55×10^{-6}	AAAGATCATAACCACTGTG
all7016	4.22×10^{-6}	TAATCTCAATTCGATTTTG
all2165	6.55×10^{-6}	TAAAAGCAAAATGAGTACA
psbAIV_all3572	8.67×10^{-6}	CGTAATCATAAAAACATGA
rbrA_alr1174	1.16×10^{-5}	TAATCTGAATACCAATTTG
alr9013	1.58×10^{-5}	CAACATCATGCTGAGAATA
ftsZ_alr3858	4.31×10^{-4}	CAACCTTAACAAGATTGTA
alr4028-33	7.60×10^{-4}	TATTATCACACTGATAGTG
nifH_all1455	9.50×10^{-4}	TTAACTGTTAACTAAACTG
all3914-13	1.75×10^{-3}	ATAGGTCAAATTAATTTCT

[a]Direct target genes found in this work are shown in bold.
[b]Putative FurC binding sequences obtained by FIMO (http://meme-suite.org/tools/fimo) scanning the predicted FurC box (Fig. 6) in the promoter regions of the FurC direct targets found in this and previous works.
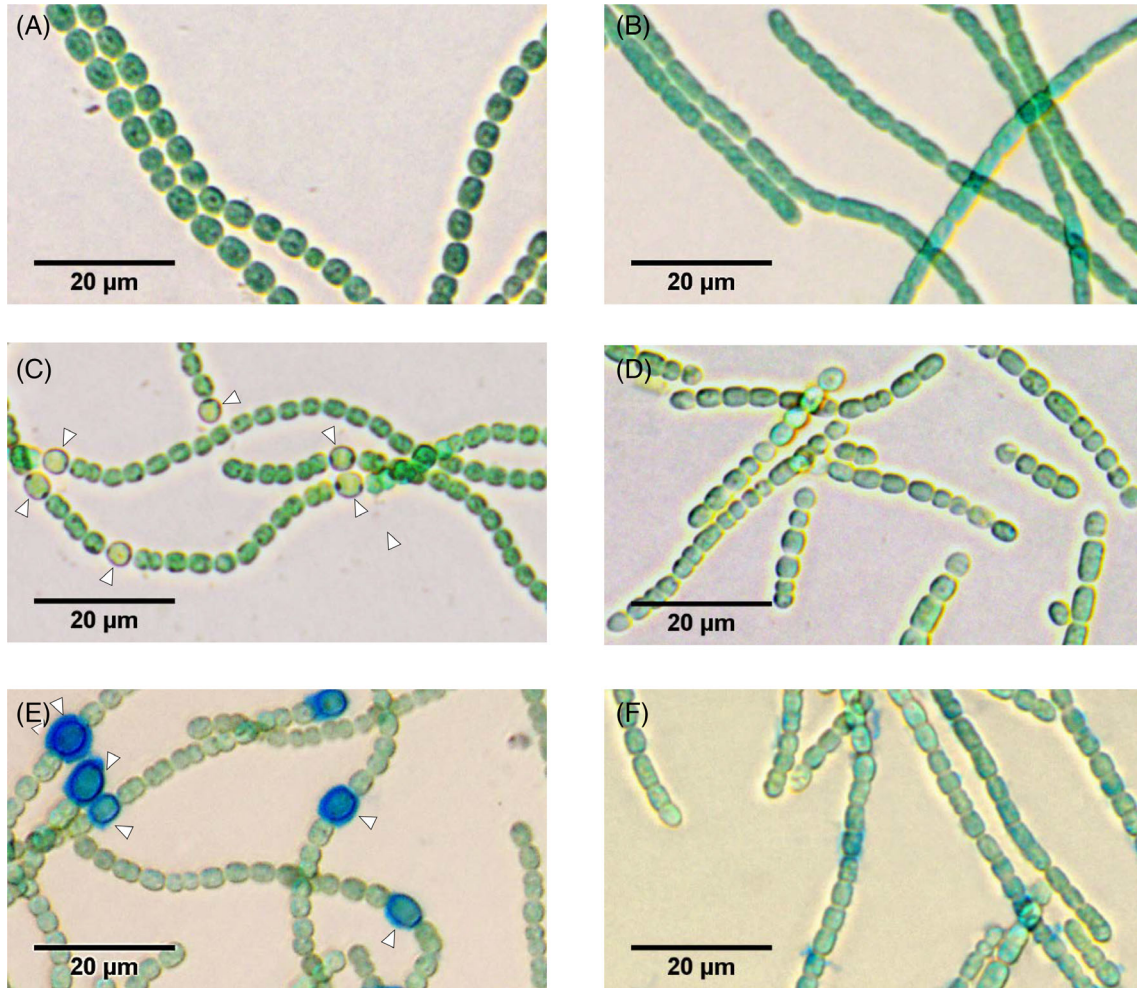
Fig 7. Bright-field micrographs of *Anabaena* sp. PCC7120 (A, C, E) and its derivative *furC*-overexpressing strain EB2770FurC (B, D, F) grown under standard conditions (A, B) and after 72 h of nitrogen step-down before (C, D, E, F). Arrows indicate positions of heterocysts. Filaments of nitrate-deprived cultures were treated with Alcian blue to stain the polysaccharide layer of heterocysts (E, F). Photomicrographs of each strain are representative of at least 10 different images from two biological replicates (scale bars: 20 μm). [Color figure can be viewed at wileyonlinelibrary.com]

regulon comprises in the first place, genes involved in detoxification of reactive oxygen species such as *katA* and *ahpC* (Chen *et al*., 1995; Bsat *et al*., 1998). The second group includes genes related to metal homeostasis such as *hemAXCDBL* (Faulkner *et al*., 2012), *mgrA* (Chen and Helmann, 1995; Chiancone and Ceci, 2010), *pfeT* (Guan *et al*., 2015) and *zosA* (Gaballa and Helmann, 2002). On the other hand, PerR also control the expression of *fur* and its own expression (Fuangthong *et al*., 2002). Similarly, in pathogens like *Streptococcus pyogenes*, *Staphylococcus aureus* or *Listeria monocytogenes*, the PerR regulons are mainly composed by genes involved in the defence against oxidative stress and iron metabolism frequently linked to pathogenesis (Horsburgh *et al*., 2001; Ricci *et al*., 2002; Brenot *et al*., 2005; Rea *et al*., 2005). To date, some

orthologues of PerR proteins have been described in cyanobacteria. The PerR regulon from *Synechocystis* sp. PC6803 comprises genes involved in oxidative stress response such as *ahpC* or *sodB* and also genes related to iron homeostasis *idiA*, *isiA* or *mrgA* (Li *et al*., 2004). It was previously reported that in *Anabaena* sp. PCC7120, FurC directly regulated the expression of genes involved in oxidative stress response like *ahpC*, *srxA*, *prxA*, *CGT3* and *alr4404* (Yingping *et al*., 2014; Sevilla *et al*., 2019). In the present work, we show that the expression of *alr2142* (oxidoreductase), *prxA* (peroxiredoxin), *alr4686* (cytochrome P450) and *alr7354* (glutathione S-transferase), all of them participating in the defence against oxidative stress, are deregulated in EB2770FurC strain. These findings corroborate the main role of FurC in the maintenance of redox homeostasis in *Anabaena*
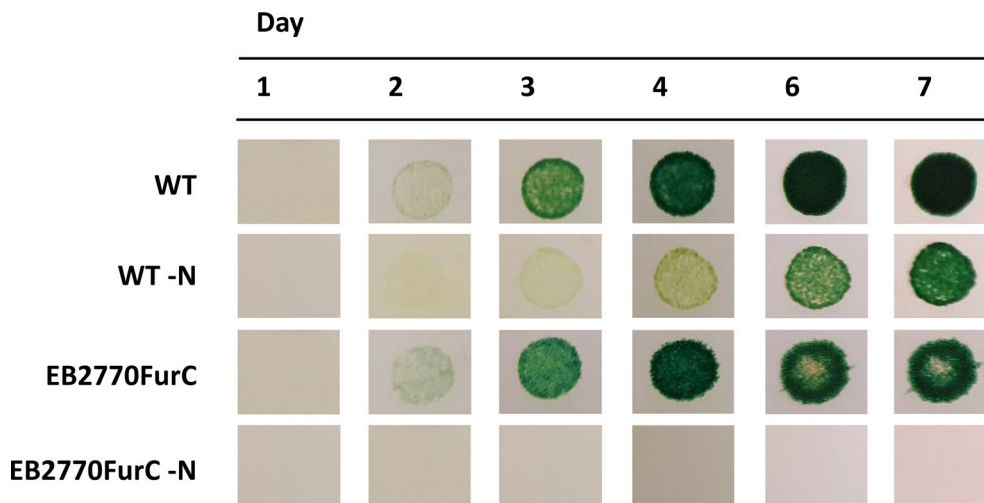
Day

	1	2	3	4	6	7
WT						
WT -N						
EB2770FurC						
EB2770FurC -N						

Fig 8. Viability analyses of *Anabaena* sp. PCC7120 and EB2770FurC on BG11 agar plates in the presence and absence of combined nitrogen ($-NO_3^-$). 5 μl volume of liquid cultures at an OD_{750} of 0.4 were applied per spot and incubated under continuous light of 30 μmol photons m^{-2} s^{-1} for 7 days. Two biological replicates were tested separately. [Color figure can be viewed at wileyonlinelibrary.com]

sp. PCC7120. On the other hand, both the expression of *alr4028-33* (Fec system) and *futABC* operons controlling the uptake of inorganic Fe (III) (Stevanovic *et al*., 2012) are altered in EB2770FurC strain. Indeed, the expression of *alr4028-33* operon composed by a TonB-dependent receptor, a putative ferredoxine, FecB (ABC transporter, periplasmic-binding protein), FecD3 (Iron(III) ABC transporter, permease protein) and FecE3 (ferrichrome ABC transporter, ATP binding protein) seems to be directly activated by FurC. Similarly, other genes involved in iron transport named *iacT* and *schE* as well as *all1100* and *all2609* encoding iron (III) dicitrate-binding periplasmic proteins could be under the transcriptional control of FurC by an indirect regulation. Taken together, these results suggest that FurC, like other PerR orthologues, could play a key role in the transport and uptake of iron in *Anabaena* sp. PCC7120.

However, the large number of deregulated genes in EB2770FurC cells grown under nitrogen step-down that are related to photosynthesis and nitrogen metabolism points to new roles of FurC in *Anabaena* sp. PCC7120 (Fig. 1A). Some of these novel functions could be attributed to the fact that unlike *Synechocystis* sp., *Anabaena* sp. PCC7120 is a nitrogen-fixing cyanobacterium. The functional category comprising photosynthetic genes was meaningfully altered in the EB2770FurC strain. In fact, seven genes were deregulated in the EB2770FurC strain under standard conditions, while 32 genes changed their expression under nitrogen step-down. These genes included ORFs encoding proteins from both photosystems and from phycobilisomes. We previously reported the involvement of FurC in the structural and functional maintenance of photosynthetic apparatus so that these

data would be in concordance with our previous results (Sevilla *et al*., 2019). In that work, we reported that the major thylakoid membrane protease *ftsH* displaying a role in the PSII repair cycle is directly controlled by FurC. FtsH is a protease involved in the degradation of the D1 protein. In the present work we found that the expression of *psbAIV* gene which encodes a D1 protein is also under the control of FurC suggesting that the role of FurC in the management of PSII repair cycle goes further than the FtsH transcriptional control. On the other hand, the deregulation of 32 genes among photosynthesis category in the EB2770FurC strain grown under nitrogen step-down conditions appears to be quite enigmatic. It is well known that heterocysts undergo the dismantling of oxygen-producing PSII during differentiation to reduce oxygen production (Wolk *et al*., 1994) so that a potential involvement of FurC in the regulation of this process could be hypothesized.

In the present work we show that FurC from *Anabaena* sp. PCC7120 displays an important role in regulating hierarchically at different levels, the complex regulatory network of nitrogen metabolism (Fig. 9). Regarding to regulatory elements, FurC directly regulates the expression of HetZ, a key regulatory element in the heterocyst differentiation process of *Anabaena*. HetZ has been reported to be a positive effector of differentiation through protein–protein interactions with HetR and HetP (Videau *et al*., 2018). The negative regulator of heterocyst development Asr1734 and All2165, a response regulator which presumably forms an operon with other response regulator called All2164 (Wang *et al*., 2002) are also under the control of FurC. The mRNA levels of *all2165* was found to be modified in a *hepN* and *hepK* deletion mutants
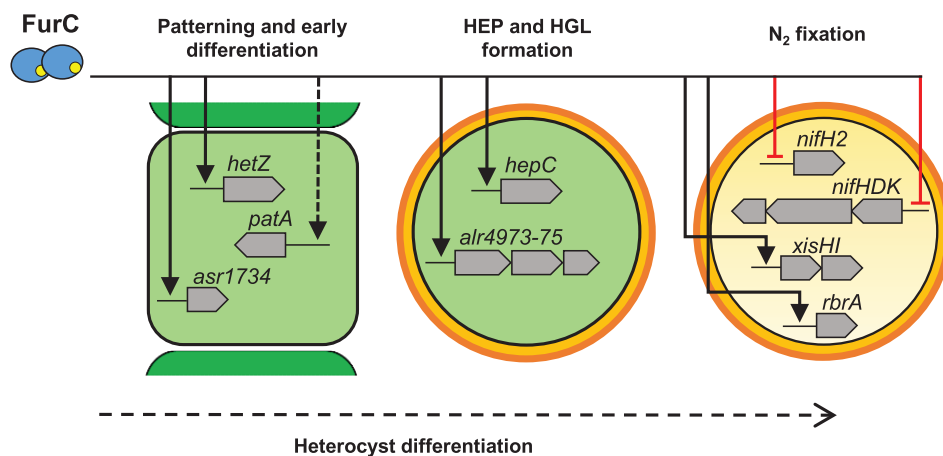
Fig 9. General scheme of the transcriptional regulation performed by FurC of its novel direct targets genes involved in the indicated phases of the heterocyst differentiation and development (pattern formation, early differentiation, HEP and HGL formation and nitrogen fixation). Due to its relevance, PatA has been included in the general scheme because its mRNA is altered in the EB2270FurC strain grown under nitrogen deficiency in spite of the fact that is not directly regulated by FurC (dotted arrows). Black arrows indicate the putative transcriptional activation performed by FurC and red blunt arrows indicate the putative transcriptional repression. [Color figure can be viewed at wileyonlinelibrary.com]

(Lechno-Yossef *et al*., 2006) so that it cannot be ruled out its potential involvement in heterocyst development. In conclusion, the control exerted by FurC on *hetZ* and *asr1734* expression suggests that this transcriptional regulator could be an important player in the regulation of the heterocyst pattern formation and early differentiation process of *Anabaena* sp. PCC7120 (Fig. 9).

One of the morphological changes preceding nitrogen fixation in the heterocyst is the formation of a thick cell envelope comprising two layers, the outer exo-polysaccharide layer (HEP layer) and the inner laminated glycolipid layer (HGL layer). The HGL layer is the barrier for gases while HEP layer protects the HGL layer (Maldener *et al*., 2014). In the present work, we evidenced that HepC, which likely plays a role in polysaccharide deposition of HEP layer, is a direct target of FurC (Wolk *et al*., 1994). In *Anabaena*, DevBCA proteins are involved in the heterocyst-specific glycolipids deposition into the HGL layer. Indeed, *Anabaena* sp. PCC7120 contains several DevBCA homologues as is the case of the *alr4973-75* operon (Staron, 2012). We found that FurC directly controls the expression of the *alr4973-75* operon. Taken together, as the biosynthesis of both, HEP and HGL layers, is influenced by FurC it seems feasible to propose that FurC is also relevant in the development of heterocyst envelope (Fig. 9).

It is noteworthy that FurC overexpression in the *Anabaena* sp. PCC7120 strain impairs heterocyst development (Fig. 7). Deregulation of key genes that take part in the regulation of heterocyst patterning, early differentiation and envelope formation can be hampering the normal developing of heterocysts. However, the inhibition of

heterocyst development observed in EB2770FurC strain could be also explained by the upregulation of the negative regulator of heterocyst development Asr1734 since the overexpression of Asr1734 in *Anabaena* sp. PCC7120 triggers this phenotype (Wu *et al*., 2007).

Regarding to nitrogen fixation, FurC seems to be able to repress the *nifHDK* operon, encoding the major structural proteins of the molybdenum-containing nitrogenase complex from *Anabaena* sp. PCC7120 (Mazur *et al*., 1980; Rice *et al*., 1982) and a copy of the *nifH* gene called *nifH2*. In spite of the fact that *nifH2* shows almost an identical sequence than *nifH*, *nifH2* is not accompanied by copies of *nifD* and *nifK* as happens with the canonical *nifH* gene (Haselkorn, 1986). In *Anabaena* sp. PCC7120, the role of *nifH2* remains unknown although its upregulation after nitrogen deprivation (Flaherty *et al*., 2011) suggests its involvement in the adaptive response of *Anabaena* sp. PCC7120 to this stress. Interestingly, the *xisHI* operon is also under the control of FurC. The *xisHI* operon encodes two proteins that together with the XisF recombinase seem to be required for the excision of a 59 428-bp fragment that interrupts the heterocyst ferredoxin gene (*fdxN*) (Ramaswamy *et al*., 1997). This excision is needed for the proper expression of the major electron donor to nitrogenase (Kumar *et al*., 2018). Finally, the expression of *rbrA* (rubrerythrin) is directly regulated by FurC. This gene encodes a peroxidase that plays an important role in nitrogenase protection against peroxide in heterocysts (Zhao *et al*., 2007). In summary, FurC seems to control nitrogen fixation at several levels, on the one hand, it downregulates the expression of the *nifHDK* operon and

nifH2 and on the other hand, FurC controls the excision of the heterocyst-specific ferredoxin gene and the induction of the nitrogenase-protecting *rbrA* gene (Fig. 9).

Finally, in the present work we show that FurC indirectly controls the expression of *patA* since a *patA* induction of 5.3-fold was observed in the EB2770FurC strain grown under nitrogen deficiency. PatA is under the control of HetR and NtcA and plays a pivotal role in the proper development of heterocyst pattern in *Anabaena* sp. PCC7120 filaments (Liang *et al*., 1992; Bastet *et al*., 2010; Flaherty *et al*., 2014; Hou *et al*., 2015). Interestingly, RNA polymerase sigma G and C factors are also indirectly regulated by FurC. *sigG* expression was slightly downregulated in EB2770FurC strain grown under nitrogen-sufficient conditions, whereas *sigC* was upregulated in EB2770FurC strain after nitrogen-step down. SigG and SigC factors have been related to nitrogen metabolism because both genes are upregulated in differentiating heterocysts and their mRNA levels are controlled by HetR (Aldea *et al*., 2007; Ehira and Miyazaki, 2015). In addition, DNA microarray analysis of a *sigC* deletion mutant revealed that the expression of genes involved in heterocyst differentiation is regulated by this sigma factor (Ehira and Miyazaki, 2015).

If the transcriptional profile of the EB2770FurC strain is analysed, it can be concluded that FurC would act as transcriptional activator in most of these FurC directly regulated genes with the exception of *nifH* and *nifH2*. In some cases, PerR has been reported to be acting as a transcriptional activator, for example in the regulation of *srfA* from *B. subtilis* (Hayashi *et al*., 2005), *csp* from *S. pyogenes* (Brenot *et al*., 2005) or *ftsH* and *ftsZ* from *Anabaena* sp. PCC7120 (Sevilla *et al*., 2019), although in these cases the underlying transcriptional mechanism is unknown. Comparing the sequences of the promoters directly regulated by FurC, we proposed a putative consensus for the FurC box. The comparison of the FurC boxes location in FurC-target promoters showed that in most cases, when FurC acts as activator the FurC box is far from the TATA box, in contrast, FurC-repressed promoters exhibit FurC boxes close to their TATA box (Fig. 6B and Supplementary Fig. S1). This observation is in agreement with the fact that transcriptional repressors usually act hampering the binding of the RNA polymerase to the TATA box (Payankaulam *et al*., 2010).

Finally, it is interesting to note that in absence of nitrogen, about 500 genes are deregulated in the EB2770FurC variant, whereas in standard conditions only about 200 genes displayed differences in their transcriptional levels with respect to those from the wild-type *Anabaena*. This differential expression under nitrogen-deficiency conditions suggests that some signal is activating the FurC response under nitrogen step-down conditions. Some key proteins with relevant roles in the regulation of nitrogen metabolism such as NtcA and FurA are able to bind 2-OG. In view of the transcriptomic profile of the EB2770FurC strain, we wondered if FurC could also be sensing nitrogen deficiency by binding 2-OG. However, when EMSA assays were carried out with the FurC-direct targets involved in nitrogen metabolism in the presence and absence of 2-OG, no effect was observed potentiating or diminishing the complex formation between FurC and the selected promoters (Supplementary Fig. S3).

There are increasing evidences of the interplay between iron deficiency, oxidative stress and nitrogen metabolism (Latifi *et al*., 2005; Saxena *et al*., 2006; Kaushik *et al*., 2015; Kaushik *et al*., 2017; Kaushik and Mishra, 2019; Pernil and Schleiff, 2019; Robles-Rengel *et al*., 2019). Therefore, a protein like PerR that integrates the levels of iron, manganese and hydrogen peroxide in the cell can display an important role in this cross-regulation. Actually, it has also been proposed an alternative mechanism in which the molecular oxygen could oxidize PerR of *B. subtilis* as it occurs with H_2O_2 (Sethu *et al*., 2016). It is interesting to note that some of the processes altered in the EB2770FurC strain, such as the heterocyst envelope formation or the nitrogenase expression are processes relying on oxygen availability.

In summary, FurC regulates the transcription of some essential regulatory elements that display different roles in heterocyst development and patterning such as HetZ, the negative heterocyst regulator (Asr1734) or PatA. In addition, FurC is directly involved in the activation of the heterocyst envelope formation affecting the synthesis and deposition of the HEP and the HGL layers. Finally, this protein controls the transcription of the *nifHDK* operon as well as the *nifH2*, *xisHI* and *rbrA* genes, evidencing that FurC is a key regulatory element in the nitrogen fixation process. Therefore, in the present work strong evidences indicating that FurC plays a crucial role in the adaptation and maintenance of nitrogen homeostasis in *Anabaena* sp. PCC7120 are presented. Overall, our results suggest that the regulatory network exerted by FurC in nitrogen metabolism seems to be complex and is only starting to be understood.

Acknowledgement

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supplementary Information