A functional study of the transcriptional regulator FurC in cyanobacteria

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“A functional study of the transcriptional regulator FurC in cyanobacteria”

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Proyecto Fin de Master dirigido por:

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Las Dras. Emma Sevilla Miguel y Maria F. Fillat Castejon, Catedrática y Profesora Titular del Departamento de Bioquimica y Biología Molecular y Celular de la Universidad de Zaragoza,

CERTIFICAN

Que la memoria de Proyecto Master titulada " A funciontal study of the transcriptional regulator FurC in cyanobacteria", presentada por Cristina Sarasa Buisán, ha sido realizada bajo su dirección en el Departamento de Bioquimica y Biología Molecular de la Univerisdad de Zaragoza y que reúne todos los requisitos para ser presentada por su autora para optar al título de Máster en Biología Celular y Molecular por la Universidad de Zaragoza.

Zaragoza, a 24 de Junio de 2016

Dra. Maria F. Fillat Castejon

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1. ABSTRACT

FUR (ferric uptake regulator) proteins constitute a large family of transcriptional regulators that exhibit a wide range of functions. FurC from the filamentous, nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120 is the regulator least studied and the one with less similarities with FUR paralogues. It has been reported that FurC is involved in a various physhiological processes including a potential modulation of the transcriptional activity of its paralogues, FurA and FurB, a clear implication in the oxidative stress processes and also it has been found that FurC is regulated by the global master of nitrogen processes, NtcA. The main goal of this work was focused on the study of these general functions, focusing on the nitrogen metabolism processes. To this aim we performed both a biochemical characterization of the recombinant FurC protein and also a phenotypical study of a *furC*-overexpressing mutant. FurC was overexpressed and purified with an optimized method showing binding activity and an abscense of metal residues in its structure. Unlike the other FUR paralogues, FurC did not required the presence of any regulatory metal to perform the DNA-protein interaction. Novel targets related to nitrogen metabolisim and oxidative stress response were described for FurC, such as *ntcA* and *nblA* and *prxA*. In conclussion, by combining the phenotypical response and transcriptional analysis, we explored the multifaceted character of FurC and we described its important role in the degradation of the phycobilisomes, an acclimation process performed during nitrogen starvation in cyanobacteria.

1.1. Resumen

Las proteínas FUR (ferric uptake regulator) constituyen una extensa familia de reguladores transcripcionales con una gran diversidad de funciones. FurC es un regulador en la cianobacterias filamentosa fijadora de nitrógeno Anabaena sp. PCC 7120 y se trata del regulador menos estudiado en la familia FUR y el que menos se asemeja a sus parálogos FurA y FurB. Se ha descrito que FurC está involucrado en varios procesos fisiológicos como la regulación de la actividad transcripcional de FurA y FurB, su clara implicación en procesos de estrés oxidativo y la participación en el metabolismo del nitrogeno, siendo regulado por el regulador global del nitrogeno, NtcA. El trabajo descrito en esta memoria se ha dirigido a estudiar estas funciones, focalizando en el papel que juega FurC dentro del metabolismo del nitrógeno. Para ello se realizó tanto una caracterización bioquímica de la proteína FurC recombinante, como un estudio fenotípico de un mutante de sobreexpresión de *furC* en *Anabaena* sp.. Se sobreexpresó y purificó FurC mediante un método optimizado en nuestro laboratorio que permitió obtener una proteína activa y la cual no mostraba átomos de Zinc en su estructura. Al contrario que los otros paralogos FUR, FurC no requirió de la presencia de metal regulador para mostrar una interacción específica con el ADN. Se describieron nuevos genes diana para el regulador FurC relacionados con el metabolismo de nitrógeno, *ntcA* y *nblA* y con el estrés oxidativo, *prxA*. En conclusión, la combinación de los estudios fenotípicos con análisis transcripcionales permitió explorar el carácter multifacético de FurC y describir su fuerte implicación en el proceso de degradación de los ficobilisomas, proceso de aclimatación que tiene lugar bajo condiciones de deficiencia de nitrógeno en cianobacterias.

2. INTRODUCTION

2.1. Basic aspects of cyanobacteria

Cyanobacteria are oxygenic photoautotrophic prokaryotes, classified as Gram negative bacteria. About 2,300-2,400 million years ago, they were responsible for the rapid oxygenation of Earth's atmosphere during an episode referred to as the Great Oxidation Event (GOE). In this period, atmospheric oxygen levels accumulated rapidly (Anbar et al., 2007; Bekker et al., 2004) as a result of cyanobacterial metabolic activity leading to great changes to the Earth system (Van Kranendonk et al., 2012), subsequently allowing for the evolutionary origin of multicellularity and complexity as we know it nowadays. (Schirrmeister, Gugger, & Donoghue, 2015)

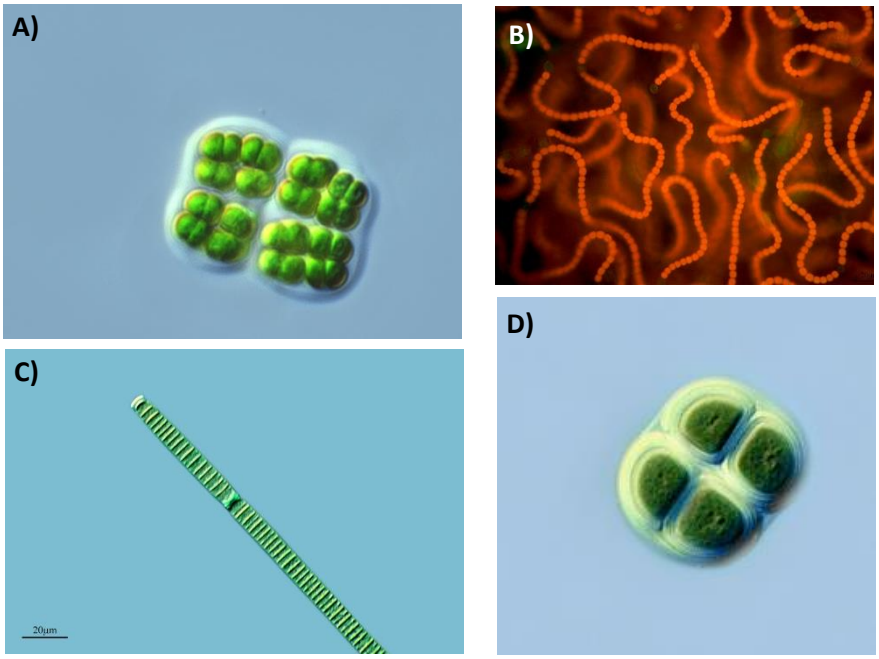

Figure 1. Morphological diversity of cyanobacteria. A) *Cyanosarcina* sp. B) *Nostoc* sp. Fluorescence microscopy. C) *Oscillatoria* sp. D) *Chroococcus tenax.* Photographs A,C,D differential interference contrast microscopy, B) fluorescence microscopy. Photographs were taken and kindly provided by Antonio Guillen from "Water Project".

This phylum is capable of surviving under a wide range of environmental conditions, this can be explained in the light of their considerable morphological diversity, the

symbiotic relationships with certain plants or even the toxicity that some species present. Such high level of complexity allows some vegetative cells from filamentous species to differentiate into functional specialized cell types such as, hormogonium, nitrogen-fixing heterocysts, and spore-like akinetes. (Singh & Montgomery, 2011) that play key roles in global carbon and nitrogen cycles.

2.2. *Anabaena* sp.

The genus *Anabaena* belongs to the cyanobacterial family Nostocaceae, order Nostocales. It is a filamentous, heterocyst-forming cyanobacterium present in both fresh and brackish water worldwide. The *Anabaena* sp. strain PCC 7120 used in this work is a non-toxic strain commonly used as a laboratory model for biological and biochemical studies. It's largely use was motivated by the sequencing of its genome, that was updated in the data base *Cyanobase* (http://genome.kazusa.or.jp/cyanobase). (Nakao et al., 2009)

2.2.1. Nitrogen metabolism

In the presence of a combined nitrogen source, *Anabaena* grows as long filament units containing hundreds of photosynthetic vegetative cells. However, in the absence of combined nitrogen some filaments of this diazotrophic cyanobacteria spatially separate photosynthesis and nitrogen fixation processes by differentiating specialized cells called heterocysts. This separation relies on the fact that the nitrogen-fixing enzyme, nitrogenase, that is irreversibly inactivated when exposed to molecular oxygen generated in photosynthetic vegetative cells. (Flores & Herrero, 2010).

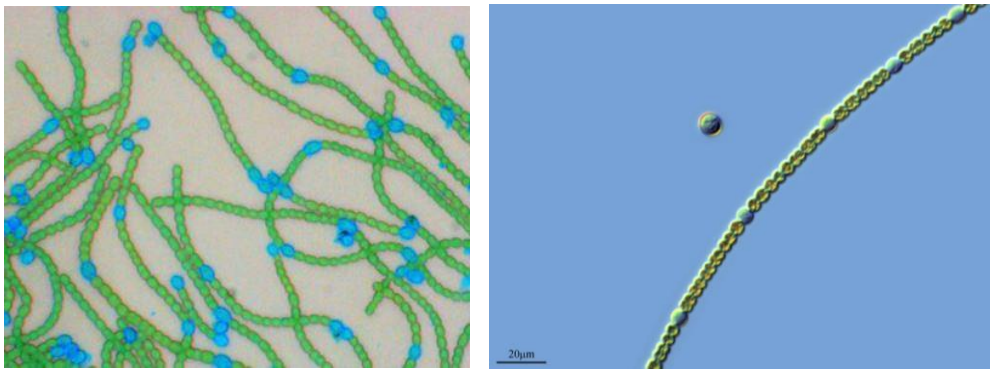

Figure 2: Left. Bright-field micrographs of filaments of Anabaena wild-type strain PCC 7120. Filaments showing heterocysts stained with Alcian Blue. Adapted from (González, Valladares, Peleato, & Fillat, 2013). **Right. *Anabaena* sp.** Differential interference contrast microscopy. Photograph was taken and kindly provided by Antonio Guillen from "Water Project".

This complex morphological and metabolic change that occurs in heterocyst differentiation is tightly controlled by transcriptional regulators, including the global nitrogen metabolism regulator NtcA protein. NtcA is necessary for the formation of heterocysts and apparently acts sensing the balance C-N in the cell by the molecule of 2-oxoglutarate (Tanigawa et al., 2002). Depending on the level of 2-oxoglutarate, NtcA modulates the transcription of most genes involved in heterocyst development and maturation, such as *hetR*, as well as those responsible for the metabolism of nitrogen, such as the *nifHDK* operon encoding the nitrogenase structural genes (Reviewed by (Herrero et al., 2001)). The gene *hetR* is central to heterocyst differentiation, its expression is the main positive regulatory factor in heterocyst development (Buikema & Haselkorn, 1991; Risser & Callahan, 2007). Moreover, this expression is negatively regulated by two genes, *patS* and *hetN*. (Figure 1).

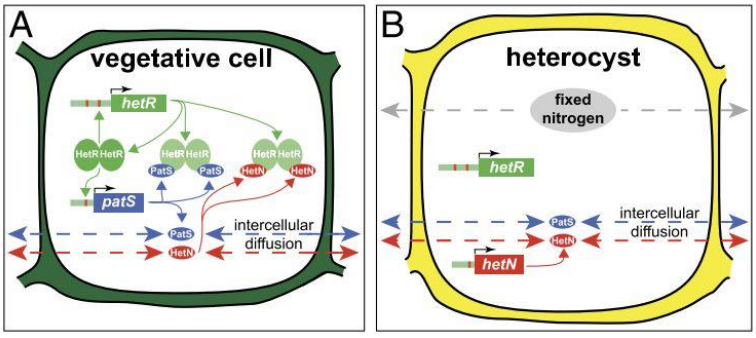

Figure 3. Minimal of the genetic regulation network in heterocyst differenciation. (A) In vegetative cells, HetR can activate the expression of *hetR* and *patS*.(B) In heterocysts, *hetN* is expressed constitutively. Active products of PatS and HetN, possibly the RGSGR pentapeptide, can diffuse between cells of any kind in the filament and bind HetR, preventing it from binding DNA.

It has been observed that *patS* is strongly expressed in developing heterocysts ;(Yoon & Golden, 1998, 2001). PatS is a short peptide, containing a carboxyl-terminal pentapeptide RGSGR that prevents DNA binding activity of HetR (Feldmann et al., 2011; Huang, Dong, & Zhao, 2004). The expression of *patS* in vegetative cells was shown to decrease the levels of HetR in adjacent cells (Risser & Callahan, 2009), suggesting that a PatS-dependent signal can diffuse along the filament (Corrales-Guerrero et al., 2013). *hetN* also performs the same function and contains the same RGSGR motif but its activated later than *patS*. When both genes are suppressed, almost all cells along the filament eventually differentiate, causing lethal levels of heterocysts (Borthakur et al., 2005). This complex intercellular processes for heterocyst differentiation have been recently observed to be tightly influenced by the physical separation between cells constituted by septa junctions, which integrity is modulated by a gene called *sepJ*

(Mariscal et al., 2016). Recent years have seen advances in the identification of the molecular mechanism regulating heterocyst formation, in which both genetic regulation and the effects of cell division and filament growth are key components. (Muñoz-García & Ares, 2016).

In recent years it has found many different processes are involved in the regulation of the metabolism of nitrogen. In fact ChIP analysis unraveled a wide distribution of DNA binding sites for the NtcA transcription factor in a *Anabaena* sp. (Picossi, Flores, & Herrero, 2014). NtcA (Master Regulator of Nitrogen Metabolism) also regulate transcriptional levels of genes implicated in other processes such iron homeostasis, including genes encoding the iron-responsive transcriptional repressor FurA or FurC.

2.2.2. Phycobilisome degradation

One of the first responses displayed in all cyanobacteria is the degradation of phycobilisomes (PBS) under nitrogen starvation. PBS are the major light-harvesting complexes of the photosynthetic apparatus exclusive from cyanobacteria and red algae and can constitute the 50% of the total cellular protein. Cyanobacterial phycobilisomes harvest light and cause energy migration usually toward photosystem II reaction centers. (MacColl, 1998). The phycobilisomes are highly organized complexes of various biliproteins (Allophycocyanin (AC), Phycocyanin (PC) and Phycoerythrin (PE) subunits) and linker polypeptides, which together with chlorophyll *a* give cyanobacteria their typical blue-green colour.

Degradation of PBS is thought to provide substrates for protein synthesis required for the acclimation process and heterocyst formation. In diazotrophic cyanobacteria, like *Anabaena sp.*, PBS degradation is also detectable spectrophotmetrically, although it occurs only transiently just before heterocyst formation (Baier, Lehmann, Stephan, & Lockau, 2004).

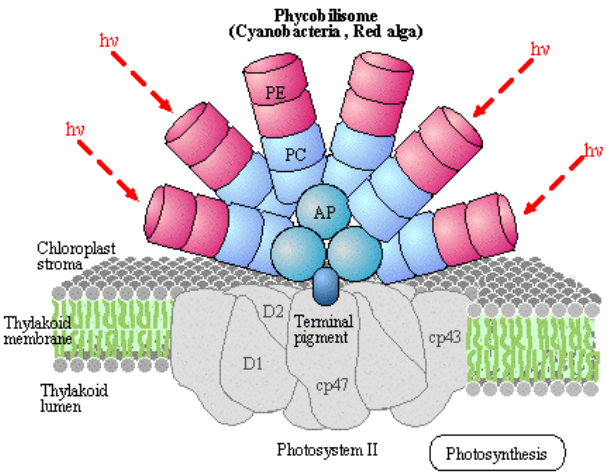

Figure 4: Structure of a phycobilisome. There is a central core of light harvesting complex, allophycocyanin (AP) which sits above the photosynthetic reaction center. Also fluorescent pigments are present in the phycocyanin (PC) and phycoerythrin (PE) subunits. *Kanehisa laboratories. Kegg project:* www.kegg.org

A protein called NblA seems to modulate this PBS degradation during early stages upon nitrogen starvation. *Anabaena* sp. has two *nblA* homologous genes, one on the chromosome, *asr4517* (*nblA*) and one on plasmid delta, *asr8504*(*nblA-p*). (Baier et al., 2004). This protein play the role of a proteolysis adaptor, it initiates protein pigment degradation by interacting with the cyanobacterial light-harvesting complexes, in particular with the trimer-trimer interface of phycocyanin, which is mainly formed by the α-subunits and the β-subunits (Figure 5) (Bienert, Baier, Volkmer, Lockau, & Heinemann, 2006)

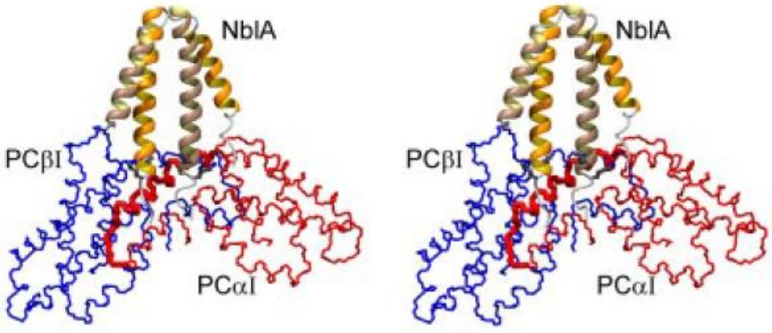

Figure 5. Model of NblA binding to phycobilisome subunits. Stereo representation of NblA binding to phycocyanin monomers PC(αβ). (α-subunits colored in red and gold; β-subunits are shown in blue). Adapted from (Bienert et al., 2006)

Although NblA itself has no detectable proteolytic activity, it was recently shown in *Synechocystis* sp. that dephosphorylation of linker proteins within the phycobilisomes requires their partial disassembly (Piven et al., 2005), therefore further interactions partners of NblA could be as yet unknown protein phosphatases (Bienert et al., 2006). A possible mechanism to the phycobilisome degradation was proposed in Baier et al in 2006 and it consisted on the proposal of that NblA tags PBS for the cellular proteolytic machinery, in analogy to e.g. ubiquitin that covalently binds to other proteins and makes them accessible to the 26 S proteasome. This mechanism suggested that NblA would also need a further interaction partners that would help to the degradation of phycobilisomes. (Bienert et al., 2006).

2.3. FUR proteins in *Anabaena* sp.

Ferric uptake regulator proteins (FUR) are ubiquitous in most prokaryotes. They were firstly identified as iron-sensing repressors, proteins that use Fe^{2+} as a cofactor to negatively regulate expression of their target genes.

Fur binds specifically to a palindromic DNA sequence called Iron Box. However, it is widely known that FUR proteins not only sense iron but display other metal selectivity, including sensors of Zn, Mn, and Ni.

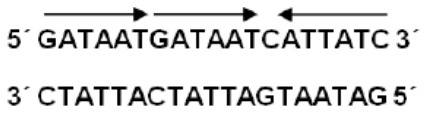

Figure 6: **Iron Box.** Fur binding site is composed of 3 repetitions of a palindromic consensus sequence GATAAT, two of these are direct and other is indirect.

In particular, FUR proteins in *Anabaena* are involved in the control of a large number of genes related to general metabolism, electron transport and defense against different stresses. Moreover, the initially considered classical repressor, Fur regulator, has also been described as activator of the expression of several genes either directly or indirectly. Is the case of FurA of *Anabaena* PCC7120 that positively regulates *asr4517* (*nblA*) and *alr2495* (*sufS*) (González et al., 2016).

A common feature of FUR proteins is the presence of a metal-binding motif rich in histidine ($H_2XHX_2CX_2C$). By searching for this sequence in *Anabaena* sp. three paralogs were identified, called *all1691* (*furA*), *all2473* (*furB*) and *alr0957* (*furC*) (Hernandez et al., 2004). **FurA** is the most abundant of the three proteins under standard culture conditions and also its structure present a certain CXXC motif specially conserved among the Fur orthologues identified in other cyanobacteria. **Zur (FurB)** has been described as a key transcriptional factor in oxidative response control in *Anabaena* sp. (Sein-Echaluce et al., 2015) and it also protects cells against oxidative stress by inespecifically binding to DNA (Lopez-Gomollon et al., 2009). Finally, **FurC** protein is the less studied in *Anabaena* sp., and the one whose basal expression is lower than those of FurA an FurB. It has been described as a potential modulator of binding activity of the other FUR paralogues (Hernandez et al., 2004) and recently as the PerR regulator in *Anabaena* sp. (Yingping et al., 2014)

2.4. FurC from *Anabaena* sp. PCC7120

2.4.1. Structure

The FurC protein, as discussed above, is the least abundant protein compared to the other Fur family proteins in *Anabaena* 7120(Hernandez et al., 2004). FurC has 149 aminoacids and a molecular weight of 17,328.3 Da. and exhibit a significant gap in multiple sequence

alignment with other FurA and FurB. As shown in Figure 7, FurC His-rich motif, HXHX2CX2T considerably differ from His-rich motifs in FurA and FurB, $H_5X_2CX_2C$ and $H_2X_2CX_2C$ respectively.

Unlike FurA and FurB having 5 cysteines residues and binding heme, FurC presents only 3 cysteine residues, of which none of them are part of the CXXC motif, that has been related to the existence of structural zinc coordinated by four cysteine residues (Lee & Helmann, 2006; Traoré et al., 2006).

Figure 7: Multiple secuences alignment. Residues conserved in all proteins FUR in *Anabaena* (FurA, FurB and FurC) are shaded yellow. The His-rich motifs of each protein is boxed in red. The alignment was performed with AlignX tool from Vector NTI Software (Invitrogen).

Its theoretical isoelectric point (pI) defined by the primary sequence is 5.38. Its theoretical extinction coefficient at 280 nm is 14565 M^{-1} cm^{-1} (assuming all pairs of Cys residues form cystines) or 14440 M^{-1} cm^{-1} (assuming all Cys residues are reduced). These data have been obtained with ExPASy ProtParam (http://web.expasy.org/protparam/) Web server. A phylogenetic tree of FurC secuences in other cyanobacterial species is shown in supplementary material, Figure 1.

2.4.2. Function

FurC (Ferric Uptake Regulator) is involved in a wide range of physhiological processes including a potential modulation of the transcriptional activity of its paralogues, FurA and FurB (Hernandez et al., 2004; Lopez-Gomollon et al., 2009), a clear implication in the oxidative stress processes (Yingping et al., 2014) and also a potential role in nitrogen metabolism (Picossi et al., 2014).

2.4.2.1. FurC implications in oxidative stress processes

Several studies have reported that when cyanobacteria are challenged by iron starvation, they also suffer from oxidative stress (Jeanjean et al., 2003; Xu et al., 2003; Yousef et al., 2003; Singh et al., 2004; Latifi et al., 2005). It is well known that an excess of free intracellular iron is extremely deleterious because of it catalyzes the formation of reactive oxygen species through Fenton reactions, leading to oxidative stress (Latifi, Ruiz, & Zhang, 2009). Iron metabolism is, therefore, tightly regulated in order to maintain the intracellular concentration within non-toxic levels. In fact, under iron stress, transcriptional analysis showed that more than 20% of the differentially expressed genes were also responsive to the ones under oxidative stress conditions. (Yingping et al., 2014)

Although expression levels of FurC are very low in *Anabaena* sp. under standard culture conditions, its expression levels markedly increase when cells are treated with H_2O_2 or methyl viologen, an electron acceptor that generates high amounts of superoxide anions (Lopez-Gomollon et al., 2009). Besides, binding assays reported that FurC specifically binds to promoters of genes related to oxidative stress such as *srxA* and *prxA*. It is also noteworthy that comparing with other cyanobacteria, similar sequences to FurC from *Anabaena* sp. are found and interestingly some of them are considered as the PerR regulator in the organism. (see Supplementary material, Figure 1).

2.4.2.2. Rol of FurC in nitrogen metabolism

It has been observed that FurC is implicated in the cross talk described between the Ferric Uptake regulator (FurA) and the global nitrogen regulator (NtcA) in *Anabaena* sp. (Hernandez et al., 2004) acting as a modulator of the binding activity of FurA. FurC seemed to be highly related to the nitrogen metabolism since an overexpression of NtcA (master regulator of nitrogen processes) led to an increase of transcriptions levels of *furC* (Picossi et al., 2014). Furthermore, although direct FurC implications in these processes remains unknown, previous studies in our laboratory indicated a potential relationship between FurC and nitrogen metabolism. Comparing a *furC*-overexpression mutant of *Anabaena sp.* PCC 7120 and the wild type strain, different phenotypic responses were observed under nitrogen deficiency (Unppublished data, Broset, 2012). In addition, the same study showed that FurC joined to two linker subunits of the Phycobilisome PBS, a photosynthetic pigment structure that is degraded as an early response upon nitrogen step down.(Baier et al., 2004). This result is very innovative because the interaction of the FUR proteins with the phycobilisome is unknown.

2.4.2.3. ¿Regulator of other FUR family members?

Previously it was reported that FurC acts modulating the binding activity of FurA and FurB to their own promoters (Hernandez et al., 2004). In the view of the results, Hernandez et al in 2004 proposed that FurC might form heterodimers through its C-terminus with the other FUR family members, and performing a combinatory effect by reducing FurB binding to DNA and intensifying FurA one. However, FurC was showed to be inactive with *furA, furB* and *furC* promoter regions. Further gel-shift assays with FurC were performed against the promoters of *furA* and *furB*, but it was not possible to discern whether there was interaction or not since the protein did not show a clear active state.

3. OBJECTIVES

The main goal of this work was focused on the study of the general functions of FurC transcriptional regulator in *Anabaena* sp.

To achieve this general goal, we have defined the following specific objectives:

- Overexpression and purification of FurC from *Anabaena* sp. providing an active state of the protein showing specific binding activity.
- Biochemical evaluation of the purified FurC studying the oligomerization state and exploring the presence of regulatory metals in its structure.
- Phenotypical characterization of a *furC*-overexpressing mutant in *Anabaena* sp.
- Identification of novel FurC-interacting partners likely involved in the oxidative stress response and nitrogen metabolism in *Anabaena* sp.

4. MATERIALS AND METHODS

4.1. MICRORGANISMS AND CULTURE CONDITIONS

4.1.1. Microorganisms

4.1.1.1. Cyanobacterial strains

In this study the axenic strain *Anabaena* sp. PCC7120 was used as wild type strain. This strain was provided by the Pasteur Culture Collection. Along with wild type the *furC*-overexpressing strain EB2770C was also used during the entire experiment. This strain was generated using the shuttle vector pAM2770 that contained an extra copy of the wild-type *furC* gene located downstream of the copper- inducible *petE* plastocyanine promoter. An overview of the properties of both strains is given in Table 1.

TABLE 1. Selected cyanobacterial strains.

Anabaena sp.	Features	Reference
PCC 7120	WT	Pasteur Culture Collection, France
EB2770C	Contains pAM2770*furC* plasmid. (for FurC overexpression)	(Broset, 2012)

4.1.1.2. *E. coli* strains

BL21 (DE3) strain was used for protein overexpression and purification and *E. coli* DH5α was used for plasmid maintenance.

TABLE 2. Selected *E.coli* strains

Strains	Features	References
BL21(DE3)	*F-, ompT, hsd*S_B ($r_B^- m_B^-$), *gal dcm, λ(DE3).*	EMD Biosciences
DH5α	*F-*, U169 *(φ80ΔlacZΔ*M15*), deo*R, *rec*A1, *end*A1, *hsd*R17(r_K^-, m_K^+), *poa*, *sup*E44, *thi*-1 *gyr*A96, *rel*A1*λ-, Δ(lacZYA-argF).*	Invitrogen

4.1.2. Growth conditions

4.1.2.1. Cyanobacteria culture conditions

Anabaena sp. strains were grown on BG-11 medium (Rippka, 1988). Composition of the media is shown in Supplementary material, Table 1. In mutant strain cultures, BG-11 medium was supplemented with neomycin 50μg/ml sterilized by filtration.

Growth in agar medium was performed in Petri dishes by the classical streaking method with BG-11 medium supplemented with 1% (w/v) agar and with neomycin 50 μg/ml for mutant strains culturing. Cultures were incubated at 25-30° C with soft white light under 50 μmol $m^{-2}s^{-1}$. To avoid evaporation of the media, the Petri dishes were introduced in a clear plastic box that maintained optimal humidity.

Liquid cultures were initiated in 250 ml Erlenmeyer's flasks filled with 50 ml of BG-11 medium. Inocula from plate cultures were used and they were grown photoautotrophically at 25-30°C under continuous illumination of 50 μmol $m^{-2}s^{-1}$ and continuous shaking to maintain constant aeration. Photo-oxidation processes at early stages were avoided by wrapping culture flasks in white paper.

Large-scale cultures were grown on 1 l capacity Roux flasks with sterile bubbling (filtered air through a sterile 0.22 μm pore-size filter unit). They were maintained at a temperature between 25-30 ° C and with side lighting (30 μmoles of photons m^{-2}. s^{-1})

i) Nitrogen starved cultures

In order to analyze FurC implications on nitrogen metabolism, different cultures under nitrogen starvation were performed, either in the wild type and the *furC* overexpression strains. Cells were grown in $BG11_0$ in absence of fixed nitrogen. Non nitrogen-starved cells (at DO 700 nm: 0.3) were collected by gentle centrifugation, 5 min at 4000 rpm at 4 ° C, washed 2 times with the same volume of $BG11_0$ medium and slowly resuspended in this medium for further cultivation.

4.1.2.2. *E. coli* culture conditions

As usual growth medium for liquid and solid *Escherichia coli* cultures, it was used the Luria-Bertani (LB) (Sambrook et al., 1989) supplemented with kanamycin 50 μg/ml when

necessary. Liquid cultures were shaked at 200 rpm continuous orbital shaking at 37ºC. Plate cultures were performed in Petri dishes supplemented with 1% agar and maintained at 37ºC.

4.2. OVEREXPRESION AND PURIFICATION OF FURC

FurC was obtained by heterologous overexpression in *Escherichia coli* and subsequent purification by Ion-exchange chromatography.

4.2.1. Transformation of *E.coli* with pET-28(a)+*furC*

Overexpression of the recombinant protein FurC *Anabaena* sp. PCC71220 in *E.coli* cells was performed with the induction plasmid pET-28(a)+ which already contained the insert *furC* (*alr0957*), cloned previously in our laboratory (Unpublished, Broset, 2012). The pET family induction plasmids was used due to its high performance as overexpression systems of recombinant proteins. These plasmids have as main feature target genes expression to the bacteriophage T7 promoter, which is strongly inducible.

4.2.1.1. Preparation of termocompetent *E.coli* cells

E. coli termocompetent cells were prepared by calcium chloride treatment. Bacteria were grown in a 10 ml falcon filled with sterile LB medium and incubated overnight at 37ºC and 200 rpm. A fresh 1 ml inoculum was grown in 200 ml of sterile LB to reach exponential phase ($D.O_{600}$ = 0.3-0.4).. After centrifugation for 15 minutes at 4000 rpm at 4ºC, the pellet was washed with precooled buffer 1 containing 0.13M $CaCl_2$, 0.15 M $MgCl_2$, 0.04 M NaAc and recentrifuged. Finally, cells were resuspended in 4 ml of cold Buffer 2 (0.13 M $CaCl_2$, 15% glycerol (v/v)). Aliquots of 200 µl were prepared and kept at -80ºC.

3.2.1.2. Heat-shock transformation of *E.coli*

Termocompetent cells were transformed by heat-shock transformation. To this aim, the plasmid pET-28 (about 100 ng) was added in an eppendorf tube with freshly thawed termocompetent cells and mixed gently avoiding pipetting, as the membrane of these cells is extremely sensitive. Cells were incubated 30 minutes on ice. Immediately, the heat-shock was

performed by immersing the tubes in a bath at 42 ° C for 90 seconds and afterwards cells were kept for 2 minutes on ice. Then, was mixed with 800 µl of sterile LB without antibiotic and incubated for 45 minutes. Finally, the culture was plated in LB plates with 50 µg/ml kanamycin. Plates were incubated overnight at 37ºC.

4.2.2. Overexpression of FurC

Conditions for inducing overexpression of the protein FurC were optimized according to several protocols used for other FUR proteins overexpressed and purified in our laboratory. (Hernandez et al., 2004; José A Hernández, Bes, Fillat, Neira, & Peleato, 2002).

Initially, 4 colonies of *E. coli* cells BL21 (DE3) freshly transformed (see section 4.2.1) were selected for an induction assay on a small-scale. Cells were grown overnight, then the culture was diluted 1: 100 and incubated at 37 ° C until reaching the exponential phase. At this time the culture was separated into two aliquots, one was kept as a control (no induction treatment) and the other was induced with 1 mM IPTG for 3 h at 37 ºC. The expression levels were analyzed by denaturing SDS-PAGE electrophoresis in a 17% polyacrylamide gel (see section 4.3.1.). A colony showing higher level of overexpression was induced and cultivated at large scale under the same conditions to obtain 5 L of *E. coli* culture overexpressing FurC. Cells were centrifuged 2 times 20 min at 20000 rpm and washed with distilled water, finally the cell pellet was weighted and stored at -20ºC.

4.2.3. Purification of FurC from *Anabaena* sp. PCC 7120.

4.2.3.1. Preliminary stability assays of FurC at different pHs

The stability of FurC at different pHs (4.5, 5.5, 6.8, 8, 8.8) was checked to optimize purification conditions in order to obtain the active form of the protein. To this end, 0,1 g of thawed cells overexpressing FurC were resuspended in an eppendorf with 500 µl of the different buffers and 1 mM of PMSF (phenylmethane sulfonyl fluoride), a serine protease inhibitor. This suspension was sonicated on an ice-bath by using 5-7 sonication cycles of 45" (0.8 amplitude and 0.5 cycle) and 30" intervals. Cell lysate was incubated for 1 hour and then centrifuged at 12,000 rpm for 20 minutes at 4 ° C. The supernatant and the pellet obtained after the treatment in each buffer were loaded on a polyacrylamide gel 17% and electrophoresed in denaturing conditions (see section 4.3.1.) to evaluate the amount of recombinant protein that remained in solution at each pH.

4.2.3.2. Obtention of crude extracts

i) Cell disruption

About 4 g of cell pellet overexpressing FurC were thawed, slowly resuspended in 20 ml of Tris-HCl 50 mM (pH 8) and supplemented with ¼ tablet containing a "cocktail" of protease inhibitors "Complete ™, EDTA-free protease Inhibitor Cocktail "(Roche). This step is essential because when cells are disrupted, crude extract is exposed to oxidative conditions that might trigger processes of proteolysis. This suspension was sonicated in a 50 ml falcon on ice-bath for 5-7 cycles of 45" (0.8 amplitude and 0.5 cycle) with 30" intervals. Cell lysate was twice centrifuged at 12,000 rpm for 20 minutes at 4 ° C. The whole process was performed on ice-bath since high temperatures might denature and inactivate protein.

i) DNase I treatment

A previous experiment showed that purified FurC was quite bound to nucleic acids. In order to eliminate nucleic acids, present in the sample, the crude extract was incubated with 100 u/ml DNaseI in the presence of incubation buffer (400 mM Tris-HCl, 100 mM NaCl, 60 mM $MgCl_2$, 10 mM $CaCl_2$, pH 7.9) for 35 min at room temperature without shaking.

4.2.3.3. Ion-exchange chromatography

The ion exchange chromatography is a method that allows the separation of molecules based on their electrical charge density. It relies on the reversible exchange of ions in solution with ions electrostatically bound to an insoluble support. The sample proteins are then added to the column and the charge opposite to the functional group are bound to exchanger, replacing buffer counter ions; any neutral charged or not bounded molecules pass through. The bound molecules are sequentially eluted. A weakly basic anionic exchanger, diethyaminoethyl (DEAE) on a DEAE-Cellulose matrix was selected since the protein was stable at pHs above the isoelectric point. The optimized procedure for purification was performed as follows:

Preparation and equilibration of the ion-exchange column

i) DEAE cellulose matrix was homogeneously added on a column of 20 ml with a glass Pasteur pipette to a volume of 10-15 ml. The following steps were performed at 4 ° C.
ii) Column was equilibrated by adding 5 column volumes (CV) of Tris-HCl 50 mM (pH 8)

Filtration and loading of crude extract

i) Crude extract obtained after cell disruption and DNaseI treatment was centrifuged for 30 min at 18000 rpm.
ii) Supernatant was filtered first through 0.8 and afterwards through 0.45 µm-pore-size (Millipore) and was loaded into the previously equilibrated column with a flow rate of 3 ml.

Washing and elution of the protein

The purpose of washing step is eluting all proteins that do not specifically interact with the matrix.

i) Matrix was washed with 3 CV of 50 mM Tris-HCl (pH8).
ii) Elution was performed with a 100 ml linear NaCl gradient from 0 to 1.5 M in 50 Mm Tris-HCl pH-8 and additional 20 ml Tris-HCl (pH 8) containing 50 mM, 1.5M NaCl. Fractions of approximately 1 ml were collected. Protein was studied in denaturing polyacrylamide gels (SDS-PAGE) (see section 4.3.1.) and grouped according to increasing degree of purity and aliquoted to keep them at -80 ° C.

4.3. BIOCHEMICAL APPROACHES AND ANALYTICAL METHODS

4.3.1. Protein electrophoresis

4.3.1.1. Denaturing polyacrilamide gel electrophoresis (SDS-PAGE)

Proteins were resolved using a 17% polyarilamide gel [30:0.8 (w/w) acrylamide/bisacrylamide] (Supplementary material, Table 3) in a Hoefer Mighty Small SE250 / SE260 (Thermo Fisher)

i) For sample preparation, 16 µl of sample and 3 µl of 6x sample buffer were heated for 5 minutes at 95ºC and then centrifugated for 1 minute at 10.600 xg . Sample buffer 1x (10 Mm Tris-HCl (pH 8), 2.5% SDS, 5% β-mercaptoethanol 5%, 10% glycerol and 0.02% bromophenol blue).
ii) The samples were loaded onto the gel and the tank was filled with Tris-HCl 25 mM (pH 8.8), glycine 200 mM y SDS 3.5 Mm. The gel was running at 35 mA until the sample front arrived at the end of the gel.
iii) For visualization of the results, gels were stained with a dye solution with 0.05% (w / v) Blue Coomassie, 6% acetic acid, 45% methanol and 49% water. Gels

were stained for 45 min with stirring at 100 rpm until protein blue bands appeared and destained with a solution of 10% acetic acid, 25% methanol and 65% water.

4.3.2. Protein determination

10 ml from *Anabaena* sp. cultures were collected in exponential phase and centrifuged for 10 min at 4000 rpm. Then supernatant was carefully decanted and cell pellet was resuspended in 4 ml of milliQ water supplemented with proteinase inhibitor PMSF 1mM. The suspension was sonicated by using 6 cycles of 45" (0.8 amplitude and 0.5 cycle) with 30" of intervals. 1 ml of this cellular extract was centrifuged for 20 min at 12000 rpm and the supernatant was used in protein quantification.

4.3.2.1. Protein quantification with "BCA" (Acid Bicinchrinomic Method)

BCA™ ProteinAssay Kit (Thermo Fisher Scientific) is a commercial kit that is used to determine protein concentration. BCA principle relies on the reaction of ITS reagent with the sample, forming complexes between copper ions and peptide bonds producing a purple end product. A calibration curve with bovine serum albumin (BSA) between 0,025 and 2 mg/ml was prepared and at least two different dilutions of the sample were prepared. 50µl of each dilution were added into a plastic cuvette and then 1 ml of "Working Reagent" A + B (50 parts of A and 1 part of B) was added and it was mixed thoroughly. It was incubated for 30 min at 37 ° C and its absorbance was measured at 562 nm. Finally, data was interpolated on the calibration curve to calculate the concentration in mg/ml.

4.3.3. Chlorophyll *a* quantification

The determination of chlorophyll *a* content, the only chlorophyll present in cyanobacteria, is a good estimate of the physiological state of cyanobacteria. For this we used the method described by (Mackinney, 1941). Cells were harvested by centrifugation of 1 ml culture and pellet was extracted with 1 ml of methanol and vortexed for 2 min. After 5 min centrifugation at 12000 rpm supernatant absorbance was measured at 665 nm. The obtained value was divided by the extinction coefficient (ε = 74.46 mg ml-1 cm-1). The whole process must be performed in darkness.

4.3.4. Phycobiliproteins quantification

For the determination of phycobiliproteins the following method for *Anabaena* sp. previously described (Bryant, Glazer, & Eiserling, 1976) was performed. 10 ml of cell suspension on exponential phase were centrifuged for 10 min at 4000 rpm and the supernatant was carefully decanted. This pellet was resuspended in 4 ml of milliQ water and 1 mM PMSF. Cells were broken by sonication by using 6 cycles of 45" (0.8 amplitude and 0.5 cycle) and with 30" of intervals. 1 ml of this crude extract was centrifuged for 5 min at 2500 x g and the supernatant absorbance was measured at 620. The extinction coefficient used was 7.41 mg^{-1} ml cm^{-1}. The phycocyanin to chlorophyll ratio was estimated from whole-cell absorbance spectra from 550 to 750 nm on a SPECORD® PLUS Analytik Jena spectrophotometer.

4.3.5. Zn^{2+}detection

4.3.5.1. PAR staining

The presence of Zn^{2+} in FurC was analyzed by SDS-PAGE electrophoresis using a specific type of staining, PAR (4- (2-pyridylazo) resorcinol). PAR solution (20 mM Tris-HCl (pH 8) 100 mM NaCl, 5% glycerol and 500 µM PAR) was prepared. Gel was stained by gently shaking for 2 minutes. Hydrogen peroxide was added to reveal the presence of Zn^{2+} when the Zn^{2+}-PAR complex is degraded. Under oxidizing conditions there is a change in absorbance, showing a maximum at 542 nm (orange light). Subsequently gel was stained with Coomassie Blue to check protein location.

4.3.5.2. ICP-OES. Trace Metal Analysis

Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) is used for the detection of trace metals. The technique relies on using a type of emission spectroscopy that uses the inductively coupled plasma to produce excited atoms and ions that emit electromagnetic radiation at wavelengths characteristic of a particular element. Samples were prepared under conditions specified by the service within the minimum detection range mixed with ICP Nitric Acid Blank and sent to CEBAS-CSIC. Servicio de Ionómica Campus Universitario, Murcia, (España).

4.4. ISOLATION AND ANALYSIS OF DNA

4.4.1. Polymerase Chain Reaction (PCR) Amplification

In general terms, each PCR reaction (50 µl) contained 100 ng genomic DNA, 5 µl 10x Taq buffer, 1.5 µl 50 mM $MgCl_2$, 1 µl 10 mM dNTPs Mix, 1,25 µL 20 Mm of each primer, 0.5 ul Taq DNA polymerase (Biootols, 1U/µl) and dH_2O up to 50 µl. Thermal cycling involved an initial denaturation step at 10 min at 95ºC, followed by 30 cycles of 95ºC for 30 s, 45-55ºC for 30 s and 72ºC for 1 min/kb and finally 10 min at 72ºC.

PCR amplification products were examined on a 1 % (w/v) agarose gel in TBE (90 mM Tris/HCl (pH 8), 90 mM Boric Acid, 2 mM EDTA and stained with 0.5 µg/ml ethidium bromide. Φx174 DNA-H*ae*II was used as molecular weight marker. Finally, PCR products were purified using Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare) and quantified by a NanoVue Plus spectrophotomer (GE Healthcare).

4.4.2. Small-scale isolation (*miniprep*) and plasmid sequencing

4.4.2.1. Miniprep extraction

"GenElute ™ Plasmid Miniprep Kit" (Sigma Aldrich) was used following the instructions recommended by the manufacturer. This kit includes different solutions for alkaline cell lysis method. All samples were obtained RNA free by RNaseI incorporated in the resuspension buffer. Final elution was performed with sterile milli-Q water.

4.4.2.2. Sequencing

Sequencing of cloned DNA fragments was carried out in sequencing services "Sistemas Genómicos" (www.sistemasgenomicos.com) Valencia Technology Park, Paterna, Valencia.

4.5. ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Gel retardation technique is one of the most used and valued in assays in order to describe DNA-protein interactions and therefore testing its biological activity. This method is

based on simulating intracellular conditions and ideal characteristics for an interaction. Numerous factors play important roles in the *in vitro* binding such as the molecular size of the protein and DNA (Fried & Crothers, 1983, 1984), percentage of polyacrylamide gel, voltage at which electrophoresis elapses and incubation time of the mixture DNA- protein. These factors were optimized with an extensive literature search in order to detect a specific interaction FurC-DNA ((José A Hernández et al., 2002; Lee & Helmann, 2006; Yingping et al., 2014). EMSA assays were performed with purified FurC obtained from purification in a DEAE cellulose column.

4.5.1. Preparation of native polyacrylamide gels

Composition of native polyacrylamide gels to a percentage of 5-7% is shown in Suplementary material, Table 3. Gels were pre-run at 60V for at least 1 hour at 4ºC in running buffer 1x (50 mM Tris-HCl pH 8 and 380 mM glycine once wells have been manually cleaned. This step is crucial to remove any polyacrylamide and ammonium persulfate present in the wells and also it helps to balance the gels with electrophoresis buffer at constant temperature.

4.5.2. Incubation and Electrophoresis

Binding reactions containing 35 ng of DNA fragments of around 200-300 bp were mixed with binding buffer (10Mm Bis-Tris Ph 7.5, 40 Mm KCl, 5% (v/v) glycerol and 0.05 mg/ml bovine serum albumin (BSA, Sigma). This mix was incubated with different protein concentrations (60 nM- 700 nM) for 30 min at 30ºC in darkness as previously described (Yingping et al., 2014). During optimization steps other components such as DTT and $MnCl_2$ were added (Mn^{2+} is commonly used as a substitute for Fe^{2+} to avoid its rapid oxidation to Fe^{3+}). DTT is frequently used to reduce the disulfide bonds of proteins.

After incubation time and wells cleaning, samples were mixed with 3 µl of loading buffer containing 50 mM Tris/HCl (pH 8), 30% (v/v) glycerol, bromophenol blue 0.25% (w / v) and slowly loaded into the precooled gel including a negative control without protein. Electrophoresis was performed at 90V at 4ºC until bromophenol blue reached the last quarter of the gel. Gels were stained with 1µg/ml SYBER Safe fluorescent dye for 30 minutes with gentle shaking in darkness at room temperature. Bands were visualized with BioRad Gel Doc 2000.

4.6. ISOLATION AND ANALYSIS OF RNA

4.6.1. Precautions for RNA handling

To ensure success in RNA experiments, it is important to maintain an RNase-free environment starting with RNA purification and continuing through analysis. Ribonucleases or RNases are ubiquitous and also are very stable and difficult to inactivate. Necessary precautions in working with RNA are shown below:

1. To prevent contamination from most common source of RNases such hands, it is crucial to wear gloves at all times and use sterile technique when handling the reagents used for RNA isolation or analysis.
2. Use of sterile, disposable plasticware for handling RNA
3. Cleaning of RNA work area and rinse disposable plasticware with 0.4M NaOH
4. Reserve chemicals for use in RNA isolation and analysis for RNA applications and kept it separate from chemicals for other uses.
5. Use of DEPC-treated or Nuclease-Free Water to make your RNA isolation-buffered solutions.
6. It is essential to work as quickly as possible on ice-bath.
7. In the stages of use of fume hood it is very important to prepare in advance the work area with the necessary material.

4.6.2. Isolation of RNA of cyanobacteria

RNA samples from the wild type *Anabaena* sp. PCC7120 and its mutant EB2770C overexpressing FurC were obtained following this protocol which was optimized for this study.

4.6.2.1. Cells Sampling

1) 25 ml of culture of cyanobacteria were collected to a desirable optical density ($D.O_{750}$=0.3) by centrifugation 3 min, 4000 rpm, 4ºC.
2) Supernatant is slowly poured and cell pellet was resuspended in remaining medium and placed in a 2 ml RNAse free eppendorf.
3) After 5 min, 12.000 rpm, 4ºC centrifugation, supernatant was removed with a pipette.
4) Cell pellet was resuspended in 600 µl of resuspension buffer (50 mM Tris-HCl, 100 Mm EDTA (pH 8) and 130 µl chloroform.

5) Tubes were inverted 4 times till cells were totally resuspended
6) 3 min incubation on ice-bath and centrifuged for 5 min, 13.000 rpm, 4ºC. At this time 3 phases were obtained:
    - Upper= aqueous
    - Intermediate=cells
    - Lower=chloroform
7) Upper phase was removed, intermediate phase crossed with the pipette tip and the lower organic phase was removed.
8) The eppendorf with the intermediate phase (cells) was introduced in liquid nitrogen and stored at -80 ºC until use.

4.6.2.2. RNA extraction

1) The cell sample obtained above was resuspended in 300 µl of resuspension buffer and 100 µl of 250 mM disodium EDTA (pH 8), 400 µl lysis buffer (2% SDS, 10 mM Na Acetate pH: 4.5) and 1000 µl of acid phenol at 65ºC.
2) At this stage cells were broken vortexing them during 30 seconds and afterwards cells were incubated 2.5 min at 65ºC on thermostated bath. This step was performed up to 3 times.
3) After to 5 min centrifugation at 13000 rpm, 4ºC. Aqueous phase (upper) was placed on a new eppendorf tube.
4) After adding 1 ml of Trizol and vortex 30 seconds and let it chill at room temperature during 5 min.
5) Add 200 µl of chloroform, vortex 30 seconds and let it chill at room temperature during 1 min.
6) Centrifuge 5 min at 13000 rpm 4ºC and place 750 µl of aqueous phase on a new eppendorf tube
7) Add 1 volume of chloroform and vortex 30 seconds. Centrifuge 5 min at 13000 rpm 4ºC and transfer aqueous phase into a new eppenforf tube
8) Add 2 volumes of pre-cooled absolute ethanol. Vortex 30 seconds. And store at -80 ºC o/n.

4.6.2.3. DNAseI treatment and RNA quantification

9) Samples were centrifuged 30 min at 12000 rpm 4ºC. Then the supernatant was carefully removed and the RNA pellet was washed with 500 µl of pre-cooled 70% ethanol.
10) Samples were centrifuged 5 min at 12000 rpm 4ºC. The supernatant was removed carefully.

11) RNA pellet was resuspended in 90 µl of DPCE-water. Afterwards 10µl of buffer DNAse and 2 µl of RNAse free DNAseI were added. The mix was incubated for 70 min at 37ºC and 10ºC at 65ºC.
12) RNA was quantified using NanoVue Plus spectrophotomer (GE Healthcare).

4.6.2.4. PCR for determine the absence of DNA in RNA samples

A common problem is the contamination of isolated RNA isolated with DNA, although it has been treated with DNase to avoid it. For this a PCR was performed using as negative control our RNA samples and genomic DNA as a positive control. A classical PCR protocol was performed (See section 4.4.1.) but using a high number of cycles (34).

4.6.3. Retrotranscription

Reverse transcription is used to obtain cDNA from a sample of isolated RNA. After quantification of isolated RNA, mixture shown in Table 3 was added into a PCR Eppendorf PCR tubes were introduced into a thermocycler with the following parameters: Sample denaturing: 10 min at 85ºC, primers hybridization: 1 h at 50ºC. After 70 min, the mixture shown in Table 4 is added into 1 sample, and program is restarted.

Table 3: Components mix of 1st step of retrotranscription.		Table 4: Components mix of the 2º step in retrotranscriptions	
Component for a RT	**Vol (µl)**	**Component** for each RT	**(µl)**
Random Primers	1µl	**dNTPs**	2µl
Hybridization buffer	2µl	**DTT**	4µl
RNA	2µg	**Buffer5α**	8µl
DEPC-H_2O	Make up 20µl	**DEPC-H_2O**	5µl
		SuperScript	**1µl**

4.6.4. Real Time PCR (qPCR)

Relative quantitation of gene-expression using Real-Time Quantitative PCR was performed in order to discern which transcription levels of different genes are influenced by FurC. Real-time PCR (qPCR) was carried out using a ViiA™ 7 Real-Time PCR System (Applied Biosystems) using SYBR® Green Dye.

4.6.4.1. Selected targets and primers design

qPCR assays were performed using specific primers for the amplification of genes of the FUR family *furA*(All1691) and *furB* (All2473) and also its own gene *furC* (*alr0957*), genes related with heterocyst differentiation (*nblA*) and related with oxidative stress processes (*prxA*). Finally the housekeeping gene *rnpB* was used as a reference for relative quantification of selected transcripts. All primers were tested functionally in quantitative analysis and the results were optimal. Optimized primers were designed using Primer Express® Software v3.0.1 (Applied Biosystems). The sequences of resulting specific primers are defined in Supplementary material, Table 2.

4.6.4.2. 96-Well plates preparation

Reactions were set up in 96-Well plates in a final volume of 15 ul containing 5µl of different dilutions of the cDNA sample and 10 µl of the following mix in Table 5.

Table 5. Reaction mix for qPCR assays.

Component	Volume (µl) for each well
SYBR® Green Dye	6.25 µl
Primer for	0.1 µl
Primer rev	0.1 µl
DEPC-H_2O	3.55 µl

All reactions were performed with two different biological samples and three technical samples. Values were normalized to the *rnpB* transcript and the relative quantification was performed according to the Comparative CT Method (ΔΔCT Method)(Livak & Schmittgen, 2001).

First, the difference between the Ct values (ΔCt) of the gene of interest and the housekeeping gene was calculated for each experimental sample. Then, the difference in the ΔCt values between the experimental and control samples ΔΔCt was calculated. The fold-change in expression of the gene of interest between the two samples was then equal to $2^{-\Delta\Delta Ct}$.

4.7. Microscopic Techniques

For microscopy experiments during short-term nitrogen starvation, exponentially growing cells were transferred by filtration into a medium lacking combined nitrogen ($BG11_0$) as described previously (Görl et al., 1998). Microscopic examination of strains of *Anabaena* sp. was performed using a Nikon Eclipse 50i microscope Epi-Fluorescence. The photographs were taken with a Nikon camera attached to the microscope DXM1200F.

4.1. BIOINFORMATIC TOOLS

Standard searches for protein sequence similarity alignments (Protein BLAST analyses) were performed using *BLASTP* program of the Basic Local Alignment Search Tool of the National Centre for Biotechnology Information (NCBI). Primers sequences for Real Time PCR were designed using *Primer Express 3.3.* (Applied Biosytems). Physico-chemical propiertes of proteins were deduced using *ProtParam* tool from Expasy (www.expasy.ch). Phylogenetic tree analysis was performed on the *Phylogeny.fr* platform. Graphical representation and edition of the phylogenetic tree were performed with *TreeDyn (v198.3).*

5. RESULTS

5.1. PURIFICATION AND PRELIMINARY CHARACTERIZATION OF FURC

Along with other FUR family proteins FurA and FurB, FurC has also been overexpressed and purified in previous studies (Hernandez et al., 2004). Nevertheless, previous methods used for FurC overexpression and purification did not achieve an active state of the protein that allowed to perform binding activity assays. In this work, the experimental procedure was successfully optimized.

5.1.1. Overexpression and partial Purification of FurC

BL21 cells were transformed with the plasmid pET-28(a)+*furC*. Expression was induced by adding IPTG. The overexpression of FurC was studied in four colonies that were disrupted by sonication and supernatants were studied using SDS-PAGE. The four colonies overexpressed FurC successfully (Figure 8).

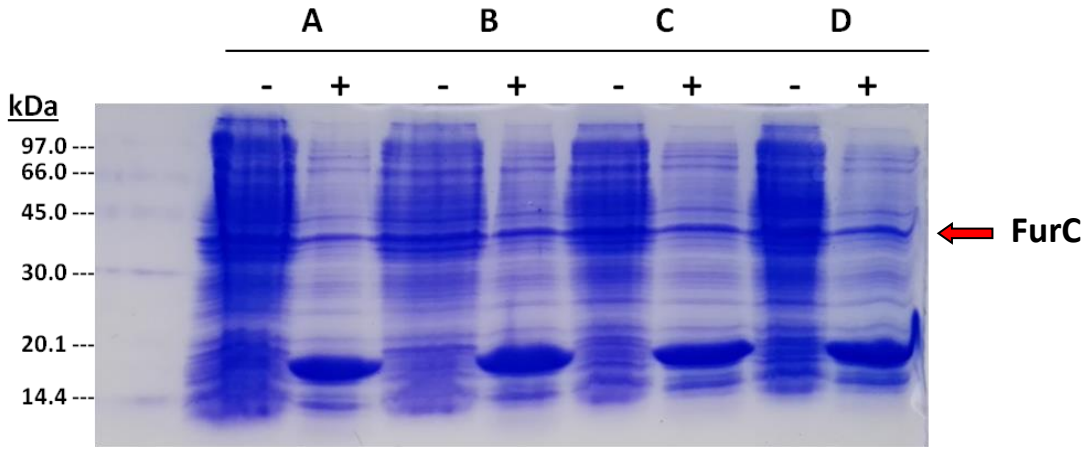

Figure 8. Overexpression of FurC from *Anabaena* PCC 7120. SDS-polyacrilamide gels (17%) showing *E.coli* BL21 (DE3) crude protein extracts of four different colonies (A,B,C,D) without (-) or with (+) adding 1 mM IPTG to induce the expression of the recombinant FurC protein. MW: Low Molecular Weight markers (Healthcare).

Since previous works resulted in a loss of FurC by precipitation, a preliminary stability assay was performed in order to adjust the purification conditions (Supplementary material, Figure 2). FurC was present in the supernatant showing better performance at pH 8 and 8.8.

Once we observed that overexpressed FurC remained soluble at pH 8, we decided to perform an Ion-Exhange Chromatography in DEAE cellulose. Previous purification with FurA and FurB were performed with Immobilized-metal affinity chromatography (IMAC), taking advantage of its higher His content in the metal-binding domain. However, FurC has only 6 histidine residues and they are spaced from each other (see Introduction section). Therefore we decided to isolate FurC by Ion-Exhange Chromatography, according to their isoelectric point (5.3). Using a pH-8 as purification buffer, FurC displayed negative charge, allowing to bind to a positively charged resin, DEAE cellulose. FurC elution profile is shown in Figure 9.

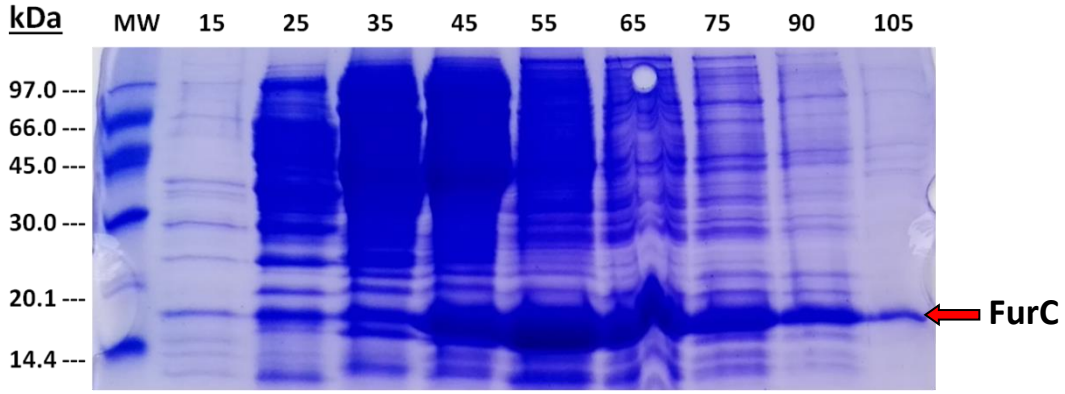

Figure 9. Purification of FurC using Ion-exchange chromatography in DEAE cellulose. SDS polyacrilamide gels (17%) showing fractions with the NaCl gradient from 0 M to 1M. Lane 1: MW: Low Molecular Weight marker (Healthcare) Lanes 2-10: different fractions of the eluted proteins.

FurC eluted widespread on the gradient, and at approximately 1.5 NaCl the protein was 90% pure. Fractions were pooled and analyzed by spectrophotometry showing a high presence of nucleic acids. This was checked by electrophoresis in agarose gel where the purest FurC fractions showed a high content of nucleic acids, while fractions with 70-80% of purity hardly showed presence of nucleic acids.

5.1.2. Activity of FurC and Biochemical properties

5.1.2.1. DNA-binding activity of FurC

A pool without nucleic acid contamination and with a FurC purity over 70% was used in activity assays. Previous work indicated that FurC bound to the promoter region of the *prxA* promoter (Yingping et al., 2014).

For this reason, this promoter was chosen, in order to ensure binding activity of FurC that would allow to perform future gel retardation assays with different targets. The promoter region was obtained as described in Materials and Methods, section 4.4.1.

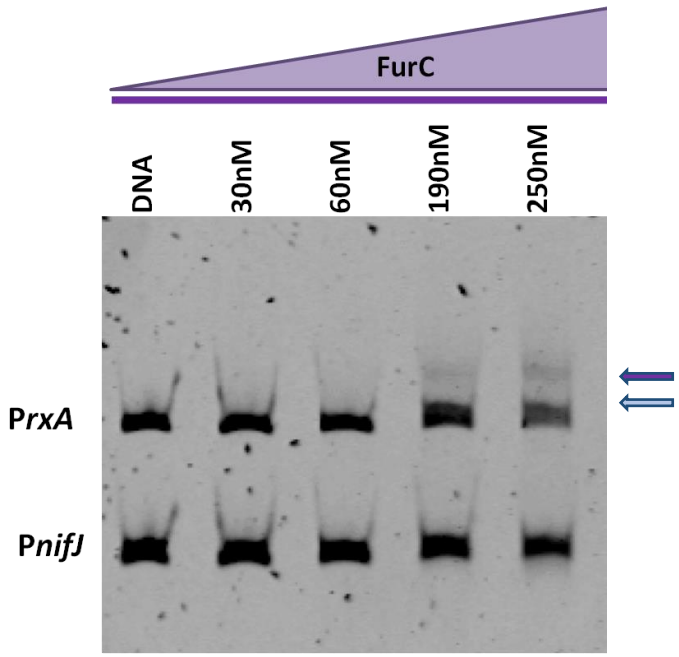

Figure 10. Electrophoretic mobility shift assays showing the ability of FurC to bind in vitro the promoter region of the *prxA* gene (P_{prxA}). Gel retardation experiments were performed with 35 ng free DNA fragments of the promoter region of *prxA* (Lane 1) or mixed with recombinant FurC protein at different concentrations (lanes 2-6). The promoter region of *nifJ* (P_{nifJ}) was used as non-specific competitor DNA in all assays. FurC-DNA complexes are indicated with arrows.

As shown in Figure 10, FurC was capable to interact with the promoter region of *prxA* which confirmed the active state of purified FurC. The protein is quite active and also it is interesting to notice that at low concentrations of FurC, an interaction protein-DNA is showed.

Gel retardation assays were carried out with purified FurC in the absence of DTT and metal, opposite conditions that the ones used in binding assays with FurA and FurB, where incubation in presence of a metal regulator is required. Also, Yingping et al., (2014) described different conditions in FurC binding assays, in which FurC was also active when incubation with Zn^2 .

5.1.2.2. Non-Reducing conditions induces monomer rearrangement to higher order aggregates of FurC

To observe if FurC was able to form oligomeric species, we performed a denaturing protein electrophoresis with non-reducing conditions.

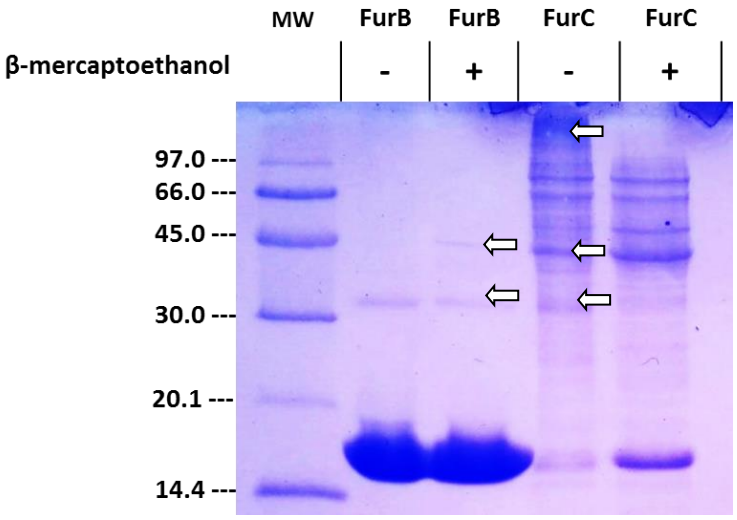

Figure 11. SDS polyacrilamide gels (17%) showing differences in oligomerization states in FurB and FurC without (-) or without (+) β-mercaptoethanol included in the Sample Buffer 6x. Oligomers of FurC are indicated with arrows.

As previously found in other members of Fur family (Pellicer et al. 2010, Lee and Helmann 2006), additional bands of FurC with different gel mobility appeared as a result of oxidation of cysteines and intramolecular disulfide bond formation. As shown in Figure 11, reducing conditions, provided by *B*-mercaptoethanol addition, induced monomer rearrangement to higher order aggregates shown with arrows. Notice that as a result of oxidation, the monomeric form of FurC disappeared and slightly displayed two bands as described in other proteins, such as PerR (Lee & Helmann, 2006).

5.1.2.3. Analysis of presence of Zn^2 in FurC.

The close connection of FurC with PerR has been studied by Yingping et al. in 2014. These authors proposed that FurC could play the role of a PerR regulator in *Anabaena* PCC7120. PerR proteins in other microorganism contain Zn^{2+}, and for this reason we decided to analyze the presence of Zn^2 in FurC. In *Bacillus subtilis* PerR presents one structural Zn^{2+} atom per

monomer that can be detected by PAR staining (Lee & Helmann, 2006). As it is shown in Figure 12 neither in oxidizing nor in reducing conditions, purified FurC appears not to have any structural Zn^2. This result was confirmed by ICP-OES analyses which also indicated the absence of Zn^2 in purified FurC protein showing a concentration of Zn^{+2} of 0.01 mg/L under the detection limit.

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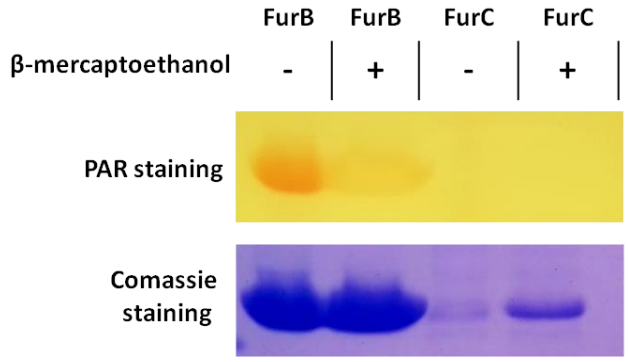

Figure 12. Measurement of Zn^2 release by H_2O_2 using PAR. FurC and a positive control of presence of Zn^2, FurB, were resolved on gel with non-reducing and reducing conditions. The SDS-PAGE gel was soaked in buffer containing 500 mM PAR for 2 min, and subsequently 50 mM H_2O_2 was added to release Zn^2.

5.2. PHENOTYPIC CHARACTERIZATION OF FURC OVEREXPRESSING MUTANT OF *ANABAENA* SP. PCC 7120

FurC has been considered as a modulator of the activity of the other Fur proteins in *Anabaena* sp. (Hernandez et al., 2004), as transcriptional regulator in processes such a oxidative stress response (Yingping et al., 2014) and related with nitrogen metabolism. (Picossi et al., 2014). In order to gain new insights into those cellular functions of FurC, we performed a phenotypic characterization and transcriptional analysis of the *furC*-overexpressing derivative strain EB2770C, previously generated in our laboratory (see Introduction section).

5.2.1. Short Term Photoautotrophic Growth and Pigment Composition

For a further understanding of the phenotypic characteristics of *furC*-overexpressing mutant, pigment and total protein contents were determined. Experimental assays were

performed with Wild type and furC-overexpressing strains in order to compare how strains adapt against standard and nitrogen starvation conditions. Measures were taken for 4 days in both standard and nitrogen starved cultures. Results are expressed in Figures 13 and 14.

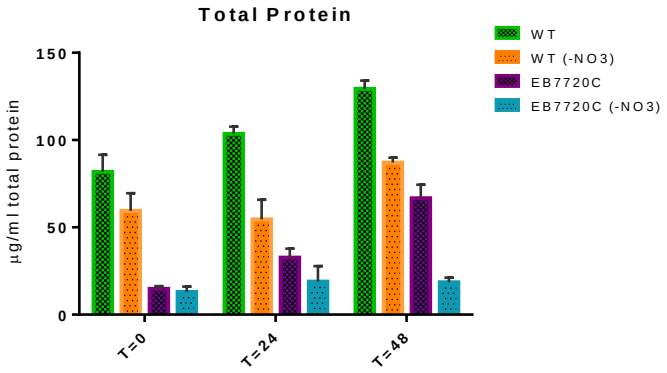

Figure 13: Total protein content of *Anabaena* sp. strains PCC 7120 and EB2770C at different stages of growth.

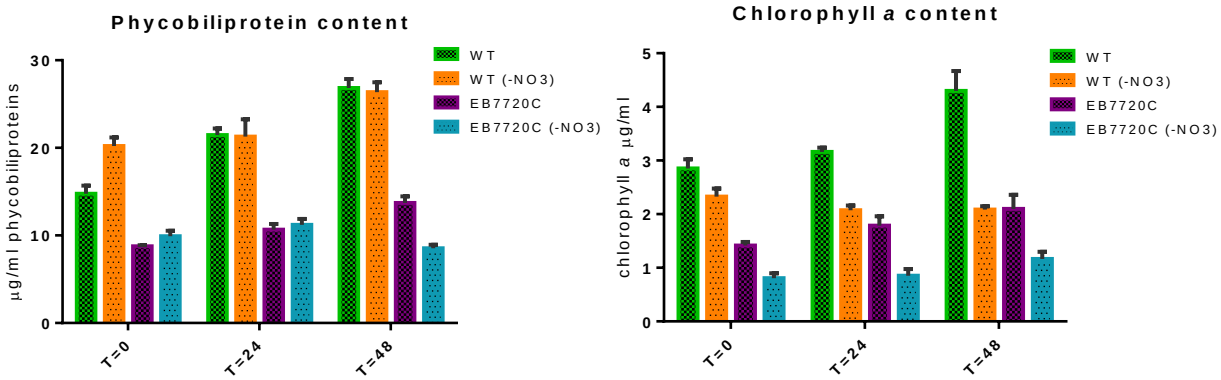

Figure 14: Pigment content of *Anabaena* sp. strains PCC 7120 and EB2770C at different stages of growth.

Interestingly, Figure 13 shows that the amount of total protein in FurC overexpressing mutant is lower than wild type one. Besides both phycobiliprotein and chlorophyll *a* content is clearly lower in the FurC-overexpressing reaching a percentage of 50% less. (Figure 14).

Considering that cultures were slightly at the same growth stage and that phycobiliproteins can constitute up to 50% of the total soluble protein of a cyanobacterial cell. (Bryant et al., 1976), we can state that the decrease of the phycobiliprotein content in the *furC*-overexpressing mutant could lead to a general decrease in total protein content.

It's worth to point out that these phenotypic changes should have been standardized to obtain a more accurate estimation, nevertheless with such a low level of total protein, the pigment to total protein ratio could give unrepresentative percentages of pigment content. This prompted us to normalize data over other types of measurement like cell counting in future experiments as observed in other studies (Sevilla et al., 2010). Nitrogen starved cultures showed different performance between both stains, whereas wild type strain maintains a similar phycobiliprotein content during nitrogen step down, EB2770C mutant shows a clear decrease up to 2-fold at 48 hours (Figure 14).

Since a clear decrease of phycobiliproteins was observed in EB277C mutant, a Whole-cell absorption spectra of *Anabaena* 7120 and its mutant was performed. Figure 15 shows that an overexpression of FurC leds to a decrease of both phycocyanin absorbance peak at ~625 nm and chlorophyll *a* peak at ~665. A similar response is reported when nitrogen starvation, because of the degradation performed by NblA (Non-Bleaching-Activator) in *Anabaena* sp. (Baier et al., 2004; Kato, Chibazakura, & Yoshikawa, 2008).

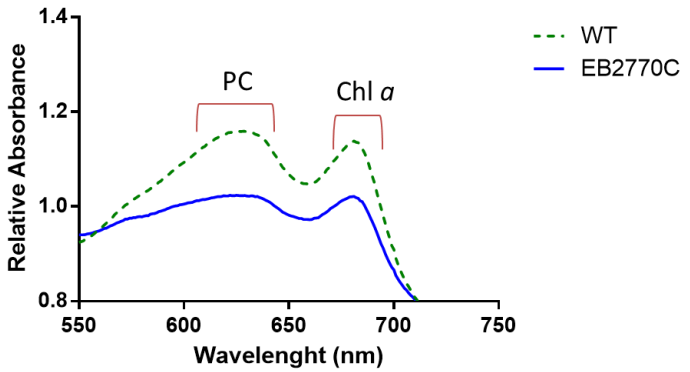

Figure 15: Whole-cell absorbance spectra of *Anabaena* 7120 and its mutant *furC*-overexpresing mutant. Measures were taken from cultures grown to the same cell densitity ($D.O_{750}$ = 0.8) The phycocyanin absorbance peak is described at ~625 (PC) nm. The chlorophyll absorbance peak is described at ~665 nm.

Note that by action of NblA, only the absorption peak of phycocyanin at ~625 is altered, but not the chlorophyll *a* one. This results reveals that overexpression of FurC could be strongly related to the phycobiliprotein degradation process. This hypothesis is investigated in the following sections.

5.2.2. Morphological changes in the *furC*-overexpresing strain.

Examination under the light microscope of cultures at the exponential phase of growth revealed significant morphological alterations in the strain EB2770C compared to its parental

strain PCC 7120. Unlike the wild type, whose filaments formed medium-size chains of rounded cells (Figure 16), the *furC*-overexpressing strain showed cells with lower size and very similar morphology between them.

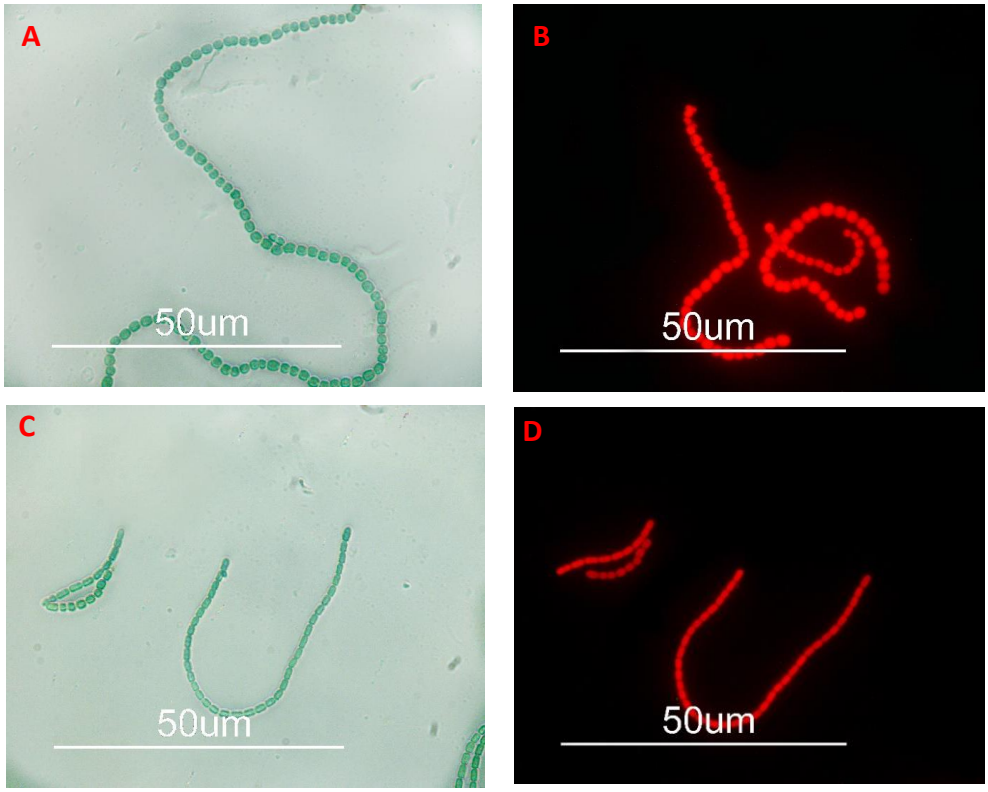

Figure 16. Wildtype and *furC*-overexpression mutant microscopy. Bright field microscopy: A) Wil type, C)furC-overexpression strain. Fluorescence microscopy: B) Wild type, D) furC-overexpresion strain. Cells were collected at a $D.O_{750}$ = ~ 0.6.

5.2.3. Response to nitrogen deficiency

This analysis was motivated by the fact that FurC is regulated by the global regulator for nitrogen control, NtcA. Therefore, a response under nitrogen starvation was expected. Nitrate from medium was removed in the early exponential phase and cultures of both strains were photographed at 48 hours after nitrogen step down.

Loss of PBS in cyanobacteria changes their appearance from the normal blue-green to yellow-green, a phenomenon known as bleaching or chlorosis (Collier & Grossman, 1992). Figure 17 shows a general process of chlorosis in both strains under nitrogen starvation, however in FurC-overexpression strain, chlorosis response seems to be more intensive.

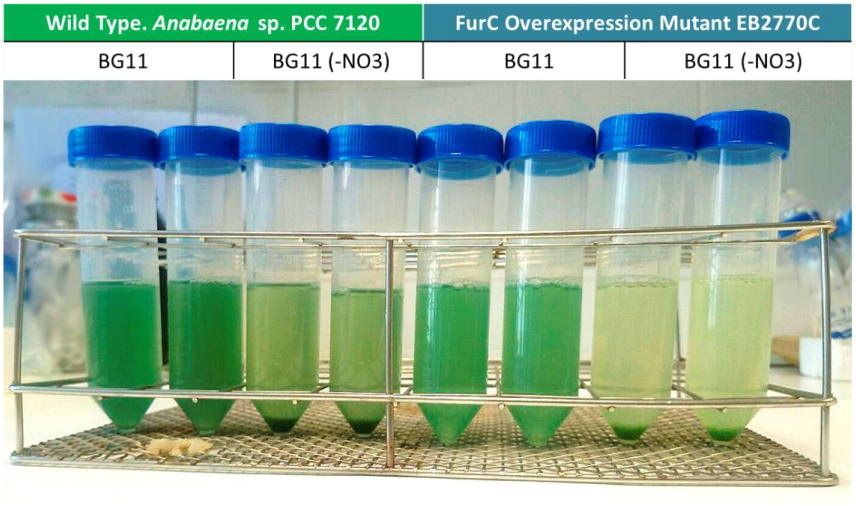

Figure 17. Wild type *Anabena* sp PCC7120 and its FurC-overexpressing mutant under standard and nitrogen starved conditions. Photographs were taken 48 h after nitrogen step down. Cultures were maintained without shaking for 5 min before photograph was taken.

This support that FurC might play an important role in phycobiliprotein degradation whose principal responsible gene in cyanobacteria is *nblA*. Moreover, it can be observed that the sedimentation rate after 5 min is higher in nitrogen starvation cultures, this is in line with a filament aggregation process present in *Anabaena* sp. PCC 7120 under nitrogen starvation led by HesF, an exoprotein identified by (Oliveira et al., 2015).

5.2.4. Transcriptional analysis

On the basis of the phenotypic changes observed in the EB2770C strain, we investigated the impact of FurC overexpression on the transcription of a variety of genes involved in several physiological processes potentially related to FurC functions such as oxidative stress defense and nitrogen metabolism.

Real-Time Quantitative PCR was used to compare the levels of transcripts of selected genes. In this study, fold changes over 1.25 were considered as representative changes. Firstly, transcription levels of FurC in EB2770C were checked. Figure 18 shows that overexpression of FurC in the *Anabaena* sp. mutant resulted in an increase of FurC transcription levels up to ~50-fold.

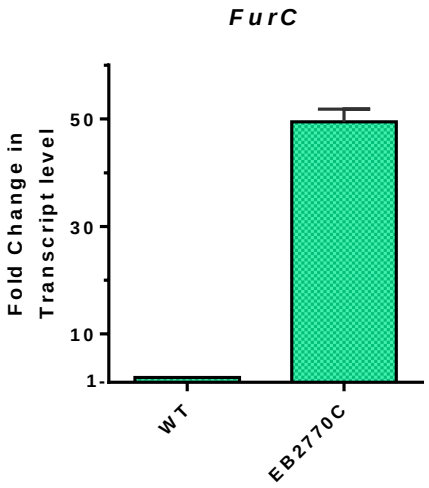

Figure 18. Quantitative RT-PCR analysis *furC* in furC-overexpressing strain EB2770C (FurC). The relative concentration of *alr0957 (furC)* mRNA was measured during growth under normal conditions in triplicate. Standard deviation is indicated by error bars. Values were normalized to the *rnpB* transcript. The fold change is then calculated using the ΔΔCT Method (Livak & Schmittgen, 2001)

5.2.4.1. *furC* overexpression does not appear to influence on transcriptional levels of the other FUR family genes, *furA* and *furB*

It is has been probed that FurC might perform a combinatory effect with other the FUR proteins in *Anabaena* by reducing FurB binding to DNA and intensifying FurA one (Hernandez et al., 2004), this encouraged us to further explore if a FurC overexpression could also trigger the transcriptional levels of *furA* and *furB*.

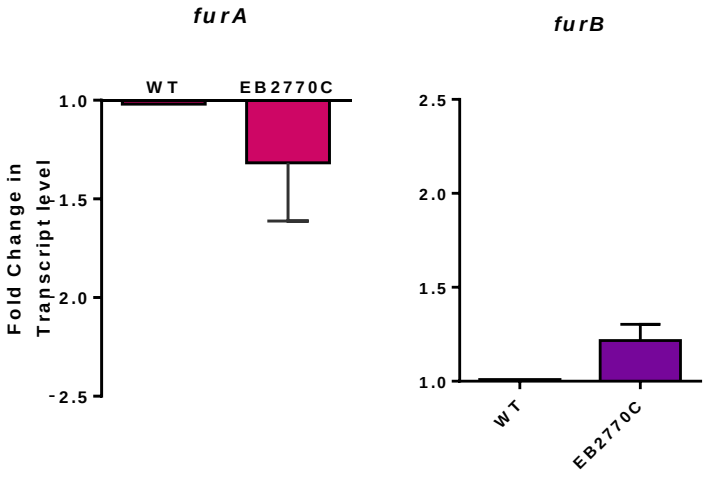

Figure 19: Quantitative RT-PCR analysis of the other two FUR genes *furA* and *furB* in *furC*-overexpressing strain EB2770C. Fold changes of all1691 (*furA*) and all2473 (*furB*) mRNAs were measured during growth under normal conditions in triplicate. Standard deviation is indicated by error bars. Values were normalized to the *rnpB* transcript. The fold change is then calculated using the ΔΔCT Method (Livak & Schmittgen, 2001)

As shown in Figura 19, A non-representative fold-change in both targets was found, showing that *furA* and *furB* are not regulated or at least not directly by FurC, this would be in line with the fact that FurC does not bind to any fur promoter region (Hernandez et al., 2004). However, due to the clear indications about heterodimer formation, further experiments will be performed in order to clarify the role of FurC within FUR family.

5.2.4.2. Overexpression of FurC in *Anabaena* sp. PCC 7120 increases transcription levels of a regulator involved in oxidative stress, *prxA*.

Although transcriptional levels of FurC are low in *Anabaena* sp., its expression is strongly upregulated when cells are treated with H_2O_2 (Lopez-Gomollon et al., 2009). Moreover, as shown Figure 10 and previously reported (Yingping et al., 2014), FurC specifically binds to the promoter region of *prxA* gene, a peroxiredoxin involved in oxidative stress response in *Anabaena* sp. These results indicated that transcriptional levels of *prxA* should be affected in FurC-overexpressing mutant. Figure 20 shows that there is a clear negative change on transcriptional levels up to -2-fold in EB2770C mutant, thus *prxA* is being downregulated by FurC.

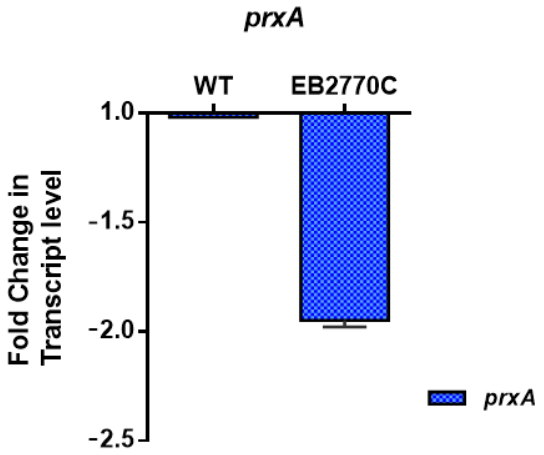

Figure 20. Fold induction of FurC target gen *prxA* in furC-overexpressing strain EB2770C (FurC). The relative concentration of alr4641 (*prxA*) mRNA was measured under normal conditions in triplicate. Standard deviation is indicated by error bars. Values were normalized using an endogenous housekeeping gene rnpB. The fold change is then calculated using the ΔΔCT Method (Livak & Schmittgen, 2001)

To better define the FurC implications in oxidative estress response, further experiments will be perform with targets that have been already explored in FurB such as the p*rxQ*, *sodA*, *sodB* genes.

5.3. ROLE OF FURC IN THE NITROGEN METABOLISM

4.3.1. Nitrogen starvation increases the expression of FurC in wild type *Anabaena sp.*

Previous analysis in our laboratory have shown that expression of *furA* is strongly induced by the global regulator of nitrogen metabolism NtcA in proheterocysts after nitrogen step-down, remaining stably expressed in mature heterocysts. Analogously, it was found that FurC was directly regulated by NtcA (López-Gomollón et al., 2007; Picossi et al., 2014). Therefore, during nitrogen starvation FurC was expected to be affected in the wild type *Anabaena* sp. PCC7120. Following this hypothesis, real-time PCR was performed using RNA extracted at different times after nitrogen step down. Figure 21 shows an increasing fold change of transcription reaching a peak of at 1.5 fold after 24h of nitrogen starvation.

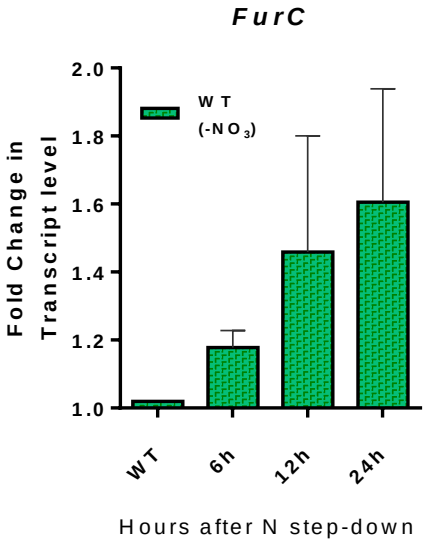

Figure 21. Change in transcript level of FurC in Anabaena sp. PCC 7120 (WT) after nitrogen step-down, as revealed by qRT-PCR analysis. RNA was isolated from ammonium-grown cells subjected to nitrogen deficiency for the number of hours indicated in each case. Standard deviation is indicated by error bars. Values were normalized using endogenous housekeeping gene *rnpB*. The fold change is then calculated using ΔΔCT Method (Livak & Schmittgen, 2001)

4.3.1. Relationship between FurC and phycobilisome degradation.

During the first hours after nitrogen deprivation all cells degrade their phycobilisomes (PBS) (Bradley & Carr, 1976). A key protein of this process is *nblA*, it specifically binds to the α-subunits of phycocyanin and phycoerythrocyanin, the main building blocks of the

phycobilisome rod structure (Bienert et al., 2006). Besides, it has been reported that in *Synechococcus elongatus* (PCC 7942), *nblA* expression is positively regulated by NblR and NtcA under nitrogen-stress conditions. (Luque, Zabulon, Contreras, & Houmard, 2001). Previous studies in our laboratory showed that FurC joined to two linker subunits of the PBS (Phycobilisome 7.8 kDa linker polypeptide, allophycocyanin-associated and Phycobilisome 34.5 kDa linker polypeptide, phycoerythrocyanin-associated) (unpublished, Broset, 2012). This result together with a decreased level of phycobiliproteins in FurC-overexpression mutant indicates that FurC must be related with the degradation process of phycobilisomes.

Therefore, the expression levels of *nblA* and its regulator *ntcA* were analysed by Real Time qPCR. Figure 22 indicates that both *ntcA* and *nblA* show an increased transcription levels in *furC*-overexpressing mutant EB2770C of about 1.5-2 fold. It has been reported that the increase in *nblA* transcript levels in *Anabaena* strains is about fivefold and it happens during early stages of nitrogen starvation (Baier et al., 2004).

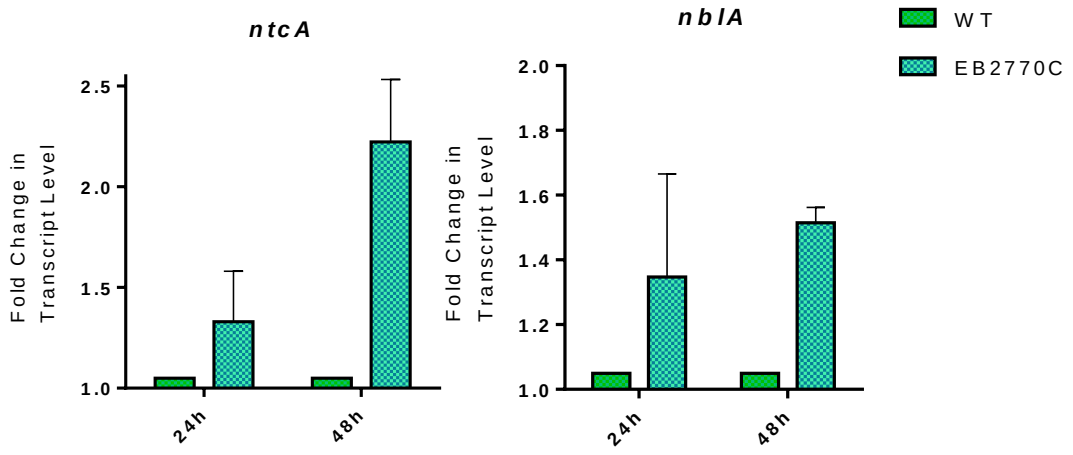

Figure 22. Quantitative RT-PCR analysis of the other two nitrogen related genes *ntcA* and *nblA* in furC-overexpressing strain EB2770C (FurC). Fold changes in mRNA abundance of (*ntcA*) and (*nblA*) mRNAs were measured during growth under normal conditions in triplicate. Standard deviation is indicated by error bars. Values were normalized to the *rnpB* transcript. The fold change is then calculated using the ΔΔCT Method (Livak & Schmittgen, 2001)

Thus, this moderate fold change shown in Figure 22 suggests indicate that FurC participates in the regulation of *ntcA* and *nblA* in some way. To confirm a direct interaction with promoters of the *nblA* and *ntcA* genes, binding assays were performed.

5.3.1.1. Binding assays of FurC with nitrogen metabolism targets: *NtcA* and *NblA*

Given all the results obtained above, gel retardation assays were performed to test if FurC bound to the promoter region of the *ntcA* and *nblA* genes. Conditions used were the same as specified in Materials and Methods section.

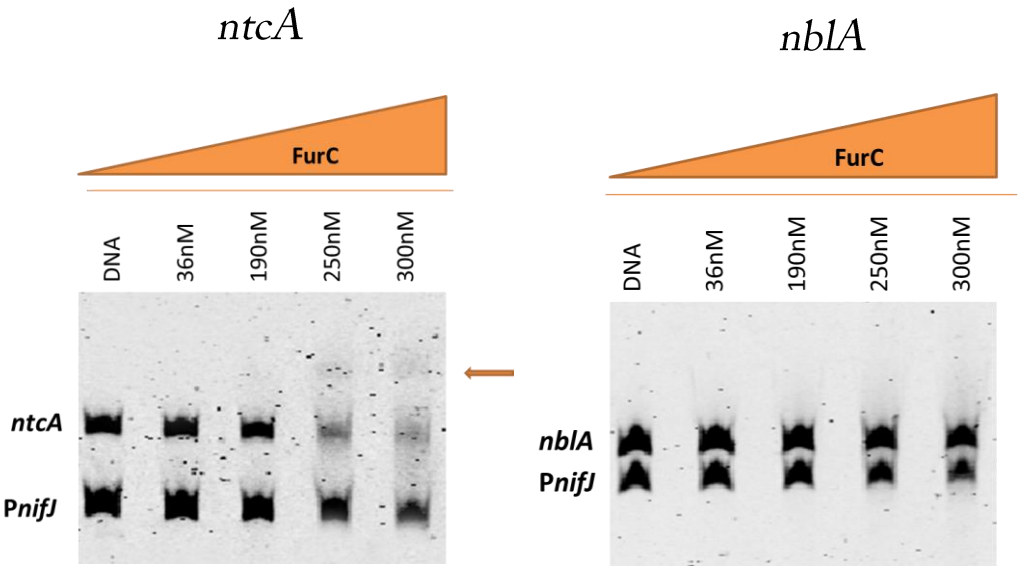

Figure 23. Electrophoretic mobility shift assays showing the ability of FurC to bind in vitro the promoters regions of *ntcA* gene (P_{ntcA}) and *nblA* gene (PnblA). Gel retardation experiments were performed with 35 ng DNA fragments free (1) or mixed with recombinant FurC protein at different concentration. The promoter region of *nifJ* (P_{nifJ}) was used as non-specific competitor DNA in all assays. FurC-DNA complexes are indicated with arrows.

As shown in Figure 23, FurC binds specifically to the promoter region of *ntcA*, complex formed are indicated by arrows. However, this bound is not found with n*blA* promoter since no delay is observed.

6. DISCUSSION

5.4. BIOCHEMICAL CHARACTERIZATION

5.4.1. Overexpression and purification of FurC as useful tool for biochemical characterization.

Previous purification of FurC paraloges, FurA and FurB were performed with Immobilized-metal affinity chromatography (IMAC), taking advatage of their higher His content in the metal-binding domain. However, FurC has only 6 histidine residues and they are spaced from each other (see Introduction section). In previous attempts to perform the purification of FurC in our laboratory small amount of protein was purified. Besides it was not known whether the purified fractions were active or not. Therefore, it was decided to isolate FurC according to their isoelectric point (5.3) by an optimized method. FurC protein was successfully overexpressed and purified using ION-exchange chromatography in which proteins are separated based on differences in their charge. The physicochemical features of FurC, previously reviewed, enabled the interaction of FurC with an anion exchanger resin, DEAE cellulose. Using this new method we obtained fractions with over 70% in the active state, which was verified by binding assays. Additionally, it should be noted that the protein fractions with greater percentage of purity contained a high presence of nucleic acids. Whereas partially purified fractions containing no nucleic acids were used, a successful activity of FurC was observed.

During biochemical characterization, electrophoretic analyses showed the presence of additional bands of FurC with different gel mobility as result of different redox states, as it was previously found in FurA from *Anabaena* sp. (Fillat, 2010), and other family members such PerR, (Lee & Helmann, 2006). In case of FurC, non-reducing conditions led to a monomer rearrangement to higher order aggregates, in fact as a result of oxidation, the monomeric form of FurC disappeared and slightly displayed two bands as described in other proteins (PerR) (Lee & Helmann, 2006). This higher order aggregates might be formed by disulfide bonds, that participate in several key cellular functions. In addition, the odd number of cysteine residues in FurC suggests that the uncoupled cysteines might be important in disulfide bonding with other proteins and/or multimerisation. Recently FurC has been considered as the PerR regulator (Yingping et al., 2014). However, unlike the ortholog PerR from *Bacillus subtilis*, ICP-OES assays and PAR staining showed that FurC did not present structural Zn^{2+}or traces of Zn^{2+}that could act as a metal regulator. This result is consistent with the absence of the CXXC motifs in

the primary structure of FurC that have been related to the existence of structural zinc coordinated by four cysteine residues (Lee & Helmann, 2006; Traoré et al., 2006).

Moreover, in absence of any regulatory metal, FurC has shown to be quite active in binding assays to DNA. Even at low concentrations, FurC showed a specific interaction with the promoter region of the *prxA* gene. Using the same promoter, Yingping and colleagues previously described that FurC specifically binds to *prxA* promoter when incubating with Zn^{2+} (Yingping et al., 2014) but they did not specify how the protein-DNA interaction behaved in absence of metal. DNA-specific interaction in paralogous proteins, FurA (J. A. Hernández et al., 2006) and FurB (Sein-Echaluce et al., 2015a) does require reducing conditions provided by DTT and also certain concentrations of regulatory metal such as Mn^{2+} or Zn^{2+}. Therefore, we could suggest that the fact that FurC does not need reducing conditions neither the presence of metal, could be related to the lack of similarity between sequences, which, as discussed in the introduction, since FurC is the protein that least resembles other orthologous FUR (Hernandez et al., 2004).

Taking all into consideration, there is still not sufficient information available to make conclusions concerning FurC mechanism of DNA-binding, thus further experiments will be performed using different redox conditions and metal concentrations.

5.5. PHENOTYPICAL CHARACTERIZATION

5.5.1.Role of the transcriptional regulator FurC in *Anabaena* sp.

It has been reported that FurC is involved in a range of physhiological processes including a potential modulation of the transcriptional activity of its paralogues, FurA and FurB (Hernandez et al., 2004; Lopez-Gomollon et al., 2009), a clear implication in the oxidative stress processes (Yingping et al., 2014) and also a key role in nitrogen metabolism (Picossi et al., 2014). In this work, we decided to address each of the possible functions of FurC, finally focusing on nitrogen metabolism which seemed to provide more relevance during the performance of the experiments. As an approach to explore the role of FurC, we combined the phenotypic characterization of the *furC*-overexpressing mutant with Real-Time PCR experiments and FurC- DNA binding assays.

5.5.1.1. FurC as potential modulator of transcriptional activity of FurA and FurB

As discussed in introduction, FurC has been previously described as a regulator of the binding activity of its paraloges FurA and FurB to its own promoters in *Anabaena* sp. It was suggested that FurC might form heterodimers through its C-terminus with other FUR family members, performing a combinatory effect by reducing FurB binding to DNA and intensifying FurA binding (Botello-Morte, González, Bes, Peleato, & Fillat, 2013; Hernandez et al., 2004). Seen these results, it was decided to compare the results by conducting transcriptional analysis in order to explore if FurC had also an effect on the expression of *furA* and *furB* genes.

Interestingly, qPCR assay showed that in the FurC-overexpressing mutant, expression levels of FurA and FurB remained unchanged. Although these results may seem contradictory, the regulation of the activity of FurA and FurB to their own promoters conducted by FurC, could be taking place only temporarily or under a specified conditions and therefore do not see a significant change in the expression of genes. Differences would also come from the fact that EMSA assays are *in vitro* results, while the real time PCR assays are a reflection of what happens *in vivo,* where these interactions could be influenced by several conditions and unknown partners.

In order to clarify how FurC regulates expression levels of its paralogues FurA and FurB, further experiments with two-hybrid screening technique will be performed to study the protein-protein interaction of FurC with FurA and FurB. Also, new culture conditions should be tested to check whether this transcriptional interaction is occurring or not.

5.5.1.2. Role of FurC against oxidative stress.

In photosynthetic organisms, the most important production of superoxide anion takes place in the photosynthetic electron-transport chain when molecular oxygen adventitiously oxidizes Photosystem I (Antoniou et al., 2013). There is substantial in vivo evidence correlating oxidative stress with alterations in the pool of intracellular free iron, as well as genetic studies showing how deregulation of iron homeostasis leads to oxidative stress (Cornelis et al., 2011; Touati, 2000). Under these oxidative-stress conditions, transcriptional levels of the three FUR (Ferric Uptake Regulator) genes (*furA, furB* and *furC*) are affected, being much stronger the affectation in the case of *furB* and *furC* mRNAs. In fact, expression of furC is enhanced specifically by addition of methyl viologen, a superoxide-anion-generating reagent (Lopez-Gomollon et al., 2009).

As observed in FurB (Sein-Echaluce et al., 2015b) and FurA (Gonzalez et al., 2011; López-Gomollón et al., 2007), previous reports demonstrated that FurC directly bound to the promoter region of the peroxiredoxin *alr4641/ prxA*, a protein implicated in detoxification and defence against oxidative response (Yingping et al., 2014). Therefore, we decided to perform FurC-DNA binding with the same promoter region, in order to prove the activity of FurC. As shown in results, FurC successfully bound to the *prxA* promoter region allowing us to perform further EMSA's assays.

In order to confirm the transcriptional regulation effect of FurC on *prxA* gene expression, analysis by qPCR in a *furC*–overexpressing mutant were performed. As shown in Results section, FurC clearly downregulated *prxA* transcription levels. It is noteworthy to point out that with a *furC*-overexpressing strain is not the desirable strain to analysed gene repression because we are observing a hyper-repressive phenotype, and clearer results would have been provided by a *furC*-deletion strain. However, a fully segregated mutant of this gene has been impossible to obtain, suggesting that seems indispensable for the viability. Theses result together with the previous studies, propose FurC as a key point in the oxidative stress adaptation of *Anabaena* sp.

5.5.1.3. Identification of novel implications of FurC related to nitrogen metabolism

FurC seemed to be highly related to the nitrogen metabolism since an overexpression of NtcA (master regulator of nitrogen metabolism) led to an increase of transcriptions levels of FurC (Picossi et al., 2014). Moreover previous studies in our laboratory suggest the possible involvement of FurC in these processes. The overexpression approach led us to identify novel direct targets of this apparently essential transcriptional regulator by combining the analysis of phenotypic changes with both transcriptional profile variation and FurC-DNA interactions. Phenotypic response in *furC*-overexpression mutant revealed that FurC was directly related to the nitrogen metabolism and in particular to the degradation of the phycobilisomes.

Spectrometrical measures of *furC*-overexpressing mutant showed decreased levels of phycobiliproteins and chlorophyll comparing with wild type strain. This difference was even more evident upon nitrogen step down, since cultures of *furC*-overexpressing mutant showed to have a stronger chlorosis response in which the loss of PBS in cyanobacteria changes their appearance from the normal blue-green to yellow-green (Collier & Grossman, 1992). Also stronger chlorosis response was observed in a *nblA*-overexpresing mutant comparing with wild type strain under nitrogen starvation, in *Anabaena* sp. (Baier et al., 2004; Kato et al., 2008). As

discussed in Introduction section NblA is a protein that specifically binds to the α-subunits of phycocyanin and phycoerythrocyanin, the main building blocks of the phycobilisome rod structure (Bienert et al., 2006) and its expression is positively regulated by NblR and NtcA under nitrogen-stress conditions. (Luque et al., 2001). A possible mechanism to the phycobilisome degradation was proposed in Baier et al 2006 and it consisted on the proposal of that NblA tags PBS for the cellular proteolytic machinery, in analogy to e.g. ubiquitin that covalently binds to other proteins and makes them accessible to the 26 S proteasome. This mechanism suggested that NblA would also need a further interaction partners that would help to the degradation of phycobilisomes. (Bienert et al., 2006).

Previous work in our laboratory revealed a similar bound between FurC and two linker subunits of the PBS (Phycobilisome 7.8 kDa linker polypeptide, allophycocyanin-associated. and Phycobilisome 34.5 kDa linker polypeptide, phycoerythrocyanin-associated) (Unpublished data, Broset, 2012). However, its noteworthy that by action of NblA, only the absorption peak of phycocyanin at ~625 (PBS) is altered, but not the chlorophyll *a* one. Therefore, The fact that a *furC*-overexpression mutant has also the chlorphyll spectra affected, could indicate that FurC could be the responsible for a complete destabilization of phycobilisome, affecting even regions of chlorophyll regions in the photosystem I. However, How both *furC* and *nblA* performe its functions is yet known, therefore further studies will be done in order to clarify the mechanism.

The relationship between FurC and nitrogen metabolism was confirmed by transcriptional assays. Firstly, it was observed that the wild type strain has an increase in the FurC expression levels under nitrogen depravation, which is in agreement with the fact that NtcA (the Global Nitrogen Regulator) directly regulates FurC (Picossi et al., 2014). These transcriptional changes sometimes occur temporarily, this is the case of NtcA, whose levels are overexpressed at early hours of nitrogen deficiency and they return to baseline at mid terms. Therefore, although an increased in *furC* levels was observed under nitrogen deficency, further experiments collecting data at additional steps of deficiency will be repeated in order to have a more representative results.

Hence, using a *furC*-overexpression strain, we could study the transcriptional levels of genes such as NblA and NtcA. Both NtcA and NblA were found to be positively regulated by FurC, changes being more marked in the case of NtcA. Subsequently, these results were combined by DNA binding assays using the promoter regions of the *ntcA* and *nblA* genes. In this experiment it was found that FurC specifically bounds to the promoter of *ntcA*, but not to *nblA*. The fact that FurC regulates the expression of NtcA confirms its direct involvement in the regulation of nitrogen metabolism. This is a very important result because NtcA is the master regulator of nitrogen and not many regulators of NtcA are known.

Regarding to NblA, we suggest that, the increased *nblA* transcription levels could be due to the up regulation of *ntcA*. However, the close relationship between FurC and degradation phycobilisomes discussed above, would not rule out the direct relationship between NblA and FurC, To explore this hypothesis, double-hybrid studies will carried out in order to study clarify a potential protein-protein interaction between NblA and FurC..

Overall, the results obtained along this work highlight the novel multifaceted character of FurC and its functional implications in essential processes such as oxidative stress response and nitrogen metabolism. All those data have provided valuable and extensible information to perform further experiments in future studies to explain the in vitro and in vivo behavior of FurC.

7. CONCLUSIONS

The findings gained throughout this work have allowed to obtain the following conclusions:

- Overexpression and purification of FurC by Ion-exchange chromatography provides a protein preparation with a 80% of purity and showing specific and unespecific DNA binding capacity.

- FurC recombinant protein obtained by the proposed method does not present Zinc atoms in its structure and does not require reducing conditions or the presence of any regulatory metal to show binding activity in gel-shift assays, unlike the other FUR paralogues.

- The furC-overexpressin mutant of *Anabaena* sp. showed altered phenotypic characteristics such a clear decrease of phycobiliproteins and chlorophyll under both standard conditions and nitrogen starvation. This allow us to relate *furC* with the process of degradation phycobilisome. Microscopy observation has determined that the filaments of *furC*-overexpressing mutant contain lower-size cells than the wild-type strain.

- The overexpression of FurC in *Anabaena* has altered the transcriptional pattern of genes related to oxidative stress response, such *prxA* and the ones involved in nitrogen metabolism such as *ntcA* and *nblA*. Its direct regulation was confirmed by binding assays for the promoter regions of *prxA* and *ntcA*.

7.1. Conclusiones

Los resultados obtenidos a lo largo del trabajo expuesto en esta memoria han dado lugar a las siguientes conclusiones:

- La sobreexpresión y purificación de FurC por cromatografía de intercambio iónico proporciona una preparación de proteína con una purificación superior al 80% de pureza. La proteína obtenida tiene además capacidad de unión al DNA, tanto específica como inespecífica.

- La proteína recombinante obtenida no presenta átomos de zinc en su estructura. Además, esta no requiere condiciones reductoras o presencia de metal regulador para mostrar capacidad de unión específica al DNA, al contrario que sus parálogos dentro de la familia FUR.

- La sobreexpresión del gen *furC* en *Anabaena* sp. provoca la alteración de características fenotípicas como la disminución de la cantidad de ficobiliproteinas y clorofila *a* bajo condiciones estándar y en deficiencia de nitrógeno. Su observación por microscopía ha determinado que los filamentos del mutante de sobreexpresión de *furC* contienen células de menos tamaño que la cepa silvestre.

- El mutante de sobreexpesion de FurC en *Anabaena* sp. presenta una alteración del patrón transcripcional de diversos genes. FurC interviene tanto en la regulación de genes implicados en la respuesta frente a estrés oxidativo como es *prxA,* como en genes relacionados con el metabolismo del nitrógeno, *ntcA* y *nblA*. La regulación directa se comprobó mediante ensayos de binding, mostrando interacción con las regiones promotoras de *prxA* y *ntcA*.

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[redacted]

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY MATERIAL

Tables

Table 1. BG-11. Medium composition

Basal medium 1x (3 L)		
Reagent	Grames	Final concentration (µM)
NaNO3	4.05	16
NaHCO3	2.214	8.8
K2HPO4 x 3h2o	0.12	0.2
CaCl2 x 2h2o	0.108	0.25
MgSO4 x 7h2o	0.225	0.3
Na2CO3	0.06	0.2
Supplement 1000x (100 ml)		
Reagent	Miligrames	Final concentration (µM)
Ferric Ammonium Citrate	600	30
Citric Acid	600	30
EDTA	100	190
H3BO3	286	46
MnCl2 X 4H2O	181	9.15
ZnSO4 X H2O	13.8	0.77
Na2MoO4 X 2H2O	39	1.61
CuSO4 X 5H20	7.9	0.32
CoCL2	4.94	0.17

Table 2. Oligonucleotids used in this work

PRIMER	SECUENCE 5' → 3'	AMPLIFIED SECUENCE	PURPOSE
rnpB for	AGCGGAACTGGTAAAAGACCAA	rpnB gen *Anabaena* sp. PCC7120	qPCR
rnpB rev	GAGAGGTACTGGCTCGGTAAACC		
furC for	GCGTTGTCAGCTGTTAGGTATGC	*furC* gen *Anabaena* sp. PCC7120	qPCR
furC rev	GCTTGCCACAGCAGTTCTAAAA		
nblA for	TTCAGCATTCGCTCATTTGC	*nblA* gen *Anabaena* sp. PCC7120	qPCR
nblA rev	CTTACGGACAACCATTTGTTC		
ntcA for	GGCCCTAGCAAATGTTTTTCG	*ntcA* gen *Anabaena* sp. PCC7120	qPCR
ntcA rev	TTTATTGCGTTCAAACGTTTCG		
prxA for	CCATCACCTACGGAACACAAGA	*prxA* gen *Anabaena* sp. PCC7120	qPCR
prxA rev	TCCTGATCAACTACAGCTGTTGCT		
furA for	TGTGTCCTTCGCCTAATTCCA	*furA* gen *Anabaena* sp. PCC7120	qPCR
furA rev	ACGATTTACCGGACGTTGAAGT		
furB for	CAGGGCATTGATGAATAGGAATAGA	*furB* gen *Anabaena* sp. PCC7120	qPCR
furB rev	AGCCTAGCGCAGCAAGATAAA		
prxA up	GTCCAGAAGGCGGATTTGTC	*prxA* promoter *Anabaena* sp. PCC7120	EMSA
prxA dw	CTTAATTCTCCTTCAACTTATATCGG		
ntcA_up	CATGGTTAGCAAAAATGATG	*ntcA* promoter *Anabaena* sp. PCC7120	EMSA
ntcA_dw	CTTGTGTCACGATCATCTCC		
nblA up	AACAGTTTTGAATAGGTAGT	*nblA* promoter *Anabaena* sp. PCC7120	EMSA
nblA dw	ATAACAGACTCCTAAAAGAC		
pnifJ up	GCCTACTCTGCGAGTTCTCCG	*nifJ* promoter *Anabaena* sp. PCC7120	EMSA
pnifJ dw	GGCCTGTGAGAGTTGCTGCAC		

Table 3 .- SDS-PAGE gel composition				
Component	**12,5% 1,5 gel**	**15% 1,5 gel**	**17% 2 geles**	**5% 1 gel stacking gel**
H2O	1,8 ml	1,06 ml	0,72 ml	1,4 ml
Tris-HCl 1,5M pH 8,8	3,36 ml	3,36 ml	4,48 ml	-
Tris-HCl 0,5M pH 6,8	-	-	-	0,25ml
Acrilamide/Bis acrilamide 30%	3,75 ml	4,5 ml	6,8 ml	0,33 ml
SDS 10%	45 µl	45 µl	60 µl	10 µl
PSA 10%	30 µl	30 µl	40 µl	10 µl
TEMED	15 µl	15 µl	20 µl	10 µl

Figures

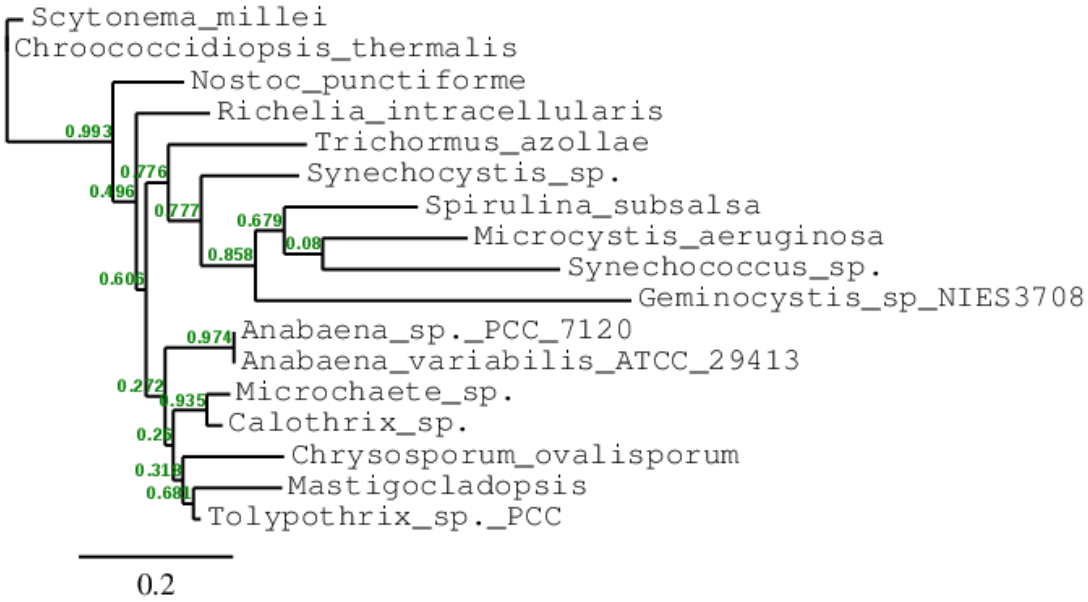

Figure 1: Phylogenetic tree of FurC secuences in other cyanobacterial species. (the branch length is proportional to the number of substitutions per site). FurC from *Anabaena* sp. is indicated with an arrow. The analysis was performed on the Phylogeny.fr platform. Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3).

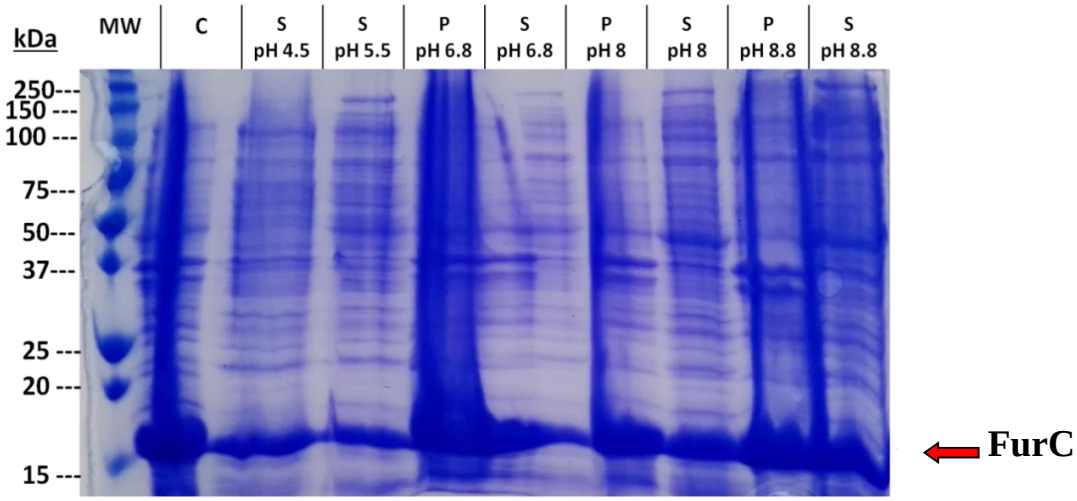

Figure 2. Preliminary stability assay. SDS-polyacrilamide gels (17%) showing supernatant (S) and pellet (P) of different cell lysates of *E.coli* BL21 (DE3) overexpressing FurC which were incubated 1 h at different pHs. FurC is indicated with an arrow. (C) is a control using crude extract with Tris-HCl pH 8 50 mM. MW: Low Molecular Weight marker (Healthcare).