OXFORD UNIVERSITY PRESS | Plant and Cell Physiology

Regulation by FurC in *Anabaena* links the oxidative stress response to photosynthetic metabolism

Journal:	*Plant and Cell Physiology*
Manuscript ID	PCP-2019-E-00193.R1
Manuscript Type:	Regular Paper
Date Submitted by the Author:	25-Apr-2019
Complete List of Authors:	Sevilla, Emma; Universidad de Zaragoza, Bioquímica y Biología Molecular Sarasa-Buisán, Cristina; University of Zaragoza and BIFI, Biochemistry and Molecular and Cell Biology Gonzalez, Andres; University of Zaragoza and BIFI, Biochemistry and Molecular and Cell Biology; Aragon Institute for Health Research , ISS Aragón Cases, Rafael; University of Zaragoza, Departamento de Física de la Materia Condensada; CSIC, Instituto de Ciencia de Materiales de Aragón Kufryk, Galyna; Grand Canyon University, College of Science, Engineering and Technology Peleato, M.; Universidad de Zaragoza, Bioiquímica y Biología Fillat, Maria; University of Zaragoza and BIFI, Biochemistry and Molecular and Cell Biology
Keywords:	cyanobacteria, FurC (PerR), cell division, ftsH, peroxiredoxins, photosynthesis

Article Title: Regulation by FurC in *Anabaena* links the oxidative stress response to photosynthetic metabolism.

Short title: FurC Links Peroxide Response To Photosynthesis

Corresponding author: M. F. Fillat; Departamento de Bioquímica y Biología Molecular y Celular and Institute for Biocomputation and Physics of Complex Systems / Universidad de Zaragoza / Pedro Cerbuna 12 / Zaragoza / 50009 / Spain / +34976841282 / fillat@unizar.es

Subject areas: (3) Regulation of gene expression, (5) Photosynthesis, respiration and bioenergetics

Black and white figures: 10

Tables: 1

Supplementary material: 1 (Table S1)

Article Title: Regulation by FurC in *Anabaena* links the oxidative stress response to photosynthetic metabolism.

Short title: FurC Links Peroxide Response To Photosynthesis

Authors: Emma Sevilla[1], Cristina Sarasa-Buisan[1], Andrés González[1,2], Rafael Cases[3], Galyna Kufryk[4], M. Luisa Peleato[1] and María F. Fillat[1,*]

[1]Departamento de Bioquímica y Biología Molecular y Celular and Institute for Biocomputation and Physics of Complex Systems, Universidad de Zaragoza, Pedro Cerbuna 12, 50009 Zaragoza, Spain.

[2]Aragon Institute for Health Research (IIS Aragón). 50009 Zaragoza, Spain.

[3]Instituto de Ciencia de Materiales de Aragón and Departamento de Física de la Materia Condensada, Facultad de Ciencias, Universidad de Zaragoza-CSIC, Zaragoza, Spain

[4]College of Science, Engineering and Technology, Grand Canyon University, 3300 W. Camelback Rd., Phoenix, AZ 85017, USA.

***Corresponding author e-mail**: fillat@unizar.es

ABSTRACT

The FUR (Ferric Uptake Regulator) family in *Anabaena* sp. PCC7120 consists of three paralogs named FurA (Fur), FurB (Zur) and FurC (PerR). *furC* seems to be an essential gene in the filamentous nitrogen-fixing strain *Anabaena* sp. PCC7120, suggesting that it plays a fundamental role in this organism. In order to better understand the functions of FurC in *Anabaena*, the phenotype of a derivative strain that overexpresses this regulator (EB2770FurC) has been characterized. The *furC*-overexpressing variant presented alterations in growth rate, morphology and ultrastructure, as well as higher sensitivity to peroxide than *Anabaena* sp. PCC7120. Interestingly, the overexpression of *furC* led to reduced photosynthetic O_2 evolution, increased respiratory activity, and had a significant influence in the composition and efficiency of both photosystems. Comparative transcriptional analyses, together with electrophoretic mobility shift assays allowed the identification of different genes directly controlled by FurC, and involved in processes not previously related to PerR proteins, such as the cell division gene *ftsZ* and the major thylakoid membrane protease *ftsH*. The rise in the transcription of *ftsH* in EB2770FurC cells correlated with reduced levels of the D1 protein, which is involved in the PSII repair cycle. Deregulation of the oxidative stress response in EB2770FurC cells led to the identification of novel FurC targets involved in the response to H_2O_2 through different mechanisms. These results, together with the effect of *furC* overexpression on the composition, stability and efficiency of the photosynthetic machinery of *Anabaena,* disclose novel links between PerR proteins, cell division and photosynthesis in filamentous cyanobacteria.

Key words: Cell division, cyanobacteria, *ftsH*, FurC (PerR), peroxiredoxins, photosynthesis.

Introduction

Cyanobacteria contain a unique combination of light-harvesting complexes composed by membrane-bound antenna present in both photosystems and by phycobilisomes bound to the surface of the thylakoid membrane that transfer light energy to chlorophyll a (Chl a). Some strains, such as *Anabaena* sp. PCC7120, can reduce atmospheric nitrogen into ammonia, supplying fixed nitrogen to plants. Because of its photosynthetic metabolism, cyanobacteria have high transition metal demands. In addition to Mg^{2+} present in Chl a, Mn^{2+} and Ca^{2+} are essential for PSII stabilization and light-induced oxygen evolution; Zn^{2+} is required at the active site of carbonic anhydrase, and the cyanobacterial photosynthetic apparatus is composed of a number of iron-rich membrane protein supercomplexes (Liu 2016; Cavet et al. 2003). Thus, among transition metals, iron is the major cofactor needed not only for photosynthesis but for other cyanobacterial electron transport pathways involved in respiration, oxidative phosphorylation and nitrate assimilation. This makes cyanobacterial iron quota about 10-times higher than that of heterotrophic organisms (Shcolnick and Keren 2006). In the case of diazothropic cyanobacteria, nitrogen fixation is even more iron-demanding, since nitrogenase itself contains about 40 atoms of iron (Rees et al. 2005). In a paradox, iron-sulfur clusters are the primary targets of reactive oxygen species (ROS) generated as by-products of the photosynthetic metabolism in the Mehler reaction and, to a lesser extent, in PSII when the light-driven oxidation of water is not completed. In addition to iron-containing proteins, the D1 polypeptide in the core complex of PSII is the main target of photooxidative damage (Yamamoto 2001). Generation of ROS increases under environmental stress conditions, a common situation in cyanobacterial habitats, leading to the overreduction of the intersystem components of the photosynthetic electron transport chain and slowing down biosynthetic routes (Apel and Hirt 2004). As a result, to cope with oxidative stress, cyanobacteria have developed a battery of antioxidant enzymes that include Mn and Fe superoxide dismutases, catalases and peroxidases, among others (Latifi et al. 2009). Therefore, in *Anabaena*, photosynthesis, respiration, nitrogen metabolism and the oxidative stress response are tightly related to metal homeostasis and are coordinately regulated mainly by FUR (Ferric Uptake Regulator) proteins (Gonzalez et al. 2010; Gonzalez et al. 2016; Lopez-Gomollon et al. 2007; Sein-Echaluce et al. 2015).

The FUR family in *Anabaena* is composed of three members: FurA (Fur), FurB (Zur) and FurC (PerR) (Hernandez et al. 2004). FurA and FurB are redox-sensing regulators, since their interaction with their respective metal co-factors depends on the redox environment (Hernandez et al. 2006; Sein-Echaluce et al. 2018). FurA is essential, and it works as a global regulator that modulates around 33% of the *Anabaena* sp. PCC7120 genome, including a large number of genes involved in iron metabolism, as well as a major groups of genes related to photosynthesis, respiration, nitrogen metabolism, heterocyst differentiation and the oxidative stress response (Gonzalez et al. 2016). FurB controls zinc homeostasis in *Anabaena* sp. PCC7120 together with a set of key genes involved in the oxidative stress protection (Napolitano et al. 2012; Sein-Echaluce et al. 2015). FurC is the most unknown member of Fur proteins of *Anabaena* PCC7120. It was previously reported to be able to modulate the binding activity of the other FUR paralogs to their own promoters (Hernandez et al. 2004). In addition, it has been proposed that FurC corresponds to the PerR orthologue described in many Gram-positive bacteria (Yingping et al. 2014). PerR (Peroxide Response) proteins repress genes involved in oxidative stress response and sense H_2O_2 by Fe-catalyzed histidine oxidation that leads to the release of the regulator from their targets (Lee and Helmann 2006). In the unicellular, non-diazotrophic cyanobacterium *Synechocystis* PCC 6803, PerR is encoded by *slr1738*, and its disruption allowed the identification of a putative regulon that includes about 37 genes mainly involved in the oxidative stress response, transport across membranes, iron metabolism and CO_2 concentration (Kobayashi et al. 2004; Li et al. 2004). Conversely, previous attempts to inactivate the *furC* gene in *Anabaena* sp. PCC7120 were unsuccessful (Yingping et al. 2014) and our unpublished results, suggesting that either FurC might have an extra function in nitrogen-fixing cyanobacteria or it could control a wider set of genes in *Anabaena* PCC7120 than in unicellular cyanobacteria. Therefore, with the aim to better understand the role of FurC in *Anabaena* sp. PCC7120, a *furC*-overexpressing strain was produced, and its phenotype has been characterized. Constitutive expression of *furC* leads to a pleiotropic phenotype with observable changes in cell shape, increased sensitivity to oxidative stress and remodelling of the photosynthetic machinery. Furthermore, a set of novel direct targets of FurC involved in cell-division, PSII quality control and the oxidative stress response have been identified. Our results unveil that the expression levels of FurC in *Anabaena* are related to the control of cell-growth parameters as well as to the composition and efficiency of the photosynthetic machinery.

Results

FurC overexpression from the copper-inducible petE promoter

A derivative strain of *Anabaena* sp. PCC 7120 showing a high level of expression of *furC* was generated using the shuttle vector pAM2770 (Lee et al. 2003) as described previously (González et al. 2010). The resulting vector, pAM2770*furC*, contained an extra copy of the wild-type *furC* gene located downstream of the copper inducible *petE* (plastocyanin) promoter (Buikema et al. 2001). Transcription levels of *furC* and translation of the protein were verified by real-time RT-PCR and Western blot respectively (Fig. 1). Since supplementation of BG-11 with additional copper does not result in appreciable increase in the amount of the recombinant protein produced using this system (González et al. 2010), standard BG-11 was used in further analyses.

Anabaena *cells overexpressing furC exhibit altered growth and morphology*

Photoautotrophic growth of the wild-type and EB2770FurC strains in BG-11 medium was compared by following the cell number measuring optical density at 750 nm and cell count over the course of 20 days. The number of cells in an average filament was similar in both *Anabaena* strains, ranging between 30 and 40 cells. Thus, the change of the absorbance at 750 nm as well as cell counting indicated that EB2770FurC growth rate was markedly higher than that of the wild type strain (Fig. 2, panels A and B). Cells of *Anabaena* sp. PCC7120 showed a growth rate of 5.54 ± 0.2 d^{-1} whereas the EB2770FurC variant doubled at 4.78 ± 0.5 d^{-1} (Table 1).

The morphology of EB2770FurC cells was analysed by using Scanning Electron Microscopy (SEM) in comparison with cells of the wild type. At low magnification (x3000) the microphotographs showed that filaments of the EB2770FurC strain are composed by rod-shaped cells whereas cells of the wild type are elliptically-shaped (Figs. 3A and 3B). Moreover, filaments of EB2770FurC exhibited cells more homogeneous in shape and size, compared to the wild type strain (Figs. 3A and 3B). The size of the filaments was similar in both strains. On the other hand, higher magnification (x7000) microphotographs revealed that surface roughness exhibited by the wild type decreased considerably in the EB2770FurC strain (Figs. 3C and 3D).

Changes in the ultrastructure of EB2770FurC cells were analysed by Transmission Electron Microscopy (TEM). TEM microphotographs showed differences between both strains in thylakoid arrangement (Figs. 3E and 3F). In *Anabaena* sp. PCC7120, thylakoids were homogeneously distributed in cell cytoplasm (Fig. 3E) whereas in the EB2770FurC strain, they were located close to cell membrane at the periphery of the cell (Fig. 3F). A smaller diameter of EB2770FurC cells (Fig. 3F), compared to the wild type (Fig. 3E) was also observed. Furthermore, the EB2770FurC variant exhibited a peptidoglycan layer with higher density and a smoother surface than the wild type. Finally, the septa between EB2770FurC cells seemed to be wider than those in the wild type *Anabaena* sp. PCC7120 (Figs. 3C, 3D, 3G and 3H). Pectic acids or mucopolysaccharides forming the mucilaginous sheath of *Anabaena* appeared to be differently distributed on the septum boundaries (Figs. 3G and 3H).

ftsZ *and* ftsH *genes are controlled by FurC in Anabaena PCC7120*

In the light of these results, we investigated the potential involvement of FurC in the modulation of some of the genes related to morphology and growth processes such as the the *mreBCD* operon, encoding the bacterial actins MreB, MreC and MreD (Hu et al. 2007), responsible for changes in cell morphology, as well as the cell division genes *ftsZ* and *ftsH* (*all4776*). The binding of FurC to the promoter regions of these genes was tested by electrophoretic mobility shift assays (EMSA). Figure 4A shows that FurC bound specifically to the promoter regions of both *ftsZ* and *ftsH* genes whereas it did not bind to the promoter of the *mreBCD* operon. Afterwards, the influence of FurC overexpression in the transcriptional levels of *ftsZ* and *ftsH* was investigated. Relative changes in mRNA levels were determined by real-time RT-PCR. Figure 4B shows that transcription of both *ftsH* and *ftsZ* genes was up-regulated in EB2270FurC strain (1.62- and 1.87-fold respectively), indicating that FurC could work as a transcriptional activator of both genes.

Anabaena *cells overexpressing* furC *contain reduced levels of D1*

Since *ftsH* codes for a protease involved in the degradation of the D1 polypeptide, we sought to investigate in what extent the activation of *ftsH* by FurC would influence the levels of D1. Western blot analyses of the *Anabaena* sp. PCC7120 wild type and the EB2770FurC strain confirmed that the total amount of D1 was reduced in EB2770FurC cells. Figure 5 shows that both strains present

a 32 kDa form corresponding to native D1 and the processed peptide at 14 kDa. Furthermore, a faint band of higher molecular weight that could correspond either to a D1/D2 heterodimer (Yamamoto 2001) or to D1 aggregates (Kale et al. 2017) can be observed only in both biological replicates of the wild type *Anabaena* sp. PCC7120.

Overexpression of furC *leads to modifications in total pigment content of* Anabaena *sp. PCC7120*

Whole-cell spectra of *Anabaena* sp. PCC7120 and the EB2770FurC strain evidenced that the amount of phycobiliproteins (maximum of absorbance at ~625 nm) and Chl a (maximum of absorbance at ~665 nm) was lower in EB2770FurC cells than in the wild type (Fig. 6A). With the aim to better evaluate the effect of an excess of FurC, contents of phycobiliproteins, Chl a, carotenoid and total protein were determined in both strains at the exponential and the stationary phases of growth. These data were normalized by dividing the amount of pigments by the number of cells present in the samples. The levels of Chl a, phycobiliproteins as well as total protein were significantly lower in the EB2770FurC strain, both in exponential and stationary phases (Figs. 6B, 6C, 6D). However, the carotenoid content decreased in EB2770FurC cells in the exponential phase but increased in the stationary phase, compared to the wild type (Fig. 6E).

Levels of FurC affect transcription of phycobilisome genes

The lower content of phycobilisomes observed in the EB2770FurC variant could be due to a decrease in the expression of phycobilisome proteins. Phycobilisomes of *Anabaena* sp. PCC7120 are composed of an allophycocyanin core and several rods made of stacked disks of phycocyanin and allophycocyanin connected through hydrophobic linker peptides (Singh et al. 2015). In order to get a deeper knowledge about the influence of *furC* overexpression in the photosynthetic machinery, transcription of the *apcA* α-subunit of allophycocyanin (*alr0021*), the *cpcB* β-subunit of phycocyanin (*alr0528*), as well as *apcC*, *apcE*, *cpcG,* and *pecC* linkers was analyzed by real-time RT-PCR in the EB2770FurC strain relative to the wild type (Fig. 6F). The transcription levels of the *apcA*, *cpcB* and *cpcG* genes decreased 2.47-fold, 2.8-fold and 2.1-fold, respectively, in the EB2770FurC strain. In contrast, the expression of *apcC*, *apcE* and *pecC* remained unaltered. In order to assess whether the *apcA*, *cpcB* and *cpcG* genes were directly controlled by FurC, EMSA assays were conducted. FurC did not bind to any of the promoter regions

of these genes (not shown), indicating that downregulation of *apcA*, *cpcB* and *cpcG* in the *furC*-overexpressing EB2770FurC strain is not controlled directly by FurC.

furC-*overexpressing cells display reduced photosynthetic O_2 evolution and increased respiratory activity*

Net photosynthesis and respiratory activities of EB2770FurC and *Anabaena* sp. PCC7120 were measured in cells at the exponential phase of growth by using an oxygen electrode. The oxygen-evolving activity of the EB2770FurC strain was around 30% of that of the wild type (Table 1). The decrease in photosynthetic activity was in agreement with the fact that the pigment content (phycobiliproteins and Chl a) as well as the amount of the PSII protein D1 was notably reduced in the EB2770FurC strain. Nevertheless, the respiration rate was increased in the EB2770FurC variant compared to the wild type (Table 1).

Overexpression of furC *affects the efficiency of photosystems*

To monitor the consequences of *furC*-overexpression on the content of both photosystems, as well as their interactions with the light-harvesting systems, analyses of the fluorescence spectra at 77K were carried out. Efficiency of energy transfer in both photosystems to Chl a was tested with 440 nm excitation light. Fluorescence emission at 680-695 nm is higher in the EB2770FurC strain than in *Anabaena* sp. PCC7120 (Fig. 7A), indicating that the efficiency of energy transfer in PSII is lower in EB2770FurC cells. A similar picture was observed when cells where excited at 580 nm (Fig. 7B). The higher peaks at 695 and 730 nm (corresponding to the emission of the antennae of PSII and Chl a associated with PSI, respectively) in the spectrum of EB2770FurC indicated a lower efficiency of energy transfer to both photosystems in the *furC*-ovexpressing strain, which is in good agreement with the lower photosynthetic oxygen evolution observed in this strain, compared to the wild type.

PSII displays higher sensitivity to the lack of calcium in the EB2770FurC strain

The influence of the overexpression of FurC in the stability of PSII was investigated under different stress conditions. A successive transfer of cyanobacterial cultures to the Ca^{2+}-less BG-11 medium caused a gradual depletion of Ca^{2+} in the cells, and produced a substantial decrease in the Fv/Fm ratio in the wild type *Anabaena* sp. PCC7120 cells over time (Fig. 8). This is a reflection of the

increased stress caused by the lack of Ca^{2+}, and the decline of the photochemical quenching capacity of PSII. FurC overexpression produced an important decline in the Fv/Fm ratio in the mutant cells, compared to the wild type, upon the decrease of Ca^{2+} concentration (Fig. 8). This has not been observed in the cultures subjected to other stress factors, namely high temperature and chloride deficiency (data not shown). Hence, it appears that the *furC* overexpression has a Ca^{2+}-mediated effect on the functional stability of PSII.

FurC differentially regulates oxidative stress response genes in the presence of H_2O_2

The effect of increasing concentrations of H_2O_2 in cultures of *Anabaena* sp. PCC7120 and the EB2770 *furC*-overexpressing variant is shown in Fig. 9. The tolerance of both strains to this oxidant remained similar at concentrations up to 1 mM. However, the presence of higher concentrations of peroxide led to a considerable decrease in the tolerance of EB2270FurC after 24 h of incubation compared to the *Anabaena* wild type (Fig. 9A). At 48 h the differences in the tolerance of the wild type and the EB2270FurC strain to peroxide were clearly observed at 3 mM. However, under higher concentrations of H_2O_2, namely 7 and 10 mM, both strains were highly affected (Fig. 9B).

On the basis of the differences in the tolerance to H_2O_2 observed in the EB2770FurC strain, quantification of the relative transcription of a variety of genes involved in oxidative stress in presence and absence of 250 µM H_2O_2 was determined by real-time RT-PCR. The selected genes included peroxiredoxins (*prxA/alr4641*, *ahpC*/*all1541*, *alr4404*, *CGT3/alr2375, CGT1* and *alr2503*), sulfiredoxin (*srxA*), the Mn-catalase *alr0998* (*catA*) and superoxide dismutase *sodA* (Fig. 10A). In addition, the ability of FurC to bind specifically to the promoter regions of these genes was tested. Apart from the previously reported direct targets, *prxA* and *srxA* (Yingping et al. 2014), we found that FurC was able to bind to the promoter regions of the three peroxiredoxin genes *ahpC* (*all1541*), *CGT3* and *alr4404* although with different affinities (Fig. 10B). In contrast, FurC did not bind to the other studied promoters (data not shown). Quantification of the relative transcription of these genes in presence and absence of 250 µM H_2O_2 unveiled that the genes directly controlled by FurC, including *prxA, srxA, ahpC and CGT3* (Fig. 10A), were upregulated in the presence of oxidative stress, while their transcription levels under normal conditions were similar to these in the wild type (*srxA* and *CGT3*) or even downregulated (*prxA* and *ahpC*) (Fig. 10A). In contrast, *all4404* showed a reversed pattern of expression in EB2770FurC, displaying an increment in transcription in normal

conditions that decreased in the presence of H_2O_2, suggesting that in this case FurC could be acting as an activator. The peroxiredoxins *CGT1* and *alr2503* were upregulated around 1.7-fold in EB2770FurC cells under both normal and oxidative stress conditions. Since no specific binding was observed with these promoters by EMSA, we can assume that there might be an indirect regulation by FurC which is not dependent on the oxidation state of the cell. Transcription of the superoxide dismutase *sodA* increased under normal conditions around 2.5-fold, while under oxidative stress remained similar to the level determined in *Anabaena* sp. PCC7120. In the case of the catalase *catA*, no transcriptional change was observed under normal conditions, but when incubating with H_2O_2, mRNA levels of *catA* dramatically decreased to around 4-fold. Furthermore, EMSA analyses showed that FurC did not bind to *sodA* and *catA* promoters (not shown).

Discussion

Most FUR proteins play a key role in the physiology of cyanobacteria and alterations in their expression affect multiple essential metabolic and signaling processes. Since previous attempts to inactivate *furC* in *Anabaena* sp. PCC7120 have failed, the production of a *furC*-overexpressing strain of *Anabaena* is a valuable tool for understanding the functions of this regulator in filamentous, nitrogen-fixing cyanobacteria. Our results indicate that the overexpression of *furC* leads to remarkable morphological and physiological changes in *Anabaena* sp. PCC7120 cells. Considering that FurC corresponds to the PerR orthologue, it is feasible that *furC* overexpression will affect the global redox status of the cell. Therefore, several redox-regulated processes are likely to be altered in the EB2770FurC strain. Although some of the changes observed could be associated to pleiotropic effects, many others are caused by direct or indirect miss-regulation by FurC of genes involved in cell division, photosynthesis or the response to oxidative stress.

Some noticeable features associated to *furC* overexpression are a faster growth rate and the different cell shape of EB2770FurC cells, compared to the wild type *Anabaena* sp. PCC7120. Some of these differences could be related to the direct activation of *ftsZ* and *ftsH* by FurC. The tubulin homolog FtsZ is present in most bacteria and chloroplasts and it plays an important role in cell division (Osteryoung et al. 1998). In *Anabaena* cells FtsZ accumulates at the growing edge of the division septa to form the characteristic prokaryotic Z-ring structure (Klint et al. 2007). Depletion of

FtsZ in an *Anabaena* sp. PCC7120 variant caused reduced and asymmetric cell division, as well as filaments with altered morphology (Corrales-Guerrero et al. 2018). These results suggest that the activation of *ftsZ* transcription by FurC observed in EB2770FurC cells could be related with the differences in the morphology observed in this strain. In addition, since light and other redox-related signals, such as the presence of reactive oxygen species (ROS) have a deep impact on cyanobacterial morphology, adaptive responses of the EB2770FurC strain to such factors could not be ruled out (Montgomery 2015).

FtsH is the major metalloprotease in photosynthetic membranes and is essential to maintain functional protein homeostasis by removing photodamaged and misfolded proteins (Kato and Sakamoto 2018). Proteolysis is required for cell division and the reorganization of novel septa and photosynthetic membranes. Since EB2770FurC cells divide faster than the wild type, the increase in *ftsH* transcription would correlate well with the physiological needs of the cells.

Furthermore, in situations of photooxidation FtsH is involved in the selective degradation of damaged D1 polypeptide as part of a PSII repair cycle, remodeling photosystem II and cytochrome b_6f complexes (Kato and Sakamoto 2018; Malnoe et al. 2014). In *Synechocystis, ftsH* plays a crucial role in maintaining the activity of both photosystems, and is necessary to prevent chronic photoinhibition (Bečková et al. 2017; Mann et al. 2000; Silva et al. 2003). Disruption of *ftsH* (*slr0228*) caused a 60% of reduction of functional PSI and changes in the phycocyanin/Chl a ratio (Mann et al. 2000). Despite these important functions of FtsH, little is known about the factors involved in the regulation of its expression. Bečková et al, recently described that in *Synechocystis* the Psb29 subunit physically interacts with FtsH and this interaction results in the accumulation of FtsH protein (Bečková et al. 2017). In the present work we describe that FurC is a transcriptional activator of *ftsH* unveiling an interesting link between an oxidative stress sensor protein (FurC) and the maintenance of a suitable photosynthetic electron chain. Our results evidence that, under standard growth conditions, photosynthesis is affected in the EB2770FurC strain. PSII will probably be more affected by ROS in EB2770FurC cells than in *Anabaena* sp. PCC7120 due to the misregulation of pivotal antioxidant genes in the *furC*-overexpressing strain. Consequently, EB2770FurC cells will require a faster PSII turnover involving D1 cleavage by FtsH, which is in good agreement with the enhanced expression of *ftsH* and the lower content of D1 in these cells compared to the wild type. This is also supported by several features of the *furC*-overexpressing

strain, such as the decrease in energy transfer in PSII observed in the fluorescence spectra at 77K, the reduced capacity of PSII to the photochemical quenching and its higher sensitivity to Ca^{2+}. Furthermore, under regular growth conditions, EB2770FurC cells exhibit a higher Fv/Fm ratio, compared to the wild type that may reflect a difference in the redox state of the plastoquinone pool in the two strains caused by the marked difference in their respiratory rate. Another contributing factor may be a lower molar ratio of phycocyanin to Chl a that was reported to have an inverse correlation with the Fv/Fm values (Misumi et al. 2016). Accordingly, the EB2770FurC strain had markedly lower phycobiliprotein content and exhibited a lower ratio of phycobiliprotein to Chl a content per cell compared to the wild type.

The present study shows that the EB2770FurC strain is more sensitive to H_2O_2 than the wild type *Anabaena* sp. PCC7120. This could be related to the de-regulation in EB2770FurC cells of key genes involved in the defense against oxidative stress. It is likely that decreasing photosynthetic oxygen evolution, together with a higher dark respiratory activity, would relieve oxygen tension in EB2770FurC cells. This increase in respiration could be also a compensatory effect in order to balance the ATP level of the cell.

Moreover, our results show that FurC plays a major role in the regulation of the detoxification mechanisms of *Anabaena* in response to H_2O_2. It has been proposed that peroxiredoxins, a type of peroxidases, could be the main proteins involved in detoxification of H_2O_2 in *Anabaena* (Pascual et al. 2011). In this work we have shown that four peroxiredoxins *alr2375* (*CGT3*), *all1541* (*ahpC*), *alr4404* and *alr4641* (*prxA*) are under the direct control of FurC that regulates their expression in response to H_2O_2. A different regulatory mechanism seems to operate for peroxiredoxins CGT1 and *alr2503,* whose transcription is enhanced in the EB2770FurC variant regardless of the presence or absence of peroxide, suggesting that, if any, the role of FurC in their regulation should be indirect since it does not bind to CGT1 and *alr2503* promoters. Conversely, transcription of *sodA* and *cat* is downregulated in EB2770FurC cells in response to H_2O_2, though FurC is not directly acting on these promoters according to EMSA. Our results suggest that FurC indirectly regulates the expression of *sodA* and *cat* genes, but also that this regulation is dependent on the redox state of the cell since their transcription is affected by the presence of H_2O_2. Thus, the different responses to peroxide observed in the EB2770FurC strain, compared to the wild type *Anabaena* sp. PCC7120 indicate that FurC mainly acts as a repressor using different mechanisms.

In summary, our results allowed the identification of *ahpC*, *CGT3*, *alr4404*, *ftsZ* and *ftsH* as novel direct targets of FurC in *Anabaena* sp. PCC7120. Furthermore, evidences of the *in vivo* direct activation by a PerR/FurC protein of genes involved in the PSII repair cycle, namely *ftsH,* are presented, unveiling a novel strategy of a photosynthetic organism to link the quality control of the photosynthetic machinery to the redox status of the cell.

Materials and methods

Construction of a FurC-overexpression strain from Anabaena sp. PCC7120

Plasmid pAM2770FurC containing the *furC* gene downstream the copper-inducible *petE* (plastocyanin) promoter (Buikema and Haselkorn 2001) was constructed from the shuttle vector pAM2770 (Lee et al. 2003). Chromosomal DNA was extracted from *Anabaena* sp. PCC7120 (Cai and Wolk 1990) and used as a template to amplify *furC* gene using primers FurC-pAM2770_up and FurC-pAM2770_dw (Supplemental Table S1). The amplification product was digested with the enzymes BamHI and NdeI and cloned into the same restriction sites of the vector pAM2770. The construction was sequenced to ensure that amplification and cloning procedures were successful (Service of Sequencing and Functional Genomics, University of Zaragoza). Plasmid pAM2770FurC was transferred to *Anabaena* sp. PCC 7120 by triparental mating (Elhai et al. 1997). Conjugation was carried out using the *E.coli* conjugant donor strain ED8654, carrying the plasmid pRL443, and the *E.coli* conjugal helper strain CPB1893 which contains the plasmid pRL623. Selected colonies were plated in BG-11 plates supplemented with 50 µg/ml neomycin. *furC* overexpression was estimated by real-time RT-PCR and Western blot. The clone that showed higher levels of FurC was selected and named EB2770FurC.

Cell counting

The process was carried out using three biological replicates as follows. In each growth phase, an appropriate dilution was applied to the culture in order to achieve a suitable counting of *Anabaena* cells. The number of cells present in *Anabaena* filaments was determined in the two top and the two bottom squares of a Neubauer chamber. After counting, the average number of cells in the larger

square was calculated. Finally, the cell density per ml was obtained as the average of cells counted in the large square x 10^4 x dilution factor.

Growth measurement and pigment content determinations

Photoautotrophic growth of three independent cultures of wild-type *Anabaena* sp. strain PCC7120 and its derivative *furC*-overexpressing strain EB2770FurC was measured spectrophotometrically using a SPECORD® PLUS Analytik Jena spectrophotometer. The optical density was recorded at 750 nm for 20 days and total cell count was determined using a hemocytometer. Doubling time was calculated as previously described (Stein 1973). Pigment content and total protein were quantified at exponential and stationary phases of growth. Chl a (Mackinney 1941), phycobiliproteins (Glazer 1976) and carotenoids (Davies 1976) were quantified as previously described. Total protein content was determined by using the BCA™ Protein Assay kit (Thermo Fisher Scientific).

Microscopy

Scanning electron microscopic examinations of *Anabaena* sp. filaments were carried out with a JEOL JSM 6360-LV microscope. Cells were harvested at early exponential phase of growth and fixed with 2.5% glutaraldehyde in phosphate buffer (66 mM Na_2HPO_4, 66 mM KH_2PO_4, pH 6.8) for 2 h at room temperature washed three times for 5 min in phosphate buffer, fixed with 2% OsO_4 for 1 h and washed three times for 5 min in distilled water and processed by Service of Electronic Microscopy of Biological Systems (University of Zaragoza). Samples for Transmission Electron Microscopy were fixed following a different method. Cells were harvested at early exponential phase of growth and washed twice with Sorensen buffer 0.1M (Na_2HPO_4 2 H_2O, NaH_2PO_4 H_2O, pH 7.2). Pellets were soaked in 2% agar in 0.1M Sorensen buffer, homogenized and allowed to polymerize at room temperature. The agar samples were cut into cubes of approximately 1 mm in edge size and fixed for 3 h by immersion with 3.1% glutaraldehyde in 0.1 M Sorensen buffer. Sample processing and transmission electron microscopic examinations were performed in the Research Interdepartmental Service of the Autonomous University of Madrid, Madrid, Spain.

Tolerance to oxidative stress induced by H_2O_2

Cells were exposed to increasing concentrations of H_2O_2 for 24 and 48 h. Cultures at late log phase of growth (O.D. at 750 nm around 1.0) were adjusted to an optical density of 0.5 with fresh BG-11 medium, and 200 µl of culture were displayed into each well of a microtiter plate. H_2O_2 was added to the wells at a final concentration of 0-10 mM. The plates were incubated for 24 h in darkness at room temperature. The results were documented by photography, and chlorosis was estimated by reading the absorbance at 620 nm with a Multiskan EX microplate photometer (Thermo Fischer Scientific).

Determination of net photosynthesis and dark respiration rates

Net photosynthesis rate of exponentially growing cells of *Anabaena* sp. PCC7120 and EB2270FurC strains were measured at room temperature with a Clark-type oxygen electrode model Chlorolab 2 (Hansatech). Net photosynthesis defined as true photosynthesis minus photorespiration and dark respiration (Wohlfahrt and Gu 2015), was determined by measuring O_2 increase during 3 min illuminating cell suspensions with white light at saturating intensity (400 µE m^{-2} s^{-1}). Dark respiration was estimated from O_2 uptake by cells incubated in the dark. Both, net photosynthesis and dark respiration rates were expressed as nmol O_2 per million $cells^{-1}$ s^{-1}. Data were analyzed with O_2 View (Hansatech) software.

Fluorescence emission measurements at 77 K

Fluorescence emission spectra were obtained at 77 K by exciting the *Anabaena* sp. PCC7120 and EB2770FurC samples with a 1000W ORIEL 66187 tungsten halogen lamp and a double 0.22 m SPEX 1680B monochromator. Samples of cyanobacterial cultures at exponential phase of growth were set to a Chl a concentration of 10 µg ml^{-1}. Excitation was carried out either at 440 nm to monitor energy transfer from PSs to Chl a, or at 580 nm to check energy transfer from phycobilisomes to Chl a. Spectra were normalized to identical concentration of Chl a and phycobiliproteins, respectively. Fluorescence emission was detected through a 0.5 JARREL-ASH monochromator with a Hamamatsu R928 photomultiplier tube. All the measurements were corrected from the system response. The spectral line widths (FWHM) for the excitation and the emission were 3.6 and 1.7 nm, respectively.

PSII response to stress by calcium depletion

Liquid cyanobacterial cultures were grown on Ca^{2+}-less BG-11 medium, in which $CaCl_2$ was replaced by the NaCl in 1:2 molar ratio. The final concentration of Ca^{2+} in this medium was the same as in the doubly distilled water that was used for these experiments. The medium for the EB2770FurC strain was supplemented with 50 µg/ml neomycin. Upon subculturing, cells at mid-exponential phase (OD at 730 nm = 0.4-0.6) were diluted to OD at 730 nm = 0.03 to start the successive culture. The initial cyanobacterial culture (0) was grown in a standard BG-11 medium. From this culture a series of 6 successive subcultures were grown on the Ca^{2+}-less BG-11 medium. Fluorescence measurements were recorded with a Walz fluorometer. Samples of cyanobacterial cultures at exponential growth phase were prepared with a Chl a concentration of 5 µg ml^{-1}. Fv/Fm ratio was determined according to (Genty et al. 1989). The data were analyzed with FIP software (Q_A-Data, Turku, Finland).

RNA extraction

Three independent cultures of each strain (*Anabaena* PCC7120 and EB2770FurC) were grown at 28ºC in BG-11 medium at 180 rpm with 50 µmol photons.m^2.seg^{-1} Cells were harvested at mid-exponential phase and pellets were frozen at -70ºC until the analyses of the expression of selected genes. Similar experiments were conducted in presence and absence of 250 µM H_2O_2 to study differential expression under oxidative stress conditions. In this experiment, cells were harvested after 1 hour of H_2O_2 treatment. RNA was purified from each pellet following a method adapted from (Olmedo-Verd et al. 2005). The absence of DNA in the RNA samples was checked by real-time PCR, using oligonucleotides for the housekeeping gene *rnpB* (Vioque 1992). RNA was quantified spectrophotometrically using a SPECORD® PLUS Analytik Jena spectrophotometer.

Real time RT-PCR

2 µg of total RNA were reverse-transcribed using SuperScript retrotranscriptase (Invitrogen) following the manufacturer's conditions. Real-time PCR was performed using the ViiA™ 7 Real-Time PCR System (Applied Biosystems). The specific primers are included in Supplemental Table S1 of Supplementary Material. Each reaction was set up mixing 12.5 µl of SYBR Green PCR Master Mix with 0.4 µl of 25 µM primer mixture and 10 ng of cDNA template in a final volume of 30

µl. The extension of PCR products was performed at 60°C. The relative mRNA levels of the target genes were normalized to the housekeeping gene *rnpB* (Vioque 1992). Relative quantification was performed according to the comparative Ct method (ΔΔCt Method) (Livak and Schmittgen 2001). The minimum fold-change threshold was set up to ± 1.5 fold.

Overexpression and purification of recombinant FurC (PerR)

FurC was overexpressed in *E.coli* BL21 (DE3) Gold cells as previously described (Hernandez et al. 2004). FurC purification method was adapted from the procedure used for the purification of *B. subtilis* apo-PerR-Zn (Traore et al. 2006) including the following modifications. Cells overexpressing FurC were harvested and resuspended in ice-cold 50 mM Tris–HCl 7.5, 10 mM EDTA supplemented with ¼ tablet of Complete™, EDTA-free protease Inhibitor cocktail (Roche) prior to 10 cycles of sonication. Cell lysate was centrifuged for 20 min at 20,000 x g. The resulting supernatant was filtered through 0.45 µm-pore-size (Millipore) and loaded onto a DEAE-cellulose column. Protein was eluted using a linear gradient of 0-0.5 M NaCl in 50 mM Tris–HCl pH 7.5, 10 mM EDTA. Fractions containing FurC were dialyzed in Tris 50 mM pH 7.5. For the experiments that required higher purity of FurC, fractions were subjected to a second purification step on a heparin column and eluted with a linear gradient of 0-0.5 M NaCl in Tris 50 mM pH 7.5. Finally, the protein was stored at -20ºC in 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 5% glycerol.

Electrophoresis and immunoblotting

Two biological replicates of *Anabaena* sp. PCC7120 and EB2770FurC strains were cultured and harvested at the exponential phase of growth (OD750nm = 0.7-0.9). In the case of D1 immunodetection, crude extracts were prepared by sonication followed by centrifugation at 12000 rpm in a microcentrifuge Eppendorf 5427R to remove cell debris. Total protein content was determined by using a bicinchoninic acid protein assay reagent (Pierce). Then, 30 µg of total protein belonging to the different strains, were loaded and separated onto a SDS/17% polyacrylamide gel. For immunoblotting, the proteins were transferred onto a PVDF membrane (0.45 mm pore size transfer membrane from Waters) as previously described (Towbing et al 1979). Chicken polyclonal antibodies against D1 protein (Agrisera) were used at 1:4000 dilution and finally rabbit anti-chicken

polyclonal antibodies conjugated to horseradish peroxidase were applied at 1:10000 dilution. The blot was visualized by using a Bio-Rad ChemidocTM imaging system.

For immunodetection of FurC, 80 µl of each culture of *Anabaena* sp. PCC7120 and EB2770FurC were boiled with 20 µl of sample buffer 5X (Tris-HCl 300 mM pH 6.8, glycerol 50%, SDS 5%, β-mercaptoethanol 10%, 0.01% bromophenol blue) for 5 min at 95ºC. Afterwards extracts were centrifuged for 30 min at maximum speed in a microcentrifuge Eppendorf 5427R. Then 20 µl of each supernatant were loaded onto a SDS/17% polyacrylamide gel and electrophoresis was conducted. Proteins were transferred onto a PVDF membrane, probed with rabbit polyclonal antibodies against FurC (1:1000 dilution) and visualized using goat anti-rabbit polyclonal antibodies conjugated to horseradish peroxidase (1:10000 dilution). The blot image was obtained with a Bio-Rad ChemidocTM imaging system.

Electrophoretic Mobility Shift Assays (EMSA)

Gene promoters used in the analyses consisted of a 150–350 bp DNA fragment upstream of ATG and were obtained by PCR, using the *Anabaena* sp. PCC 7120 genome as template. Primers used in PCR amplifications are included in Supplemental Table S1. EMSAs were performed as follows, purified FurC supplemented with 100 µM of $MnCl_2$ was mixed in a final volume of 20 µl with 50 ng of DNA promoters. Reactions were performed in a binding buffer containing 10 mM Bis Tris/HCl, pH 7.5, 40 mM KCl, 0.1 mg/ml BSA, 1 mM DTT (1,4-dithiothreitol) and 5% (v/v) glycerol. Specifity of FurC binding to the studied promoters was checked including an internal fragment of the *pkn22* gene (if*pkn22*) as a non-specific competitor DNA in all reactions, while binding of FurC to the promoter region of the *prxA* gene was used as a positive control. The resulting mixture was incubated for 30 min at room temperature and loaded into non-denaturing 6% polyacrylamide gels. Gels were stained with SYBR ® Safe (Invitrogen) and visualized in a GelDoc 2000 device (Bio-Rad).

Funding

This work was supported by grants E35_17R from Gobierno de Aragón, BFU2012-31458/FEDER and BFU2016-77671-P/FEDER from MINECO.

Disclosures

The authors have nothing to disclose

Acknowledgements

Authors thank Dr. Rafael Picorel for helpful discussions. We acknowledge the use of Servicio General de Apoyo a la Investigación-SAI, Universidad de Zaragoza.

References

Apel, K. and Hirt, H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55: 373-399.

Bečková, M., Yu, J., Krynicka, V., Kozlo, A., Shao, S., Konik, P., et al. (2017) Structure of Psb29/Thf1 and its association with the FtsH protease complex involved in photosystem II repair in cyanobacteria. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 372.

Buikema, W.J. and Haselkorn, R. (2001) Expression of the *Anabaena hetR* gene from a copper-regulated promoter leads to heterocyst differentiation under repressing conditions. *Proc. Natl. Acad. Sci. USA* 98: 2729-2734.

Cai, Y.P. and Wolk, C.P. (1990) Use of a conditionally lethal gene in *Anabaena* sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences. *J. Bacteriol.* 172: 3138-3145.

Cavet, J.S., Borrelly, G.P. and Robinson, N.J. (2003) Zn, Cu and Co in cyanobacteria: selective control of metal availability. *FEMS Microbiol. Rev.* 27: 165-181.

Corrales-Guerrero, L., Camargo, S., Valladares, A., Picossi, S., Luque, I., Ochoa de Alda, J.A.G., et al. (2018) FtsZ of Filamentous, Heterocyst-Forming Cyanobacteria Has a Conserved N-Terminal Peptide Required for Normal FtsZ Polymerization and Cell Division. *Front. Microbiol.* 9: 2260.

Davies, B.H. (1976) Carotenoids. In *Chemistry and biochemistry of plant pigments*. Edited by Goodwin, T.W. pp. 38-165. Academic Press, New York.

Elhai, J., Vepritskiy, A., Muro-Pastor, A.M., Flores, E. and Wolk, C.P. (1997) Reduction of conjugal transfer efficiency by three restriction activities of *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* 179: 1998-2005.

Genty, B., Briantais, J.M. and Baker, N.R. (1989) The relationship between the quantum yield of photosynthetic electron-transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta* 990: 87-92.

Glazer, A.N. (1976) Phycocyanins. In *Photochemical and photobiological reviews*. Edited by Smith, K.C. pp. 71-115. Plenum Press., New York.

Gonzalez, A., Bes, M.T., Barja, F., Peleato, M.L. and Fillat, M.F. (2010) Overexpression of FurA in *Anabaena* sp. PCC 7120 reveals new targets for this regulator involved in photosynthesis, iron uptake and cellular morphology. *Plant Cell Physiol*. 51: 1900-1914.

Gonzalez, A., Bes, M.T., Peleato, M.L. and Fillat, M.F. (2016) Expanding the Role of FurA as Essential Global Regulator in Cyanobacteria. *PloS One* 11: e0151384.

Hernandez, J.A., Lopez-Gomollon, S., Bes, M.T., Fillat, M.F. and Peleato, M.L. (2004) Three *fur* homologues from *Anabaena* sp. PCC7120: exploring reciprocal protein-promoter recognition. *FEMS Microbiol. Lett.* 236: 275-282.

Hernandez, J.A., Lopez-Gomollon, S., Muro-Pastor, A., Valladares, A., Bes, M.T., Peleato, M.L., et al. (2006) Interaction of FurA from *Anabaena* sp. PCC 7120 with DNA: a reducing environment and the presence of Mn^{2+} are positive effectors in the binding to *isiB* and *furA* promoters. *Biometals* 19: 259-268.

Hu, B., Yang, G., Zhao, W., Zhang, Y. and Zhao, J. (2007) MreB is important for cell shape but not for chromosome segregation of the filamentous cyanobacterium *Anabaena* sp. PCC 7120. *Mol. Microbiol.* 63: 1640-1652.

Kale, R., Hebert, A.E., Frankel, L.K., Sallans, L., Bricker, T.M. and Pospíšil P. (2017) Amino acid oxidation of the D1 and D2 proteins by oxygen radicals during photoinhibition of Photosystem II. *Proc. Natl. Acad. Sci. USA* 114:2988-2993.

Kato, Y. and Sakamoto, W. (2018) FtsH protease in the thylakoid membrane: Physiological functions and the regulation of protease activity. *Front. Plant Sci.* 9: 855.

Klint, J., Rasmussen, U. and Bergman, B. (2007) FtsZ may have dual roles in the filamentous cyanobacterium *Nostoc/Anabaena* sp. strain PCC 7120. *J. Plant Physiol.* 164: 11-18.

Kobayashi, M., Ishizuka, T., Katayama, M., Kanehisa, M., Bhattacharyya-Pakrasi, M., Pakrasi, H.B., et al. (2004) Response to oxidative stress involves a novel peroxiredoxin gene in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. *Plant Cell Physiol*. 45: 290-299.

Latifi, A., Ruiz, M. and Zhang, C.C. (2009) Oxidative stress in cyanobacteria. *FEMS Microbiol. Rev.* 33: 258-278.

Lee, J.W. and Helmann, J.D. (2006) The PerR transcription factor senses H_2O_2 by metal-catalysed histidine oxidation. *Nature* 440: 363-367.

Lee, M.H., Scherer, M., Rigali, S. and Golden, J.W. (2003) PlmA, a new member of the GntR family, has plasmid maintenance functions in *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* 185: 4315-4325.

Li, H., Singh, A.K., McIntyre, L.M. and Sherman, L.A. (2004) Differential gene expression in response to hydrogen peroxide and the putative PerR regulon of *Synechocystis* sp. strain PCC 6803. *J. Bacteriol.* 186: 3331-3345.

Liu, L.N. (2016) Distribution and dynamics of electron transport complexes in cyanobacterial thylakoid membranes. *Biochim. Biophys. Acta* 1857: 256-265.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Δ ΔC(T)) Method. *Methods* 25: 402-408.

Lopez-Gomollon, S., Hernandez, J.A., Pellicer, S., Angarica, V.E., Peleato, M.L. and Fillat, M.F. (2007) Cross-talk between iron and nitrogen regulatory networks in *Anabaena* (*Nostoc*) sp. PCC 7120: identification of overlapping genes in FurA and NtcA regulons. *J. Mol. Biol.* 374: 267-281.

Mackinney, G. (1941) Absorption of light by chlorophyll solutions. *J. Biol. Chem.* 140: 109-112.

Malnoe, A., Wang, F., Girard-Bascou, J., Wollman, F.-A. and de Vitry, C. (2014) Thylakoid FtsH protease contributes to photosystem II and cytochrome b6f remodeling in *Chlamydomonas reinhardtii* under stress conditions. *Plant Cell* 26: 373-390.

Mann, N.H., Novac, N., Mullineaux, C.W., Newman, J., Bailey, S. and Robinson, C. (2000) Involvement of an FtsH homologue in the assembly of functional photosystem I in the cyanobacterium *Synechocystis* sp. PCC 6803. *FEBS Lett.* 479: 72-77.

Misumi, M., Katoh, H., Tomo, T. and Sonoike, K. (2016) Relationship Between Photochemical Quenching and Non-Photochemical Quenching in Six Species of Cyanobacteria Reveals Species Difference in Redox State and Species Commonality in Energy Dissipation. *Plant Cell Physiol*. 57: 1510-1517.

Montgomery, B.L. (2015) Light-dependent governance of cell shape dimensions in cyanobacteria. *Front. Microbiol.* 6: 514.

Napolitano, M., Rubio, M.A., Santamaria-Gomez, J., Olmedo-Verd, E., Robinson, N.J. and Luque, I. (2012) Characterization of the response to zinc deficiency in the cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* 194: 2426-2436.

Olmedo-Verd, E., Flores, E., Herrero, A. and Muro-Pastor, A.M. (2005) HetR-dependent and -independent expression of heterocyst-related genes in an *Anabaena* strain overproducing the NtcA transcription factor. *J. Bacteriol.* 187: 1985-1991.

Osteryoung, K.W., Stokes, K.D., Rutherford, S.M., Percival, A.L. and Lee, W.Y. (1998) Chloroplast division in higher plants requires members of two functionally divergent gene families with homology to bacterial *ftsZ*. *Plant Cell* 10: 1991-2004.

Pascual, M.B., Mata-Cabana, A., Florencio, F.J., Lindahl, M. and Cejudo, F.J. (2011) A comparative analysis of the NADPH thioredoxin reductase C-2-Cys peroxiredoxin system from plants and cyanobacteria. *Plant Physiol.* 155: 1806-1816.

Rees, D.C., Akif Tezcan, F., Haynes, C.A., Walton, M.Y., Andrade, S., Einsle, O., et al. (2005) Structural basis of biological nitrogen fixation. *Philos. Trans. A. Math. Phys. Eng. Sci*. 363: 971-984.

Sein-Echaluce, V.C., Gonzalez, A., Napolitano, M., Luque, I., Barja, F., Peleato, M.L., et al. (2015) Zur (FurB) is a key factor in the control of the oxidative stress response in *Anabaena* sp. PCC 7120. *Environ. Microbiol.* 17: 2006-2017.

Sein-Echaluce, V.C., Pallares, M.C., Lostao, A., Yruela, I., Velazquez-Campoy, A., Luisa Peleato, M., et al. (2018) Molecular basis for the integration of environmental signals by FurB from *Anabaena* sp. PCC 7120. *Biochem. J.* 475: 151-168.

Shcolnick, S. and Keren, N. (2006) Metal homeostasis in cyanobacteria and chloroplasts. Balancing benefits and risks to the photosynthetic apparatus. *Plant Physiol.* 141: 805-810.

Silva, P., Thompson, E., Bailey, S., Kruse, O., Mullineaux, C.W., Robinson, C., et al. (2003) FtsH is involved in the early stages of repair of photosystem II in *Synechocystis* sp PCC 6803. *Plant Cell* 15: 2152-2164.

Singh, N.K., Sonani, R.R., Rastogi, R.P. and Madamwar, D. (2015) The phycobilisomes: an early requisite for efficient photosynthesis in cyanobacteria. *EXCLI J.* 14: 268-289.

Stein, J.R. (1973) Handbook of phycological methods: culture methods and growth measurements. p. 4 v. Cambridge University Press, New York.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350-4354.

Traore, D.A., El Ghazouani, A., Ilango, S., Dupuy, J., Jacquamet, L., Ferrer, J.L., et al. (2006) Crystal structure of the apo-PerR-Zn protein from *Bacillus subtilis*. *Mol. Microbiol.* 61: 1211-1219.

Vioque, A. (1992) Analysis of the gene encoding the RNA subunit of ribonuclease P from cyanobacteria. *Nucleic Acids Res.* 20: 6331-6337.

Wohlfahrt, G. and Gu, L. (2015) The many meanings of gross photosynthesis and their implication for photosynthesis research from leaf to globe. *Plant Cell Environ.* 38: 2500-2507.

Yamamoto, Y. (2001) Quality control of photosystem II. *Plant Cell Physiol.* 42: 121-128.

Yingping, F., Lemeille, S., Talla, E., Janicki, A., Denis, Y., Zhang, C.C., et al. (2014) Unravelling the cross-talk between iron starvation and oxidative stress responses highlights the key role of PerR (*alr0957*) in peroxide signalling in the cyanobacterium *Nostoc* PCC 7120. *Environ. Microbiol. Rep.* 6: 468-475.

Tables

Table 1. Physiological parameters of liquid cultures of *Anabaena* sp. strains PCC7120 and EB2770FurC.

	PCC7120	**EB2770FurC**
Growth rate (d^{-1})	5.5 ± 0.2	4.8 ± 0.5
Net photosynthesis	21.05 ± 3.08 [b]	7.5 ± 2.19 [b]
Dark O_2 respiration	0.97 ± 0.26 [b]	1.97 ± 0.15 [b]
µg Phycobiliproteins/cell	$4.87x10^{-5}$ ± $0.3x10^{-5}$	$2.15x10^{-5}$ ± $0.8x10^{-5}$
µg Chl a /cell	$6.5x10^{-7}$ ± $0.8x10^{-7}$	$3.61x10^{-7}$ ± $0.03x10^{-7}$
Phycobiliproteins / Chl a	74.92	59.55

[a] *Anabaena* strains were grown photoautotrophically in BG-11 medium to early-log phase.

[b] Values are means of three independent determinations ± SD, expressed as pmol O_2 million cells $^{-1}$.

Figure legends

Figure 1. Levels of transcription and translation of *furC* in the EB2770FurC strain compared to the wild type *Anabaena* sp. PCC7120. A. Real-time RT-PCR analyses showing the abundance of *furC* mRNA in the EB2770FurC strain relative to that observed in *Anabaena* sp. PCC7120. Values are expressed as fold change; standard deviation corresponding to three biological replicates is indicated. B. Detection of FurC protein in crude extracts of *Anabaena* sp. PCC7120 and EB2770FurC by Western blotting. Two biological replicates of *Anabaena* sp. PCC7120 called WT1 and WT2 as well as two biological replicates of EB2770FurC strain named EB1 and EB2 are shown. The location of FurC is indicated with an arrow.

Figure 2. Growth of the *fur*C-overexpressing strain EB2770FurC in comparison with *Anabaena* sp. PCC 7120, expressed as both optical density (A) and number of cells per ml (B). Values are the

averages of three independent experiments; SDs are represented by vertical bars. Please note that in some instances the error is smaller than the symbols used.

Figure 3. Overexpression of *furC* induced morphological and ultrastructural changes in *Anabaena* sp., as shown in photomicrographs of the wild-type strain *Anabaena* sp. PCC 7120 (A, C, E and G) and the *furC*-overexpressing strain EB2770FurC (B, D, F and H) taken at the exponential phase of growth. Scanning electron microscopy (images A, B, C and D) and transmission electron microscopy (E, F, G and H). Septum boundaries in transmission electron microscopy images G and H are indicated with arrows. The photomicrograph of each strain is representative of at least 10 different images from two biological replicates. Bars=1 μm.

Figure 4. Study of the regulation by FurC of *mre* actins and the cell division genes *ftsZ* and *ftsH*. (A) Electrophoretic mobility shift assays testing the ability of FurC to bind in vitro the promoter regions of *mreBCD*, *ftsZ* and *ftsH.* Binding of FurC to *prxA* promoter (left panel) was included as a positive control. DNA fragments free or mixed with the indicated concentrations of recombinant FurC (nM) were separated by 6 % PAGE. An internal fragment of the gene *pkn22* was used as non-specific competitor DNA. (B) Relative transcription of *ftsZ* and *ftsH* genes determined by real-time RT-PCR in EB2770FurC cells with respect to *Anabaena* sp. PCC7120. Values are expressed as fold change and correspond to the average of three independent assays; the standard deviation is indicated.

Figure 5. Detection of the D1 protein in crude extracts of *Anabaena* sp. PCC7120 and EB2770FurC by Western blotting. Results were generated with two biological replicates of *Anabaena* sp. PCC7120 called WT1 and WT2 as well as two biological replicates of the EB2770FurC strain named EB1 and EB2. Bands corresponding to different forms of the D1 protein observed in the membrane are indicated with arrows.

Figure 6. UV-visible spectra of whole *Anabaena* sp. PCC7120 (dark line) and EB2770FurC cells (dashed line) (A). Determination of Chl a (B), phycobiliproteins (C), total proteins (D) and carotenoid (E) contents in EB2770FurC cells (light grey bars) compared to *Anabaena* sp. PCC7120 (dark grey bars) at different stages of growth. The results were normalized to the same optical density at 750

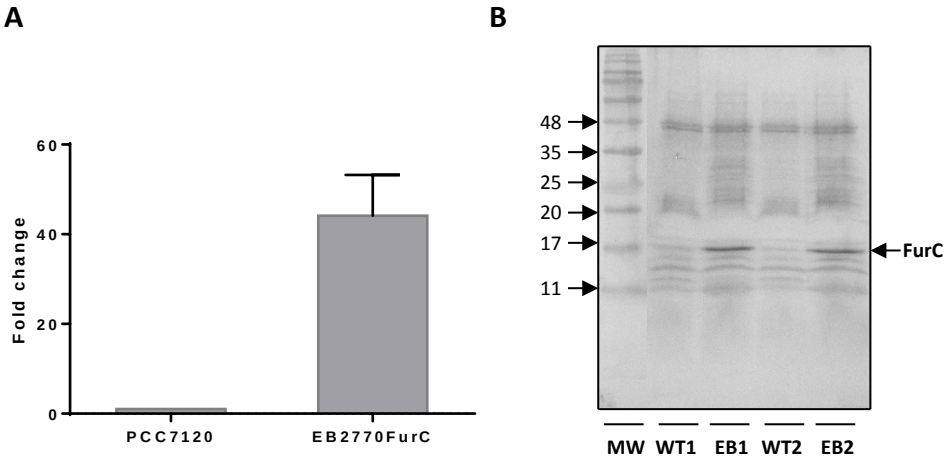
nm. (F) Relative transcription of the phycobilisome core genes *apcA*, *apcC* and *apcE* (allophycocyanin subunits A, C and E), *cpcB* (phycocyanin B) and the linker peptides *cpcG* and *pecC*. Values were determined by real-time RT-PCR in EB2770FurC cells with respect to *Anabaena* sp. PCC7120. Values are expressed as fold change and correspond to the average of three independent assays.

Figure 7. Fluorescence emission spectra at 77K of *Anabaena* sp. PCC7120 (squares) and EB2770FurC (triangles) strains harvested at the exponential phase of growth. (A) Emission spectra excited at 440 nm to monitor energy transfer from photosystems to Chl a. Spectra were normalized to identical concentration of Chl a in both strains. (B) Emission spectra excited at 580 nm to check energy transfer from phycobilisomes to Chl a. In this case, spectra were normalized to identical concentration of phycobiliproteins in both strains. The presented spectra are representative of two separate experiments.

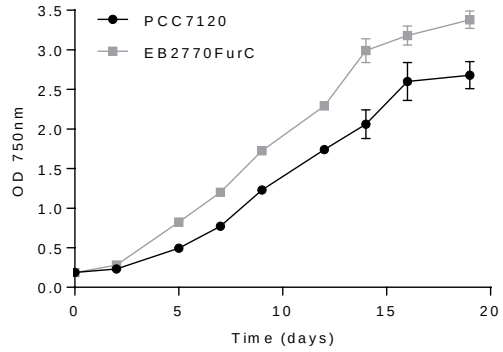
Figure 8. Fluorescence induction parameters of *Anabaena* sp. PCC7120 and the EB2770FurC derivative upon continuous cultivation on the Ca^{2+}-less BG-11 medium. During preparation of this medium $CaCl_2$ (0.24 mM) was replaced by 0.48 mM NaCl. The final concentration of Ca^{2+} was the same as the level of this ion in the double distilled water. Upon subculturing, cells in mid-exponential phase (OD_{730}=0.4-0.6) were diluted to OD_{730}=0.03 to start the successive culture. The number of each subculture is indicated in X axis. Fv/Fm ratio was determined according to (Genty et al., 1989) and plotted on Y axis. Values represent mean and standard deviation from 5 independent experiments.

Figure 9. Tolerance to oxidative stress induced by treatment with H_2O_2. *Anabaena* sp. strains were grown in BG-11 medium to late log phase, washed and adjusted to the same cell density. Cell suspensions were challenged in duplicate either to increasing concentrations of H_2O_2 up to 10 mM for 24h (A) and 48 h (C) in the dark and the estimation of chlorosis was documented by reading the microtiter plate absorbance at 620 nm at 24h (A) and 48h (B). Each experiment was done twice with similar results.

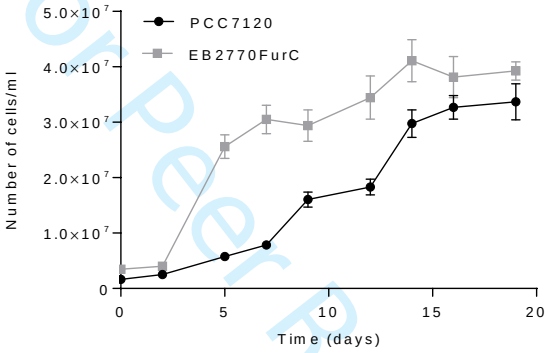
Figure 10. Study of the regulation by FurC of genes involved in the oxidative stress response. (A) Relative transcription (EB2770FurC versus *Anabaena* sp. PCC7120) of *Anabaena* peroxiredoxins, *srxA*, *sodA* and catalase *alr0998* genes and their transcriptional response to the oxidative challenge imposed by H_2O_2 measured by real-time RT-PCR. Values are expressed as fold change and correspond to the average of three independent assays; the standard deviation is indicated (B) Electrophoretic mobility shift assays showing the ability of FurC to bind in vitro the promoter regions of *prxA*, *srxA, ahpC, CGT3* and *alr4404*. DNA fragments free or mixed with the indicated concentrations of recombinant FurC (nM) were separated by 6 % PAGE. An internal fragment of the gene *pkn22* was used as non-specific competitor DNA.

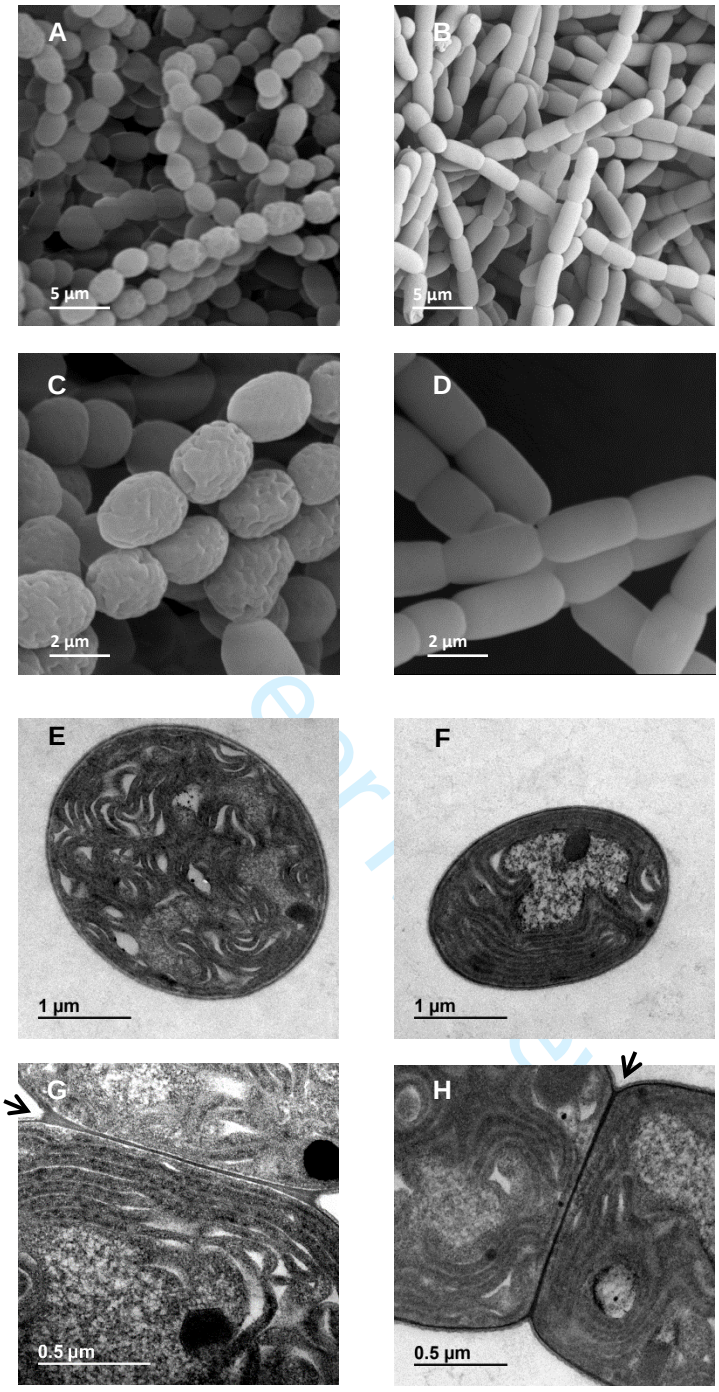


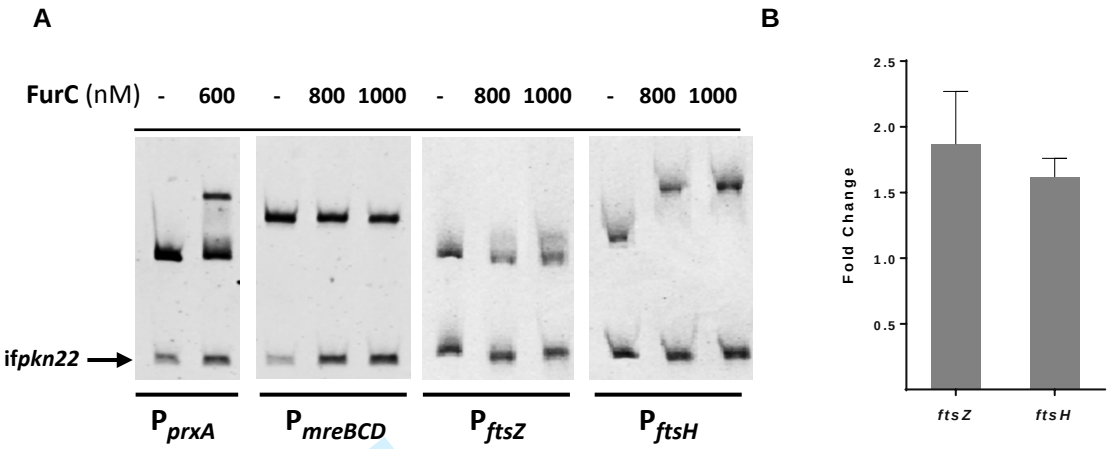
A

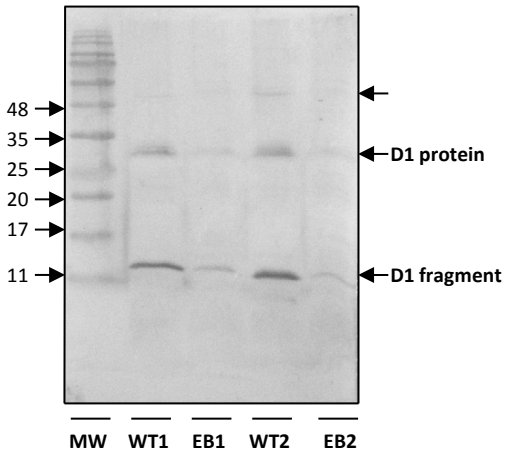


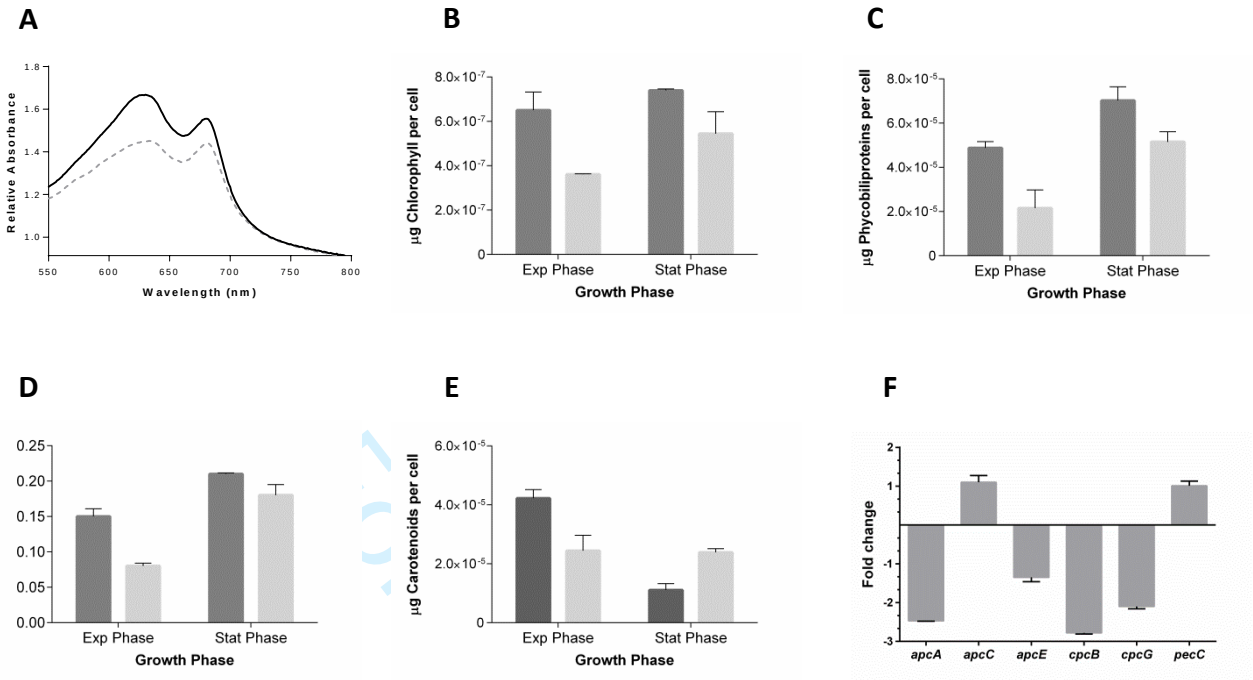
B



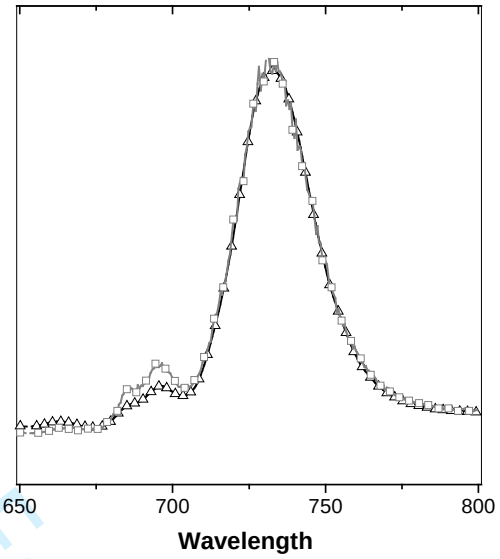

A
5 µm
B
5 µm
C
2 µm
D
2 µm
E
1 µm
F
1 µm
G
0.5 µm
H
0.5 µm



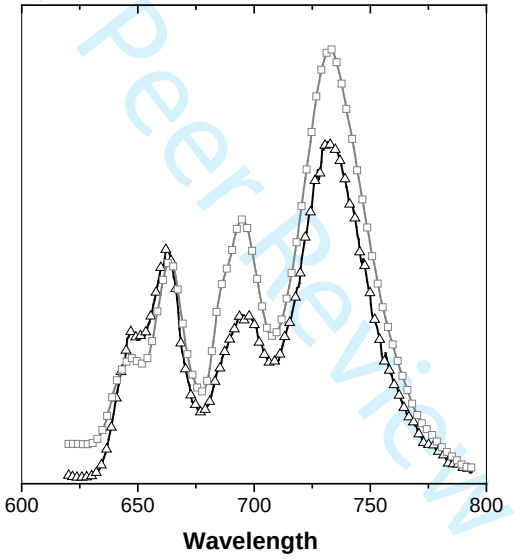


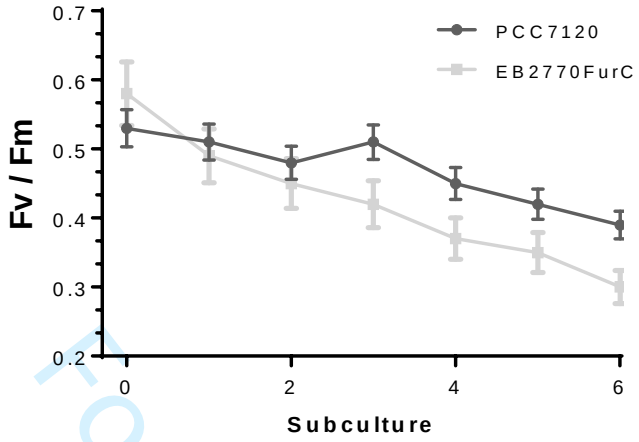


A

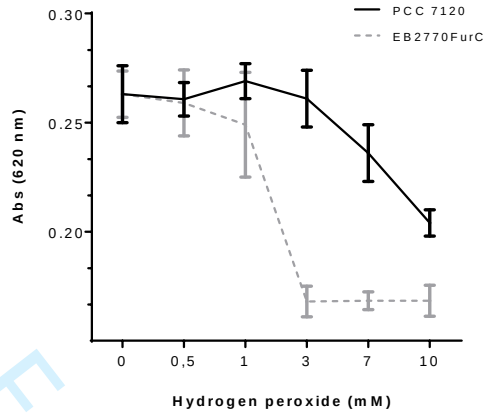


B

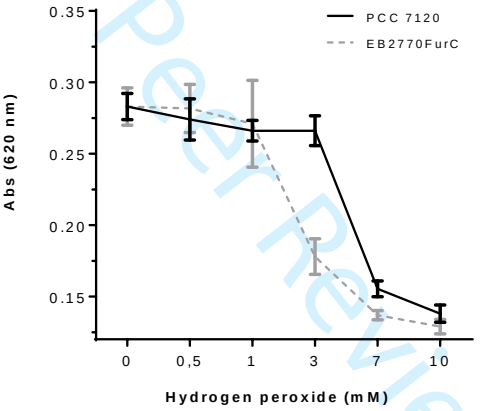




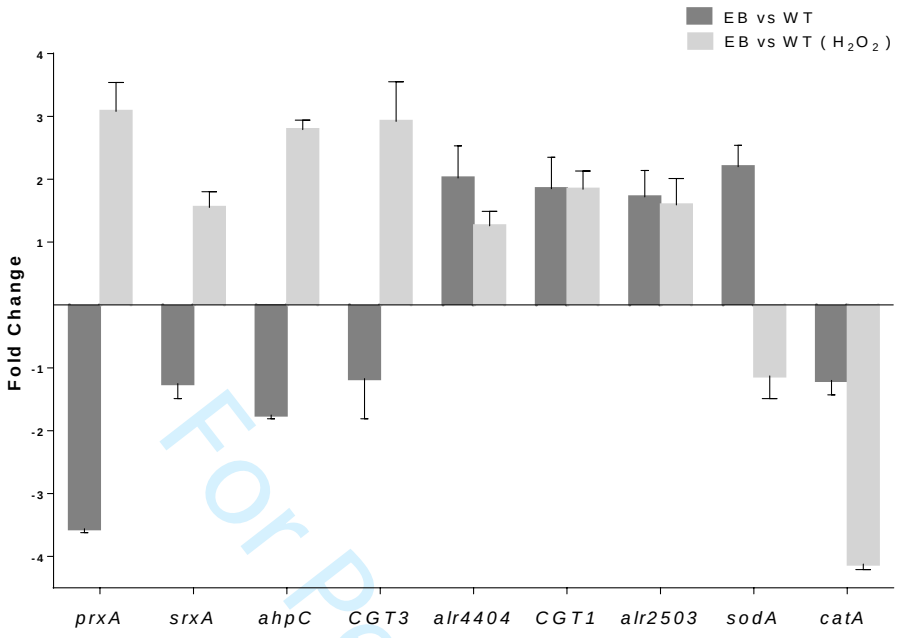
A



B



A



B