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Zur (FurB) is a key factor in the control of the oxidative stress response in *Anabaena* sp. PCC 7120.

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Running title: Role of Zur in the oxidative stress response in *Anabaena*

SUMMARY

Iron and zinc are necessary nutrients whose homeostasis is tightly controlled by members of the FUR superfamily in the cyanobacterium *Anabaena* sp. PCC7120. Although the link between iron metabolism and oxidative stress management is well documented, little is known about the connection between zinc homeostasis and the oxidative stress response in cyanobacteria. Zinc homeostasis in *Anabaena* is controlled by Zur, the FurB paralogue. When overexpressed in *Escherichia coli*, Zur (FurB) improved cell survival against oxidative stress. Zur also protected DNA against hydroxyl radical damage *in vitro*. In order to investigate the possible correlation between Zur and the oxidative stress response in *Anabaena*, *zur* deletion and *zur*-overexpressing strains have been constructed and the consequences of Zur imbalance evaluated. The lack of Zur increased sensitivity to H_2O_2, whereas an excess of Zur enhanced oxidative stress resistance. Both mutants displayed pleiotropic phenotypes, including alterations on the filament surfaces observable by scanning electron microscopy, reduced content of endogenous H_2O_2 and altered expression of *sodA*, catalases and several peroxiredoxins. Transcriptional and biochemical analyses unveiled that the appropriate level of Zur is required for proper control of the oxidative stress response and allowed us to identify major antioxidant enzymes as novel members of the Zur regulon.

INTRODUCTION

Reactive oxygen species (ROS) are unavoidable by-products of aerobic metabolism that can damage several cellular sites including iron-sulfur clusters, cysteine and methionine protein residues, lipids and DNA (Chiang and Schellhorn, 2012). The generation of toxic radicals is enhanced by a source of biometals including iron and zinc that, at the same time, are essential nutrients for the vast majority of organisms (Stohs and Bagchi, 1995). Iron is a constituent of a wide range of proteins involved in photosynthesis, respiration, nitrogen metabolism, defense against oxidative stress, DNA biosynthesis and gene regulation, among others (Cornelis and Andrews, 2010). In spite of being the fourth most abundant element in the Earth's crust, the high reactivity of ferric iron with oxygen to form insoluble oxides and hydroxides, makes this nutrient scarcely bioavailable (Andrews et al., 2003). Unlike iron, zinc is only a trace metal in the Earth's crust. Nevertheless, zinc is involved in a huge number of biological processes and its chemical properties make this metal a staple part of many proteins and enzymes, where it plays structural, catalytic or regulatory roles. Although under physiological conditions zinc is not a redox active metal, its capacity to bind and to protect free sulfhydryl groups in proteins as well as the involvement of zinc proteins in the biosynthesis of low molecular weight thiols links zinc homeostasis to the maintenance of the intracellular redox status (Ma et al., 2009; Eide, 2011). Furthermore, the occurrence of a variety of redox zinc switches coupled to thiol/disulphide exchanges enables a connection between redox status and zinc metabolism (Maret, 2006). While iron toxicity lies in the ability of this metal to effectively catalyze the Fenton reaction, zinc toxicity has been attributed to its propensity to interact adventitiously with thiol groups of many proteins, especially those involved in electron transport (Mills et al., 2002). Consequently, metal concentrations inside the cells must be finely tuned not only for adjusting metal uptake

to cell requirements, but also for keeping redox homeostasis in order to minimize oxidative stress. In prokaryotes, this control is carried out by different families of metalloregulators, which act in a coordinated way regulating metal homeostasis and preventing cell oxidative damage (Ma et al., 2009). One of the most important families of metalloregulators is constituted by the FUR (Ferric Uptake Regulator) proteins that can be divided into different functional classes according to the signal they sense: Fur (iron sensing proteins), Zur (zinc), Mur (manganese) and Nur (nickel). The PerR and Irr subfamilies, which also belong to the FUR family, sense oxidative stress and haem levels, respectively (Lee and Helmann, 2007).

Because of their photosynthetic metabolism, iron requirements of cyanobacteria are much larger than those of heterotrophic microorganisms (Shcolnick et al., 2009). On the other hand, the generation of reactive oxygen species by fortuitous electron transfer to oxygen during the photosynthetic process enhances the risk of damage to iron-sulfur clusters compromising cyanobacterial metabolism. Accordingly, metal homeostasis mechanisms and the oxidative stress defenses are tightly coordinated in cyanobacteria, where FUR proteins play major role.

Anabaena sp. PCC 7120 is a nitrogen-fixing cyanobacterium that in the absence of combined nitrogen is able to differentiate heterocysts distributed semi-regularly in the filament (Flores and Herrero, 2010). The genome of *Anabaena* sp. PCC 7120 encodes three FUR proteins, previously named as FurA, FurB and FurC (Hernandez, 2004). FurA is the master regulator of iron homeostasis and couples iron status with both nitrogen metabolism and the oxidative stress response (Lopez-Gomollon et al., 2007b; Lopez-Gomollon et al., 2007a; Gonzalez et al., 2010; González et al., 2011; González et al., 2012). FurC has been identified as a PerR protein in *Anabaena* sp. PCC 7120 (Yingping et al., 2014), while FurB controls zinc homeostasis acting as a Zur protein

(Napolitano et al., 2012). Zur (FurB) binds to DNA in a zinc-dependent manner and represses transcription of target genes under zinc-sufficient conditions. Zur controls a regulon which includes genes encoding putative metallochaperones (e.g. All4722, All1751), paralogues of zinc metalloproteins (e.g. All4725/HemE, All4723/ThrS), components of plasma membrane ABC transport systems (e.g. ZnuABC), and several outer membrane proteins (e.g. Alr3242, Alr4028) (Napolitano et al., 2012). Similar targets have been found or predicted to be regulated by Zur in other cyanobacteria (Barnett et al., 2012) as well as in non-photosynthetic bacteria, such as *Escherichia coli* (Patzer, 2000), *Staphylococcus aureus* (Lindsay and Foster, 2001), *Bacillus subtilis* (Fuangthong and Helmann, 2003) and *Mycobacterium tuberculosis* (Maciag et al., 2006), among others.

Besides its role as a metal regulator, Zur from *Anabaena* sp. has been shown to enhance cell survival under oxidative stress conditions when it is overexpressed in *E. coli*. In addition, *in vitro* assays have shown the ability of this protein to unspecifically bind DNA, protecting it against both, oxidative damage and DN*ase*I digestion (López-Gomollón et al., 2009). Hence, a dual role for *Anabaena* sp. Zur has been previously suggested depending on the protein concentration into the cell. At low concentrations, Zur works as a transcriptional regulator binding to the promoters of target genes in a specific manner. At higher concentrations of the protein, maybe induced by oxidative stress, Zur would bind unspecifically to DNA, protecting it from oxidative damage (López-Gomollón et al., 2009).

Correlation between oxidative stress and iron starvation has been well established in cyanobacteria (Latifi et al., 2005; Shcolnick et al., 2009). However, the elements linking zinc homeostasis to oxidative stress management by the cell remain to be identified. In this study, the molecular bases of the connection between Zur and the oxidative stress

response have been investigated. Our analyses of the phenotypes of two *Anabaena* sp. derivative strains, a *zur* deletion mutant and a *zur*-overexpressing strain, show that changes in *zur* expression levels deeply affect cyanobacterial phenotype, including alterations in septum morphology and the organization of the outmost cell layers, among other features. Transcriptional and biochemical assays led to the identification of key genes involved in the oxidative stress response as novel members of the Zur regulon. Those results establish a direct connection between the control of zinc metabolism and the regulation of the antioxidant defenses in *Anabaena* sp. PCC 7120.

RESULTS

Δzur and zur overexpressing mutants of Anabaena *sp. PCC 7120 exhibit a pleiotropic phenotype*

To achieve a better understanding of the alternative functions of Zur in *Anabaena* sp., a *zur* overexpressing strain (VCS2770) was generated and its phenotype evaluated in comparison with those from a *zur* deletion mutant (Δ*zur*) and the parental wild type *Anabaena* sp. PCC 7120. Photoautotrophic growth under standard culture conditions of Δ*zur* was slower than that observed in the wild type strain (doubling time 10 days versus 8.4 days). Conversely, the *zur*-overexpressing strain VCS2770 doubled in only 7.8 days. (Figure 1). Despite the *zur*-overespressing strain exhibited higher chlorophyll content than Δ*zur* and the wild type control, photosynthetic and respiratory activities were similar in the three *Anabaena* strains. (Table 2).

Cyanobacterial morphology of exponentially growing cultures was visualized using different microscopy techniques. Bright-field and fluorescence microscopy analyses showed that there were not noticeable differences in filament length and intrinsic

fluorescence between the wild type and the derivative strains. Under absence of combined nitrogen ($BG11_0$), cultures of Δ*zur* and VCS2770 strains displayed similar heterocyst development patterns than those observed in *Anabaena* sp. PCC 7120 (data not shown). However, scanning electron microscopy (SEM) analyses showed that Δ*zur* cells displayed a different shape and appeared to be connected by narrower septa compared to *Anabaena* sp. PCC 7120 and VCS2770 (Figure 2A). A severe disruption of Δ*zur* filaments after treatment for transmission electron microscopy (TEM) studies in comparison to *Anabaena* wild type and VCS2770 strains was also observed (not shown). Those features, as well as the release of phycobiliproteins of the photosynthetic antenna in Δ*zur* when cultures were left to decant overnight without bubbling (Fig. 2B) could be indicative of a more fragile junction between cells. On the other hand, the cell surface of the VCS2770 strain was visibly affected (Figure 2A), suggesting that not only the slime sheath enclosing filaments might be influenced by *zur* overexpression, but also the organisation of the outer membrane.

Oxidative stress tolerance in* Anabaena *sp. is strongly influenced by Zur expression levels

Prior to the identification of FurB as a Zur regulator in *Anabaena* sp., it was found that overexpression of FurB/Zur in *E. coli* increased its tolerance to ROS (López-Gomollón et al., 2009). In the present study, we sought to investigate whether the expression levels of Zur could influence tolerance to oxidative stress imposed by exogenous hydrogen peroxide (H_2O_2) in *Anabaena* cells. As shown in Figure 3, the *Anabaena* sp. strain lacking Zur was much more sensitive to oxidative challenge than its parental strain. In contrast, Zur overexpression increased cyanobacterial tolerance to hydrogen peroxide as it was observed when this protein was overproduced in *E. coli*.

Δzur and VCS2770 derivative strains exhibit altered superoxide dismutase (SOD) and catalase activities, as well as diminished H_2O_2 contents

To gain more insights about the mechanism underlying tolerance to H_2O_2, SOD and catalase activities, as well as the endogenous content of H_2O_2 were measured in the three cyanobacterial strains. As shown in Fig. 4, the Δ*zur* strain showed increased SOD and catalase activities (about 120% and 156%, respectively) compared to *Anabaena* sp. PCC 7120. However, catalase activity appeared diminished in the *zur*-overexpressing strain VCS2770 compared to the *Anabaena* sp. wild type (70%), while SOD activities were similar in both strains. Surprisingly, the amount of endogenous H_2O_2 dropped dramatically in both, Δ*zur* and VCS2770 *Anabaena* strains, whose values were less than 10% of this from the parental *Anabaena* sp. control (Figure 5). Those results suggest that the reduction in the amount of endogenous H_2O_2 in Δ*zur* and VCS2770 strains takes place through different pathways.

Changes in Zur levels affects the Anabaena sp. oxidative stress response machinery

These results prompted us to investigate a potential connection of Zur with the transcription of main genes related to oxidative stress tolerance. Genes under study were the two Mn-catalases (*alr0998* and *alr3090*) encoded by *Anabaena* sp. PCC 7120, superoxide dismutases and peroxiredoxins *prxA*, *gct1* and *gct3*. Since FurB/Zur was previously described as a DNA protecting protein (López-Gomollón et al., 2009), transcription of several genes coding for DNA-binding proteins related to the oxidative stress response, namely *dpsA*, *hanA* and *all4145* (probable DNA-binding stress protein) was also analysed. Finally, because of the implication of *furA* and *furC* (*perR*) in cyanobacterial redox homeostasis, the influence of *zur* expression in these paralogs was investigated. Changes in mRNA levels were determined by semi-quantitative reverse

transcription-PCR (RT-PCR), as described previously (González et al., 2012). To obtain accurate data, determinations for each gene were performed at the early exponential phase of the PCR. The *rnpB* housekeeping gene was included in all RT-PCR analyses to ensure that equivalent amounts of total RNA were being used in all reactions. As shown in Figure 6 and table 3, transcription of *furA* was up-regulated in the absence of Zur, while *furA* expression appeared down-regulated in the VCS2770 strain. Since the final expression of *furA* in *Anabaena* is modulated by an antisense RNA (Hernandez et al., 2006), Western analyses were performed to verify that the amount of FurA in *Anabaena* showed an inverse correlation to *zur* expression levels (data not shown).

Transcription of *sodA* and catalase *alr0998* was strongly enhanced in Δ*zur*. A different pattern was observed with catalase *alr3090*, whose transcription was higher in the wild type and the VCS2770 strains. The expression of peroxiredoxin *gct3* was also significantly higher in Δ*zur*, whereas *gct1* expression was affected in a similar way in the two *Anabaena* mutants, suggesting that other proteins, in addition to Zur, are involved in its regulation. Finally, *prxA* appeared downregulated in a *zur*-overexpressing background. Regarding transcription of the DNA-binding proteins tested, only a slight change was observed in the *dpsA* levels, while no significant differences were appreciated in the transcription of *hanA* and *all4145* among the three cyanobacterial strains.

Zur regulates key genes involved in the oxidative stress response in* Anabaena *sp.

In order to discern which of the genes whose transcription levels appeared strongly influenced by Zur were direct targets of this regulator, EMSA analyses were performed in the presence of the unspecific control DNA P_{nifJ}. The *all4725* promoter, where Zur has been found to bind with high affinity, was used as control (Napolitano et al., 2012).

The results shown in Fig. 7 indicate that Zur recognises Mn catalase *alr0998, sodA* and *prxA* promoters. Binding of Zur to catalase *alr3090* and peroxiredoxin *gct3* promoters was much fainter. It is also noteworthy that binding of Zur to those promoters did not yield gel defined DNA-protein complexes as in the case of the binding to *all4725* promoter, indicating a lower affinity of Zur for those oxidative stress related gene promoters.

DISCUSSION

In addition to controlling zinc homeostasis, zinc-responsive factors have also been shown to regulate the expression of genes than can be critical for an organism to survive, such as those involved in the oxidative stress defense (Choi and Bird, 2014). Despite the increasing evidences relating alterations in zinc metabolism to increased levels of oxidative stress (Bonet et al., 2012; Graham et al., 2012; Choi and Bird, 2014; Eijkelkamp et al., 2014), the potential participation of Zur in this process remains to be uncharacterized. Beyond the control of zinc homeostasis, Zur from *Anabaena* sp. PCC 7120 has been proposed to protect *E. coli* cells by direct interaction with DNA, similarly to Dps proteins. In this work, the potential implication of Zur in the oxidative stress response in *Anabaena* sp. PCC 7120 has been investigated. Analyses of the phenotypes of Δ*zur* and *zur*-overexpressing (VCS2770) strains in combination with transcriptional and EMSA assays led us to identify some of the molecular basis of the protective effect of Zur against oxidative stress in *Anabaena* sp. PCC 7120. The lack of Zur delayed cyanobacterial growth under standard culture conditions. The impairing in photoautotrophic growth of the Δ*zur* strain in BG-11 medium supplemented with 25 μM zinc sulphate has been previously observed (Napolitano et al., 2012). Our results showed that the growth of this strain is diminished even in BG-11 medium (0.77 μM

zinc). In the absence of Zur, de-regulation of the machinery involved in the control of zinc uptake likely leads to the increase of intracellular free zinc. This metal can interact with thiol groups of proteins, blocking the binding of other metals and thus impairing essential reactions, especially in electron transport systems (Mills et al., 2002). Conversely, doubling time of the VCS2770 strain was similar to that of the wild type. The increased expression of Zur, around 5 times of the value found in *Anabaena* sp. PCC 7120, might not be enough for a complete repression of target genes. Alternatively, an over-repression of the Zur regulon could led to the use of unspecific or low affinity transporters (Panina et al., 2003; Gabriel and Helmann, 2009; Sankaran et al., 2009). The morphological differences observed in the filaments of those strains with respect to *Anabaena* sp. PCC 7120, namely alteration of cell septum and cell surface in Δ*zur* and VCS2770 respectively, could be caused in part by the misregulation of Zur targets encoding outer-membrane proteins (e. g. the TonB-dependent receptors Alr3242 and Alr4028), among other unidentified causes. The *mreBCD* operon, which encodes for the bacterial actin MreB and the cell wall synthetic proteins MreC and MreD, plays a critical role in the determination of cell morphology in several species of bacteria (Singh and Montgomery, 2011). Since the interaction between TonB-dependent receptors (TBDRs) and MreC has been previously reported in *Caulobacter crescentus* (Divakaruni et al., 2005), misregulation of TBDRs could lead in an indirect way to the alteration of cell morphology. Curiously, the *mreB*CD operon is a direct target of FurA in *Anabaena* sp. PCC 7120 and alterations in FurA levels also induced changes in the *Anabaena* sp. cell morphology (Gonzalez et al., 2010).

Photosynthetic oxygen evolution and respiration measurements gave similar values in the three strains though VCS2770 displayed higher chlorophyll *a* content. Probably, the "extra" chlorophyll present in this strain could be mainly bound to peripheral antenna

proteins. Those data, together with the transcription patterns of *sod* genes and the lower expression of *furA* in VCS2770 suggest that overexpression of Zur might enhance iron uptake in *Anabaena*.

Anabaena sp. PCC 7120 contains a wide range of enzymes directly involved in the oxidative stress response including two superoxide dismutases, two catalases, several peroxiredoxins and Dps proteins, among others (Latifi et al., 2009; Banerjee et al., 2013). Our experiments revealed interesting differences in the global catalase activity of strains with different levels of expression of Zur, as well as an altered pattern of expression of catalases Alr0998 (Banerjee et al., 2012) and Alr3090/KatB (Bihani et al., 2013). Global catalase activity was significantly higher in the Δ*zur* strain, in consonance with the strong transcriptional induction of *alr0998* in this mutant. Therefore, the increased expression of catalase *alr0998* and the increase in global catalase activity in the Δ*zur* strain could explain its low concentration of intracellular H_2O_2. It is remarkable that, in spite of having decreased catalase activity, the VCS2770 strain exhibited even a lower content of intracellular hydrogen peroxide and the highest tolerance against exogenous H_2O_2. Those results suggest that this strain was subjected to lower oxidative stress and, hence, the requirement for catalase was lower. Similar results were reported when FurA was overexpressed in *Anabaena* sp. PCC 7120. A raise in FurA expression down-regulated antioxidant activities in cyanobacterium but did not lead to an oxidative stress situation (Gonzalez et al., 2010). In addition to these two catalases, the genome of *Anabaena* sp. PCC 7120 encodes two peroxidases, namely Alr1585 and Alr0672, which could account for detoxification of H_2O_2 in VCS2770, though other alternative pathways related to the excess of Zur cannot be discarded. These results are in good agreement with the hypothesis that Zur acts as a protective protein by itself when present at high concentrations (López-Gomollón et al., 2009).

Whether Zur works as a Dps protein in *Anabaena* or alleviates oxidative stress taking part in an alternative electron transport chain is an interesting issue that deserves to be investigated.

Semi-quantitative RT-PCR analyses of other major genes involved in the concerted response against oxidative stress led us to identify novel putative targets for Zur, namely *sodA*, peroxiredoxins *prxA*, *gct1* and *gct3*, and the stress-induced DNA-binding protein *dpsA* (*alr3808*). The analysis of transcriptional patterns and EMSA assays allowed us to discriminate different groups of genes according to the effect of Zur on their control. Zur seems to play a key role in the direct transcriptional repression of *sodA*, catalase *alr0998* and peroxiredoxin *gct3.* While the de-repression of *alr0998* in Δ*zur* is in good concordance with the increase of catalase activity, the strong induction of the *sodA* transcript in Δ*zur* contrasts with the moderate differences between the values of superoxide dismutase activity observed in the three strains. Manganese is a required cofactor for SodA activity. As it has been observed in *Streptococcus pneumoniae* (Eijkelkamp et al., 2014), the excess of zinc likely present in Δ*zur* could compromise manganese uptake in *Anabaena.* Consequently, Mn deficit would impair the assembly of the total induced apoprotein to yield fully active holo-SodA. Consistently with EMSA results, the promoter regions of this group of genes contain multiple AT-rich regions that partially match with the Zur consensus sequence described previously (Napolitano et al., 2012). A different set of genes is composed by *prxA* and *dpsA* that are mildly repressed by an excess of Zur (VCS2770 strain) that binds to their promoters. However, their transcription levels in Δ*zur* were similar to those in the wild type *Anabaena* sp. strain, indicating that *prxA* and *dpsA* are modulated by additional regulatory factors, including other FUR paralogs (Hernandez et al., 2007; Yingping et al., 2014). Co-regulation of Zur with other regulatory proteins has also been

reported in heterotrophic bacteria (Kallifidas et al., 2009). Finally, RT-PCR analyses suggest that Zur modulates catalase *alr3090* indirectly, since its binding to this promoter is very poor.

Regulation of FurA from *Anabaena* sp. is a rather complex process that is controlled at every single step in the flow of genetic information (Botello-Morte et al., 2013). The expression pattern of *furA* in both mutant strains confirms that Zur participates directly in this regulation. Binding assays to the *furA* promoter carried out in this work and in previous studies (Hernandez, 2004) support that hypothesis. The *furA* promoter also contains two AT-rich regions partially matching with the consensus sequence described for Zur. However, these boxes are distant from the transcription start sites (-52 bp and -115 bp) and *furA* expression is not fully repressed in the VCS2770 mutant, suggesting that Zur might be just a mild repressor of *furA*. An analogous expression pattern is observed in a *furA* overexpressing mutant, where the expression of Zur is severely decreased (Gonzalez et al., 2010). Those data support our previous results suggesting an inter-regulation between those two members of the Fur family in *Anabaena* sp. PCC 7120 (Hernandez, 2004).

Previous reports demonstrate that FurA also participates in the control of metal homeostasis and the oxidative stress response. FurA has been shown to directly regulate peroxiredoxins Alr4641/PrxA and All1541, and the DpsA protein (Hernandez et al., 2007; González et al., 2011). Regulation by Fur of genes involved in redox homeostasis has been also found in heterotrophic bacteria (Hassan and Sun, 1992; Hasset et al., 1997; Lee et al., 2004; Li et al., 2009). Our *in vivo* and *in vitro* assays unveil that Zur plays a key role in the control of the oxidative stress response in *Anabaena* sp. PCC 7120. The identification of novel Zur-regulated genes in this cyanobacterium sheds some light on the elements linking zinc homeostasis with oxidative stress management

in *Anabaena* sp. PCC 7120, showing an interesting overlap between the FurA and Zur regulons. In summary, those results evidence that Zur is a multifunctional regulatory protein that connects zinc metabolism to oxidative stress management in *Anabaena* sp. PCC 7120.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions

Bacterial strains used in this study are described in Table S1. *Anabaena* sp. PCC 7120, the *zur*-overexpressing derivative mutant VCS2770 and the deletion-insertion mutant Δ*zur* were grown photoautotrophically in BG-11 medium (Rippka et al., 1978) at 28°C under a constant illumination of 50 $\mu E\ m^{-2}\ s^{-1}$. Culture medium was supplemented with neomycin 50 μg ml^{-1} for strain VCS2770 and with streptomycin and spectinomycin 2-5 μg ml^{-1} for strain Δ*zur*. Cultures were performed using Erlenmeyer flasks at a constant shaking of 120 r.p.m.

Escherichia coli strains used for cloning procedures were grown at 37°C in Luria-Bertani medium, supplemented with the appropiated antibiotics at the following concentrations: kanamycin 50 μg ml^{-1} , ampicillin 50 μg ml^{-1} , chloramphenicol 30 μg ml^{-1}, streptomycin 25 μg ml^{-1} and spectinomycin 100 μg ml^{-1}.

Construction of the Anabaena sp. PCC 7120 derivative strains

The *zur* deletion-insertion strain was described elsewhere (Napolitano et al., 2012). The *zur*-overexpressing strain was constructed as follows. Chromosomal DNA was extracted from *Anabaena* sp. PCC 7120 (Cai and Wolk, 1990) and used as a template to amplify the *zur* gene, using primers 2770FurB_up and 2770FurB_dw (Table S2). These primers contained the restriction sites for BamHI and NdeI enzymes, so that the PCR product was double digested and cloned into those restriction sites in the pAM2770

shuttle vector (Lee et al., 2003). The resulting plasmid, pAM2770::*zur*, contained the *zur* gene downstream the *petE* promoter, which is inducible by copper (Buikema and Haselkorn, 2001). The construction was sequenced to verify that the cloning procedure was successful. Plasmid pAM2770::*zur* was transferred to *Anabaena* sp. PCC 7120 by triparental mating (Elhai et al., 1997). Three exconjugant clones were cultured in BG-11 medium to an optical density of 0.5 at 750 nm and then collected to test the overexpression of Zur by Western blot (Figure S1). As previously described, the amount of copper in BG-11 medium was enough to activate the *petE* promoter (Gonzalez et al., 2010). Therefore, no additional copper was added to enhance the overexpression of *zur*. Finally, the exconjugant clone with the highest expression of *zur* was selected as the *zur*-overexpressing strain, named VCS2770.

Cyanobacterial growth and pigment measurements

In order to analyze cyanobacterial growth, all strains were cultured in Erlenmeyer flasks and the optical density was measured at 750 nm every 2-3 days during 40 days. Growth parameters such as the specific growth rate and doubling time were calculated as previously described (Stein, 1973). Measurements were carried out using a Cary 100 Bio UV-visible spectrophotometer (Varian).

Quantification of photosynthetic pigments and total protein content was performed in cultures at the exponential phase of growth. Chlorophyll *a* (Nicolaisen et al., 2008), phycobiliproteins (Glazer, 1976) and carotenoids (Davies, 1976) were quantified as previously described. Total protein content was determined by using the bicinchoninic acid protein assay (Pierce). Pigment and protein content were expressed as micrograms per microliter of packed cell volume (PCV), where the PCV was determined by centrifuging 5 milliliters of each culture for 5 minutes at 2,000xg in a special graduated tube.

Photosynthetic and respiratory activities

Photosynthetic and respiratory activities were measured in exponentially growing cells with a Clark type electrode (Oxylab model by Hansatech) at 21°C and a constant shaking of 65%. Photosynthetic activity was determined by measuring the oxygen rate at light saturating conditions (400 µE m^{-2} s^{-1}), while the respiratory activity was determined at dark conditions, covering the electrode chamber with an aluminum paper. Data were processed with the provided Hansatech software and the results were expressed as nmol $O_2 \cdot ml^{-1} \cdot min^{-1} \cdot$mg Chl$a^{-1}$.

Purification of Zur and Western blot analyses

To obtain the recombinant Zur protein, the *zur* gene was amplified using all2473N-2 and all2473C primers described in Table S2 and cloned between the NdeI and HindIII sites of plasmid pET 28a(+). The resulting His-tagged protein was purified using a zinc affinity column (Matrix Chelating Sepharose™ Fast Flow, Amersham) and conserved in a 100 mM NaH_2PO_4, 300 mM NaCl pH 6 solution. For Western blot analysis, cyanobacterial liquid cultures were collected by centrifugation at 4°C and cells were resuspended in cold phosphate buffer 50 mM pH 8. The suspension was sonicated five times during 45 seconds with cooling intervals of 30 seconds and then centrifuged to remove cell debris. Protein concentration in crude extracts was determined by using the bicinchoninic acid method (BCA™ Protein Assay Kit, Thermo Fischer Scientific). For each sample, 10-30 micrograms of total proteins were loaded and separated by electrophoresis with 17% SDS-PAGE gels. Proteins were transferred to a PVDF membrane (0.45 µm pore size Immobilon® transfer membrane from Millipore) and immunodetection was carried out using rabbit polyclonal antibodies raised against Zur.

Catalase and superoxide dismutase activities determinations

Fifty milliliters of each cyanobacterial culture were collected by centrifugation and cells were resuspended in one milliliter of phosphate buffer 50 mM pH 8. The cell suspension was sonicated and then centrifuged to remove cell debris. Protein concentration in the extract was quantified by using the BCA method and antioxidant activities were immediately determined. Catalase activity was determined as decribed previously (Beers and Sizer, 1952), following the hydrogen peroxide dissociation by measuring the optical density at 240 nm. Breafly, 300-600 micrograms of protein extract were rapidly mixed in a quartz cuvette with hydrogen peroxide to a final concentration of 20 mM. The reaction was followed spectrophotometrically at 240 nm with a Cary 100 Bio (Varian) device during five minutes. Catalase activity was expressed as Units per milligram of total proteins, defining a Unit as the amount of enzyme that catalyzes the dissociation of 1 microgram of hydrogen peroxide per minute. Superoxide dismutase (SOD) activity was determined by a modification of the method by Winterbourn (Winterbourn et al., 1975), which is based on the ability of SOD to inhibit the reduction of nitro-blue tetrazolium (NBT) by superoxides. Reaction mixtures contained 600 micrograms of protein extract, 6.4 mM EDTA, 41 µM NBT, 2.3 µM riboflavin and 23.5 µM TEMED. The control of the assay, with a maximum NBT reduction, contained phosphate buffer instead of the protein extract. Optical density at 560 nm was measured before and after illuminating the mixtures for 10 minutes with UV light. Superoxide dismutase activity was expressed as Units per milligram of total proteins, defining a Unit as the amount of enzyme that inhibited the maximum reduction in a 50%.

Endogenous hydrogen peroxide measurement

Intracellular hydrogen peroxide was determined in the cyanobacterial strains using the ferrithiocyanate method (Thurman et al., 1972). Fifty milliliters of each culture were

collected to obtain 50-100 milligrams of fresh cells. Trichloroacetic acid (TCA) at a final concentration of 5% was added to the cells and the mixture was centrifuged to remove cell debris. 800 microliters of the supernatant were mixed in a plastic cuvette with 160 µl of $Fe(NH_4)_2(SO_4)_2$ 10 mM (1.3 mM final concentration), 80 µl of KSCN 2.5 M (167 mM final concentration) and 160 µl of TCA 50%. The absorbance at 480 nm was measured using a Cary 100 Bio spectrophotometer (Varian) to determine the hydrogen peroxide content.

Hydrogen peroxide tolerance assay

To test the tolerance of the cyanobacterial strains to hydrogen peroxide, filaments were exposed to increasing concentrations of hydrogen peroxide for 24 hours. Cultures with an approximate optical density of 1.0 at 750 nm were washed once with fresh BG-11 medium and 200 µl of culture were displayed into each well of a microtiter plate. Hydrogen peroxide was added to the wells at a final concentration of 0, 0.5, 0.7, 1 and 1.3 mM. The plate was incubated for 24 hours in dark conditions and 28°C. Chlorosis was estimated by reading the absorbance at 620 nm with a Multiskan EX microplate photometer (Thermo Fischer Scientific).

Microscopy

Bright-field and fluorescence microscopy analysis of exponentially growing cells were carried out using a Nikon Eclipse 50i Epi-fluorescence microscope coupled to a Nikon DXM 1200F camera. For scanning electron microscopy, cells were harvested at the exponential phase of growth and fixed with 2.5% glutaraldehyde in phosphate buffer (66 mM $NaH_2PO_4.2H_2O$, 66 mM KH_2PO_4, pH 7) for 1 h at room temperature, washed three times for 5 min each in phosphate buffer, fixed with 2% OsO_4 and washed three

times for 5 min each in distilled water. Scanning electron microscopy was performed in a SEM JEOL JSM 7001FA. Pictures were processed using the Photoshop 6.0 program.

Semi-quantitative reverse transcription (RT-PCR)

Total RNA was isolated from exponentially growing cultures as previously described (Olmedo-Verd et al., 2005) and residual DNA was removed by treating the samples with RNAse-free DNAseI (Roche). The successful removal of genomic DNA was tested by PCR. RNA was heated at 85°C for 10 minutes and reverse transcription was carried out using the SuperScript Reverse Transcriptase kit (Invitrogen) and following the manufacturer's conditions. The *rnpB* gene was used as an internal control to normalize the amounts of cDNA in the PCR reactions. The results of the PCRs were visualized in 1-1.5% agarose gels stained with ethidium bromide in a GelDoc 2000 device (Bio-Rad).

Electrophoretic Mobility Shift Assays (EMSAs)

Gene promoters were obtained by PCR, using the *Anabaena* sp. PCC 7120 genome as a template using the primers described in Table S2. To ensure the specific binding of the protein to the studied promoters, the promoter of *nifJ* (*alr1911*) gene was used as a competitor DNA in all reactions. Reaction mixtures with a final volume of 20 µl contained 50 ng of each promoter, binding buffer (10 mM bis-Tris pH7.5, 40 mM KCl, 2 mM $MgCl_2 \cdot 6H_2O$, 5% glycerol), 0.05 mg/ml BSA, 1 mM DTT, 5µM $ZnSO_4 \cdot H_2O$ and 100-300 nM recombinant Zur. Resulting mixtures were incubated for 30 minutes 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).

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REFERENCES

Andrews, S.C., Robinson, A.K., and Rodríguez-Quiñones, F. (2003) Bacterial iron homeostasis. *FEMS Microbiology Reviews* **27**: 215-237.

Banerjee, M., Ballal, A., and Apte, S.K. (2012) Mn-catalase (Alr0998) protects the photosynthetic, nitrogen-fixing cyanobacterium *Anabaena* PCC7120 from oxidative stress. *Environmental Microbiology* **14**: 2891-2900.

Banerjee, M., Raghavan, P.S., Ballal, A., Rajaram, H., and Apte, S.K. (2013) Oxidative stress management in the filamentous, heterocystous, diazotrophic cyanobacterium, *Anabaena* PCC7120. *Photosynthesis Research* **118**: 59-70.

Barnett, J.P., Millard, A., Ksibe, A.Z., Scanlan, D.J., Schmid, R., and Blindauer, C.A. (2012) Mining genomes of marine cyanobacteria for elements of zinc homeostasis. *Front Microbiol* **3**: 142.

Beers, R.F., and Sizer, I.W. (1952) A spectrophotmetric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry* **195**: 133-140.

Bihani, S.C., Chakravarty, D., and Ballal, A. (2013) Purification, crystallization and preliminary crystallographic analysis of KatB, a manganese catalase from *Anabaena* PCC 7120. *Acta Crystallographica Section F Structural Biology and Crystallization Communications* **69**: 1299-1302.

Bonet, B., Corcoll, N., and Guasch, H. (2012) Antioxidant enzyme activities as biomarkers of Zn pollution in fluvial biofilms. *Ecotoxicol Environ Saf* **80**: 172-178.

Botello-Morte, L., Gonzalez, A., Bes, M.T., Peleato, M.L., and Fillat, M.F. (2013) Functional Genomics of Metalloregulators in Cyanobacteria. *Genomics of Cyanobacteria* **65**: 107-156.

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. *Proceedings of the National Academy of Sciences* **98**: 2729-2734.

Cai, Y., and Wolk, P. (1990) Use of a Conditionally Lethal Gene in *Anabaena* sp. strain PCC 7120 To Select for Double Recombinants and To Entrap Insertion Sequences. *Journal of Bacteriology* **172**: 3138-3145.

Cornelis, P., Andrews, S. C., eds. (2010) Iron Uptake and Homeostasis in Microorganisms. *Caister Academic Press, Norfolk, UK*.

Chiang, S.M., and Schellhorn, H.E. (2012) Regulators of oxidative stress response genes in *Escherichia coli* and their functional conservation in bacteria. *Arch Biochem Biophys* **525**: 161-169.

Choi, S., and Bird, A.J. (2014) Zinc'ing sensibly: controlling zinc homeostasis at the transcriptional level. *Metallomics* **6**: 1198-1215.

Davies, B.H. (1976) Carotenoids. In *Chemistry and Biochemistry of Plant Pigments*. Goodwin, T.W. (ed). New York: Academic Press, pp. 38-165.

Divakaruni, A.V., Loo, R.R.O., Xie, Y., Loo, J.A., and Gober, J.W. (2005) The cell-shape protein MreC interacts with extracytoplasmic proteins including cell wall assembly complexes in *Caulobacter crescentus*. *Proceedings of the National Academy of Sciences* **102**: 18602-18607.

Eide, D.J. (2011) The oxidative stress of zinc deficiency. *Metallomics* **3**: 1124-1129.

Eijkelkamp, B.A., Morey, J.R., Ween, M.P., Ong, C.L., McEwan, A.G., Paton, J.C., and McDevitt, C.A. (2014) Extracellular zinc competitively inhibits manganese uptake

and compromises oxidative stress management in *Streptococcus pneumoniae*. *PLoS One* **9**: e89427.

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

Flores, E., and Herrero, A. (2010) Compartmentalized function through cell differentiation in filamentous cyanobacteria. *Nat Rev Microbiol* **8**: 39-50.

Fuangthong, M., and Helmann, J.D. (2003) Recognition of DNA by Three Ferric Uptake Regulator (Fur) Homologs in *Bacillus subtilis*. *Journal of Bacteriology* **185**: 6348-6357.

Gabriel, S.E., and Helmann, J.D. (2009) Contributions of Zur-controlled ribosomal proteins to growth under zinc starvation conditions. *J Bacteriol* **191**: 6116-6122.

Glazer, A.N. (1976) Phycocyanins. In *Photochemical and Photobiological Reviews*. Smith, K.C. (ed). New York: Plenum Press, pp. 71-115.

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 and Cell Physiology* **51**: 1900-1914.

González, A., Bes, M.T., Peleato, M.L., and Fillat, M.F. (2011) Unravelling the regulatory function of FurA in *Anabaena* sp. PCC 7120 through 2-D DIGE proteomic analysis. *Journal of Proteomics* **74**: 660-671.

González, A., Bes, M.T., Valladares, A., Peleato, M.L., and Fillat, M.F. (2012) FurA is the master regulator of iron homeostasis and modulates the expression of tetrapyrrole biosynthesis genes in *Anabaena* sp. PCC 7120. *Environmental Microbiology* **14**: 3175-3187.

585 Graham, A.I., Sanguinetti, G., Bramall, N., McLeod, C.W., and Poole, R.K. (2012)
586 Dynamics of a starvation-to-surfeit shift: a transcriptomic and modelling analysis of
587 the bacterial response to zinc reveals transient behaviour of the Fur and SoxS
588 regulators. *Microbiology* **158**: 284-292.
589 Hassan, H.M., and Sun, H.-C.H. (1992) Regulatory roles of Fnr, Fur and Arc in
590 expression of manganese-containing superoxide dismutase in *Escherichia coli*.
591 *Proceedings of the National Academy of Sciences* **89**: 3217-3221.
592 Hasset, D.J., Howell, M.L., Ochsner, U.A., Vasil, M.L., Johnson, Z., and Dean, G.E.
593 (1997) An Operon Containing *fumC* and *sodA* Encoding Fumarase C and Manganese
594 Superoxide Dismutase Is Controlled by the Ferric Uptake Regulator in *Pseudomonas*
595 *aeruginosa*: *fur* Mutants Produce Elevated Alginate Levels. *Journal of Bacteriology*:
596 1452-1459.
597 Hernandez, J. (2004) Three *fur* homologues from *Anabaena* sp. PCC7120: exploring
598 reciprocal protein-promoter recognition. *FEMS Microbiology Letters* **236**: 275-282.
599 Hernandez, J.A., Pellicer, S., Huang, L., Peleato, M.L., and Fillat, M.F. (2007) FurA
600 modulates gene expression of *alr3808*, a DpsA homologue in *Nostoc* (*Anabaena*) sp.
601 PCC7120. *FEBS Lett* **581**: 1351-1356.
602 Hernandez, J.A., Muro-Pastor, A.M., Flores, E., Bes, M.T., Peleato, M.L., and Fillat,
603 M.F. (2006) Identification of a *furA* cis antisense RNA in the cyanobacterium
604 *Anabaena* sp. PCC 7120. *J Mol Biol* **355**: 325-334.
605 Kallifidas, D., Pascoe, B., Owen, G.A., Strain-Damerell, C.M., Hong, H.J., and Paget,
606 M.S.B. (2009) The Zinc-Responsive Regulator Zur Controls Expression of the
607 Coelibactin Gene Cluster in *Streptomyces coelicolor*. *Journal of Bacteriology* **192**:
608 608-611.

609 Latifi, A., Ruiz, M., and Zhang, C.C. (2009) Oxidative stress in cyanobacteria. *FEMS*
610 *Microbiol Rev* **33**: 258-278.
611 Latifi, A., Jeanjean, R., Lemeille, S., Havaux, M., and Zhang, C.C. (2005) Iron
612 Starvation Leads to Oxidative Stress in *Anabaena* sp. Strain PCC 7120. *Journal of*
613 *Bacteriology* **187**: 6596-6598.
614 Lee, H.W., Choe, Y.H., Kim, D.K., Jung, S.Y., and Lee, N.G. (2004) Proteomic
615 analysis of a ferric uptake regulator mutant of *Helicobacter pylori*: Regulation of
616 *Helicobacter pylori* gene expression by ferric uptake regulator and iron. *Proteomics* **4**:
617 2014-2027.
618 Lee, J.-W., and Helmann, J.D. (2007) Functional specialization within the Fur family of
619 metalloregulators. *BioMetals* **20**: 485-499.
620 Lee, M.H., Scherer, M., Rigali, S., and Golden, J.W. (2003) PlmA, a New Member of
621 the GntR Family, Has Plasmid Maintenance Functions in *Anabaena* sp. Strain PCC
622 7120. *Journal of Bacteriology* **185**: 4315-4325.
623 Li, Y., Qiu, Y., Gao, H., Guo, Z., Han, Y., Song, Y. et al. (2009) Characterization of
624 Zur-dependent genes and direct Zur targets in *Yersinia pestis*. *BMC Microbiology* **9**:
625 128.
626 Lindsay, J.A., and Foster, S.J. (2001) zur: a Zn2+-responsive regulatory element of
627 *Staphylococcus aureus*. *Microbiology* **147**: 1259-1266.
628 Lopez-Gomollon, S., Hernandez, J.A., Wolk, C.P., Peleato, M.L., and Fillat, M.F.
629 (2007a) Expression of *furA* is modulated by NtcA and strongly enhanced in
630 heterocysts of *Anabaena* sp. PCC 7120. *Microbiology* **153**: 42-50.
631 Lopez-Gomollon, S., Hernandez, J.A., Pellicer, S., Angarica, V.E., Peleato, M.L., and
632 Fillat, M.F. (2007b) 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.

López-Gomollón, S., Sevilla, E., Bes, M.T., Peleato, M.L., and Fillat, María F. (2009) New insights into the role of Fur proteins: FurB (All2473) from *Anabaena* protects DNA and increases cell survival under oxidative stress. *Biochemical Journal* **418**: 201.

Ma, Z., Jacobsen, F.E., and Giedroc, D.P. (2009) Coordination chemistry of bacterial metal transport and sensing. *Chem Rev* **109**: 4644-4681.

Maciag, A., Dainese, E., Rodriguez, G.M., Milano, A., Provvedi, R., Pasca, M.R. et al. (2006) Global Analysis of the *Mycobacterium tuberculosis* Zur (FurB) Regulon. *Journal of Bacteriology* **189**: 730-740.

Maret, W. (2006) Zinc coordination environments in proteins as redox sensors and signal transducers. *Antioxid Redox Signal* **8**: 1419-1441.

Mills, D.A., Schmidt, B., Hiser, C., Westley, E., and Ferguson-Miller, S. (2002) Membrane Potential-controlled Inhibition of Cytochrome c Oxidase By Zinc. *The Journal of Biological Chemistry* **277**: 14894-14901.

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. *Journal of Bacteriology* **194**: 2426-2436.

Nicolaisen, K., Moslavac, S., Samborski, A., Valdebenito, M., Hantke, K., Maldener, I. et al. (2008) Alr0397 Is an Outer Membrane Transporter for the Siderophore Schizokinen in *Anabaena* sp. Strain PCC 7120. *Journal of Bacteriology* **190**: 7500-7507.

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. *Journal of Bacteriology* **187**: 1985-1991.

Panina, E.M., Mironov, A.A., and Gelfand, M.S. (2003) Comparative genomics of bacterial zinc regulons: enhanced ion transport, pathogenesis, and rearrangement of ribosomal proteins. *Proc Natl Acad Sci U S A* **100**: 9912-9917.

Patzer, S.I. (2000) The Zinc-responsive Regulator Zur and Its Control of the *znu* Gene Cluster Encoding the ZnuABC Zinc Uptake System in *Escherichia coli*. *Journal of Biological Chemistry* **275**: 24321-24332.

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., and Stanier, R.Y. (1978) Generic assignments, strain histories and propierties of pure cultures of cyanobacteria. *Journal of General Microbiology* **111**: 1-61.

Sankaran, B., Bonnett, S.A., Shah, K., Gabriel, S., Reddy, R., Schimmel, P. et al. (2009) Zinc-independent folate biosynthesis: genetic, biochemical, and structural investigations reveal new metal dependence for GTP cyclohydrolase IB. *J Bacteriol* **191**: 6936-6949.

Shcolnick, S., Summerfield, T.C., Reytman, L., Sherman, L.A., and Keren, N. (2009) The mechanism of iron homeostasis in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 and its relationship to oxidative stress. *Plant Physiol* **150**: 2045-2056.

Singh, S.P., and Montgomery, B.L. (2011) Determining cell shape: adaptive regulation of cyanobacterial cellular differentiation and morphology. *Trends in Microbiology* **19**: 278-285.

Stein, J.R. (1973) *Handbook of Phycological Methods: Culture Methods and Growth Measurements*. New York: Cambridge University Press.

682 Stohs, S.J., and Bagchi, D. (1995) Oxidative mechanisms in the toxicity of metal ions.
683 *Free Radic Biol Med* **18**: 321-336.
684 Thurman, R.G., Ley, H.G., and Scholz, R. (1972) Hepatic microsomal ethanol
685 oxidation. Hydrogen peroxide formation and the role of catalase. *Eur J Biochem* **25**:
686 420-430.
687 Winterbourn, C.C., Hawkins, R.E., Brian, M., and Carrell, R.W. (1975) Estimation of
688 Red-Cell Superoxide-Dismutase Activity. *Journal of Laboratory and Clinical*
689 *Medicine* **85**: 337-341.
690 Yingping, F., Lemeille, S., Talla, E., Janicki, A., Denis, Y., Zhang, C.-C., and Latifi, A.
691 (2014) Unravelling the cross-talk between iron starvation and oxidative stress
692 responses highlights the key role of PerR (*alr0957*) in peroxide signalling in the
693 cyanobacterium *Nostoc* PCC 7120. *Environmental Microbiology Reports*:
694 DOI: 10.1111/1758-2229.12157.
695
696
697

FIGURE LEGENDS

Figure 1. Photoautotrophic growth of the cyanobacterial strains used in this work. Cyanobacterial growth in BG11 at standard culture conditions was analyzed by measuring optical density at 750 nm. Every measure was performed three times and the standard deviation, SD, is represented by the vertical bars.

Figure 2. Analyses of cell surface and culture sedimentation. A) Scanning electron microscopy photographs from exponentially growing cultures. The scale is indicated in the horizontal bar. B) Sedimentation of the strains at 24 hours. The exponentially growing strains were cultured with no shaking and a constant illumination of 50 $\mu E/m^2 \cdot s$. Photographs were taken after 24 hours.

Figure 3. Oxidative stress tolerance against hydrogen peroxide. A) Photograph of the microtiter plate containing the cyanobacterial strains with increasing hydrogen peroxide concentrations. B) Estimation of the chlorosis by reading the OD of the microtiter plate at 620 nm. The SD is represented by the vertical bars.

Figure 4. Superoxide dismutase and catalase activities in crude extracts of the cyanobacterial strains. A) Superoxide dismutase activity. B) Catalase activity. Activities are expressed as Units per milligram of total proteins in the extract. Every measure was performed three times and the SD is represented by the vertical bars.

Figure 5. Hydrogen peroxide content in crude extracts of the cyanobacterial strains. The results are expressed as nmol H_2O_2 per milligram of fresh weight. Every measure was performed three times and the SD is represented by the vertical bars.

724 **Figure 6. Semi-quantitative RT-PCR analyses.** Total RNA was isolated from
725 exponentially growing cultures. The *rnpB* gene was used as housekeeping to normalize
726 the amount of cDNA in each sample. Please note that the images of the gels were
727 coloured inverted in order to increase the sensitivity of detection.

728
729 **Figure 7. Electrophoretic Mobility Shift Assays with recombinant Zur protein.** *nifJ*
730 promoter was used as a competitor in all the assays and is marked with an arrow on the
731 left. Lane 1 in all gels contained free promoters. Lanes 2-4 contained the promoters with
732 Zur at final concentrations of 100, 200 and 300 nM respectively. The first gel of each
733 row contains the controls with the *all4725* promoter and the *nifJ* one. The absence (-) or
734 presence of 250 nM Zur (+) in the controls is indicated above the lanes. Optimal
735 conditions (5 µM $ZnSO_4$ and 1 mM DTT) were used in all the assays. Note that the
736 images of the gels were coloured inverted in order to increase the sensitivity of
737 detection.

738

739 ## TABLES

740
741 **Table 1. Percentage of photosynthetic pigments in the cyanobacterial strains.**
742 Pigments were measured at the exponential phase of growth. The results are the average
743 of three determinations ± SD and are expressed as the percentage related to total soluble
744 protein content.

745
746 **Table 2. Photosynthetic and respiratory activities.** Activities were measured at the
747 exponential phase of growth. The results are the average of two determinations ± SD
748 and are expressed as µmol $O_2 \cdot min^{-1} \cdot mg\ Chl a^{-1}$.

749

Table 3. Relative induction ratio of selected genes in relation to the wild type strain. Data are derived from the results of the semi quantitative RT-PCR analyses. Intensity of the DNA bands in the agarose gels was determined with ImageJ software. Values are means of two independent determinations ± SD.

SUPPLEMENTARY MATERIAL

Figure S1. Verification of *zur* deletion and overexpression by Western blot. A) Verification of *zur* deletion. Lanes contain 30 µg of *Anabaena* sp. PCC 7120 and *Anabaena* Δ*zur* protein extracts respectively. B) Verification of *zur* overexpression. Lanes contain 6.5 µg of protein extracts from *Anabaena* sp. PCC 7120, *zur*-overexpressing strain (VCS2770) and *Anabaena* Δ*zur* respectively. Molecular weight of protein marker bands is expressed in kDa.

Table S1. Bacterial strains used in this study.

Table S2. Oligonucleotides used in this study.

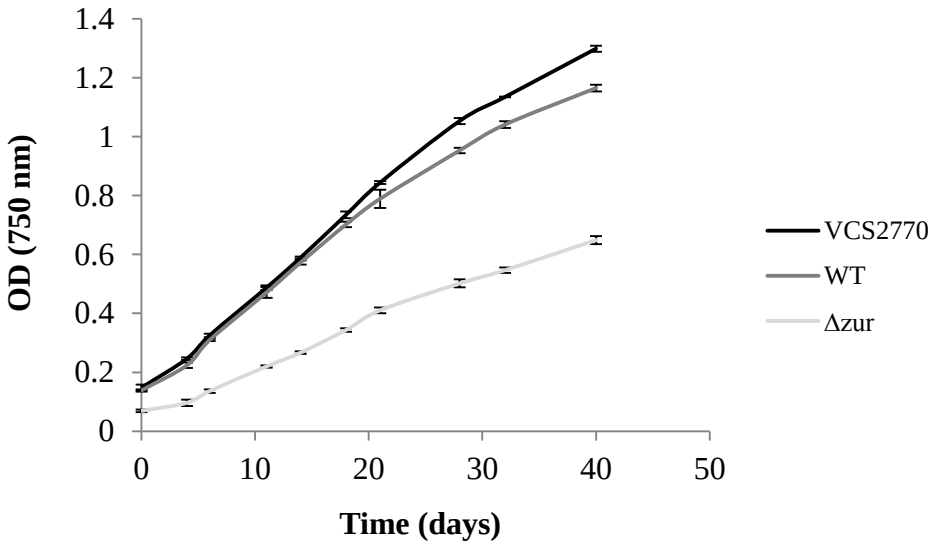
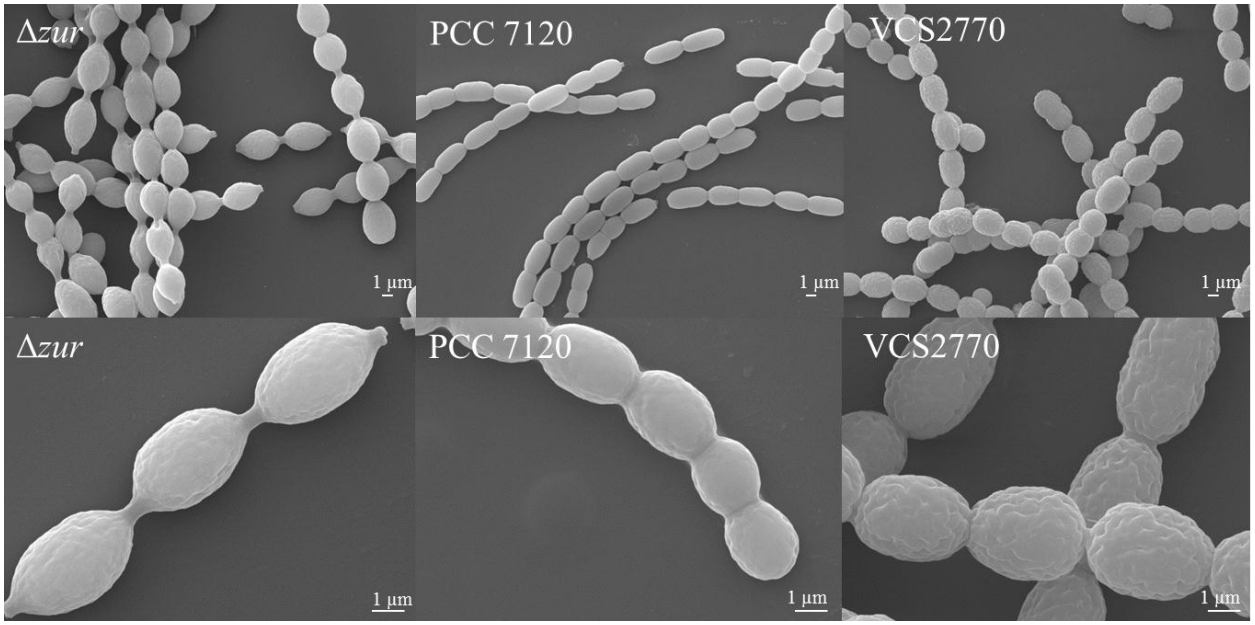

1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD (750 nm)
0
10
20
30
40
50
Time (days)
VCS2770
WT
Δzur

Figure 2

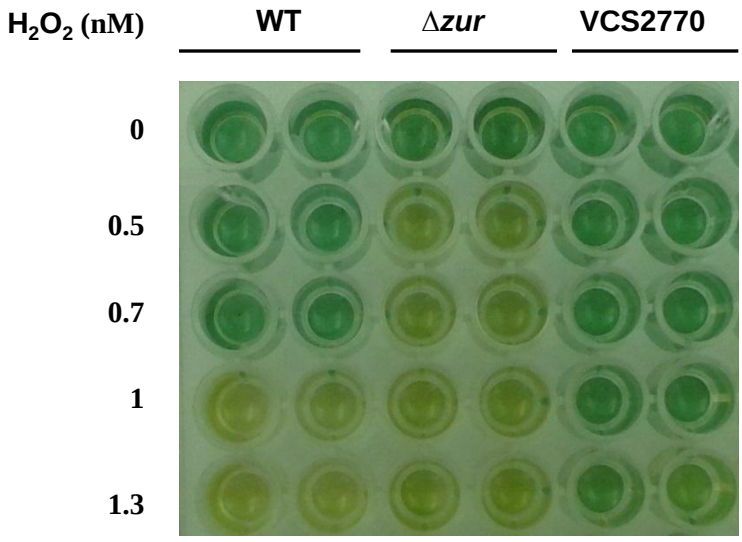
A


Δzur
PCC 7120
VCS2770
1 µm
1 µm
1 µm
Δzur
PCC 7120
VCS2770
1 µm
1 µm
1 µm

B


WT
VCS2770
Δzur

A


H2O2 (nM)
WT
Δzur
VCS2770
0
0.5
0.7
1
1.3

B

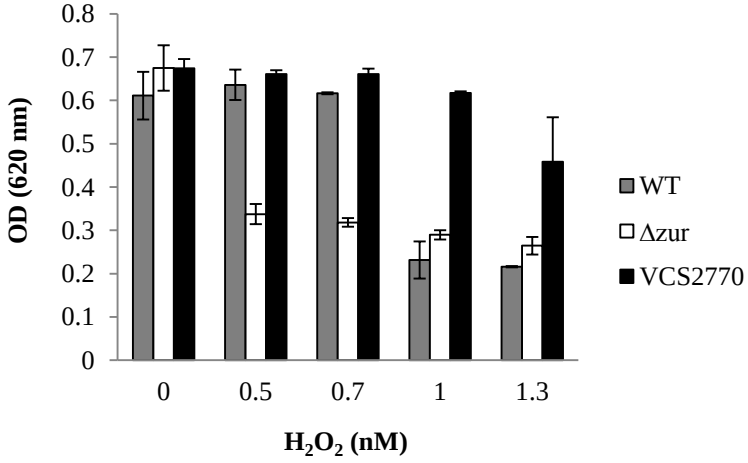
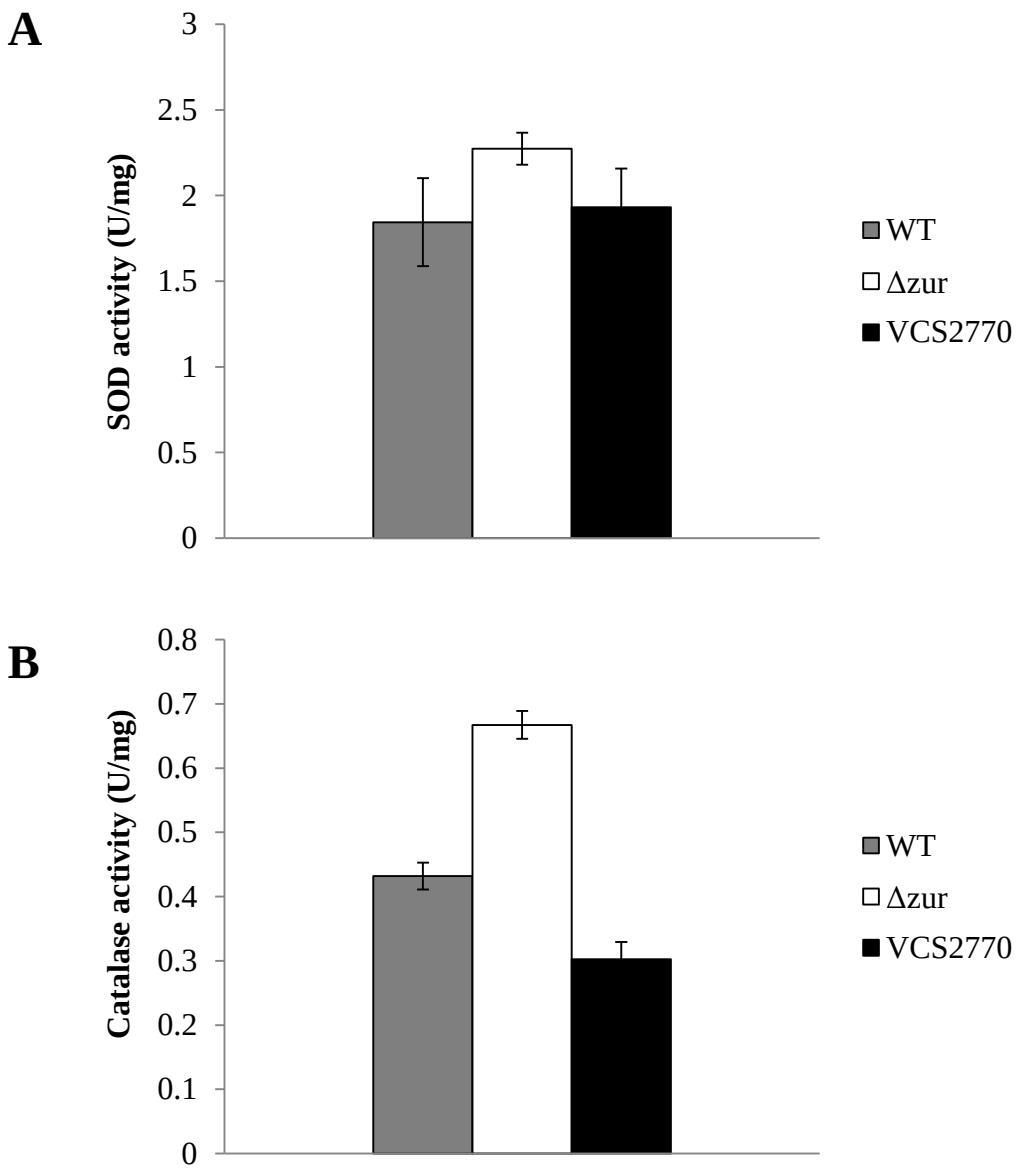

OD (620 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.5
0.7
1
1.3
H2O2 (nM)
WT
Δzur
VCS2770

Figure 4


A
SOD activity (U/mg)
3
2.5
2
1.5
1
0.5
0
WT
Δzur
VCS2770
B
Catalase activity (U/mg)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
Δzur
VCS2770

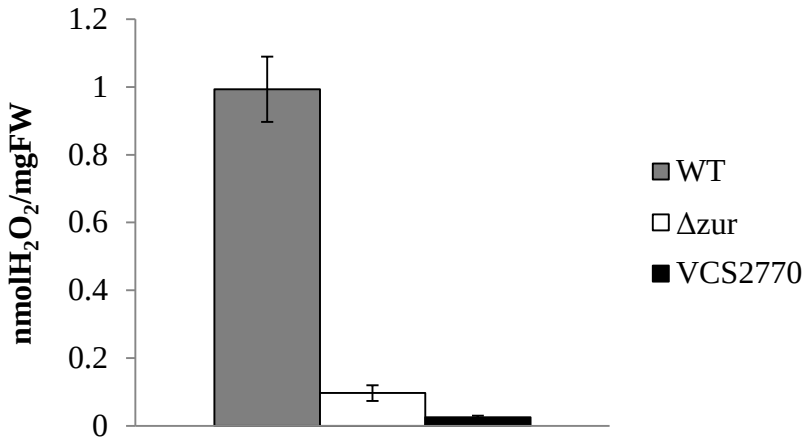
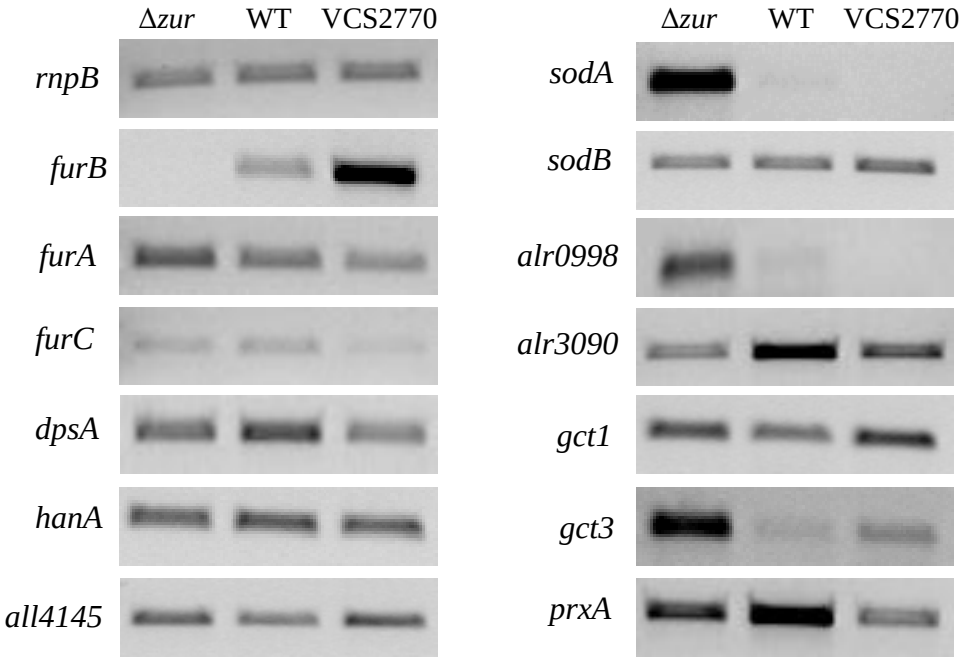

1.2
1
0.8
0.6
0.4
0.2
0
nmolH_2O_2/mgFW
WT
Δzur
VCS2770

Figure 6


Δzur
WT
VCS2770
rnpB
furB
furA
furC
dpsA
hanA
all4145
Δzur
WT
VCS2770
sodA
sodB
alr0998
alr3090
gct1
gct3
prxA

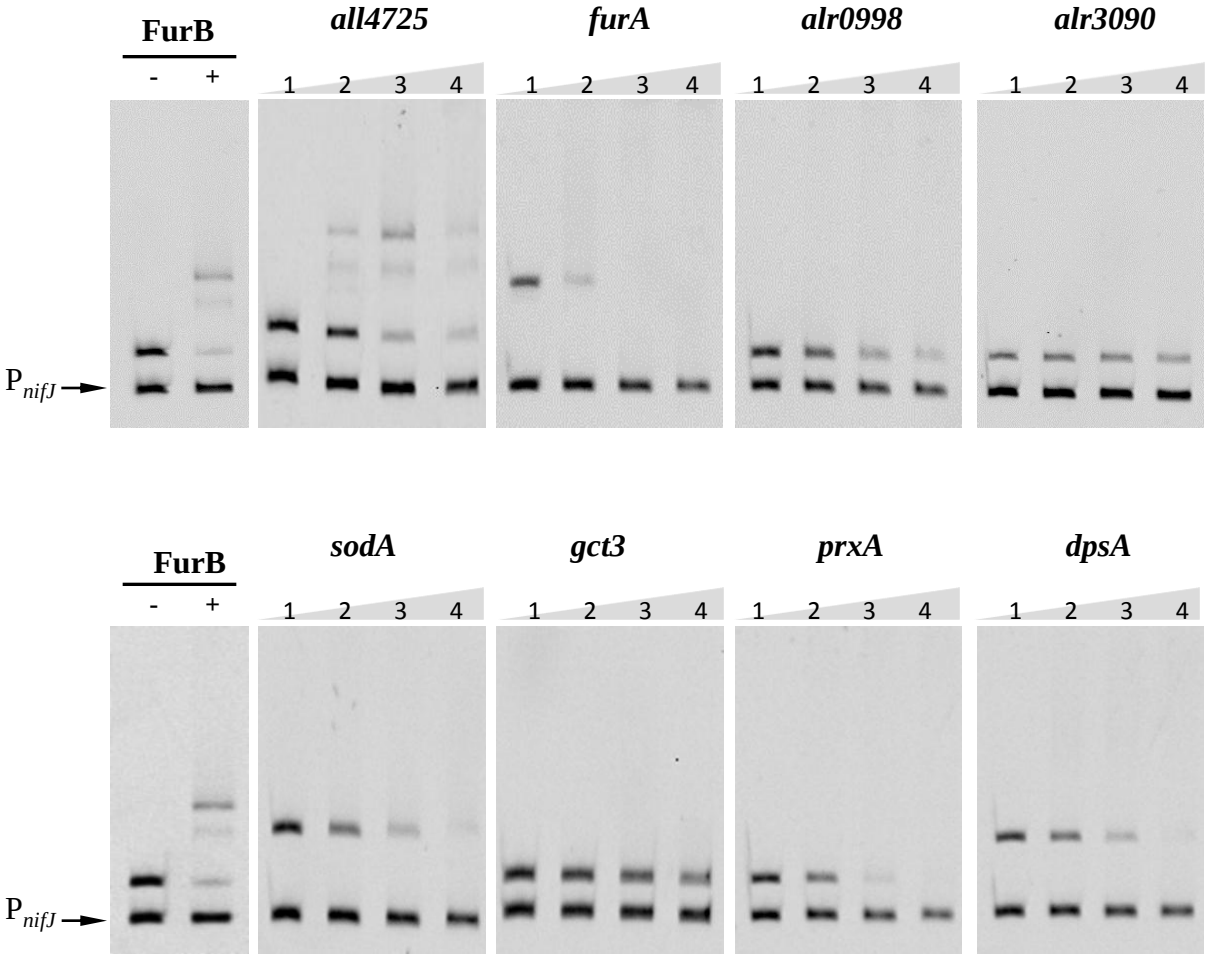

FurB
- +
all4725
furA
alr0998
alr3090
1 2 3 4
P nifJ
FurB
- +
sodA
gct3
prxA
dpsA
1 2 3 4
P nifJ

Table 1

Pigments	Strains		
	WT	**Δ*zur***	**VCS2770**
Chlorophyll *a*	2.20 ± 0.14	2.56 ± 0.61	4.06 ± 0.45
Carotenoids	0.050 ± 0.00	0.034 ± 0.00	0.052 ± 0.00
Phycobiliproteins	24.56 ± 0.03	24.64 ± 0.45	27.60 ± 0.42

Table 2

Strain	Photosynthesis	Respiration	Total oxygen evolution
WT	4.76 ± 0.17	0.68 ± 0.17	5.44± 0.17
Δ***zur***	4.29 ± 0.22	0.82 ± 0.15	5.11± 0.37
VCS2770	4.69 ± 0.28	0.75 ± 0.16	5.81± 0.44

Gene ID[a]	Protein description[a]	Δ*zur*//WT	VCS2770/WT
all2473	Ferric Uptake regulator B, FurB/Zur	0	4.69 ± 0.23
all1691	Ferric Uptake regulator A, FurA	1.25 ± 0.01	0.66 ± 0.01
alr0957	Ferric Uptake regulator C, FurC	1.16 ± 0.17	0.66 ± 0.04
all0070	Manganese superoxide dismutase, SodA	11.62 ± 0.27	0
alr2938	Iron superoxide dismutase, SodB	0.94 ± 0.01	1.27 ± 0.01
alr0998	Manganese catalase	4.12 ± 0.19	0
alr3090	Manganese catalase	0.29 ± 0.01	0.56 ± 0.05
alr3183	Peroxiredoxin, PrxQ-B, Gct1	1.36 ± 0.02	1.56 ± 0.01
all2375	Peroxiredoxin, PrxQ-C, Gct3	7.64 ± 0.14	2.34 ± 0.06
alr4641	Peroxiredoxin, PrxA	0.52 ± 0.01	0.35 ± 0.01
alr3808	DpsA	0.75 ± 0.01	0.52 ± 0.01
asr3935	DNA binding protein HU	0.83 ± 0.01	0.89 ± 0.02
all4145	Probable DNA binding stress protein	1.38 ± 0.08	1.31 ± 0.14

a. Gene identification and protein description according to the cyanobacteria genome database Cyanobase (http://genome.microbedb.jp/cyanobase/)