Functional Genomics of Metalloregulators in Cyanobacteria

Botello-Morte L, González A, Bes MT, Peleato ML and Fillat MF*

Department of Biochemistry and Molecular and Cell Biology. Faculty of Sciences and Institute for Biocomputation and Physics of Complex Systems (BIFI). University of Zaragoza, Pedro Cerbuna, 12 - 50009 Zaragoza, Spain

* Corresponding author: María F. Fillat. fillat@unizar.es. Phone: +34 976761282.

FAX: +34 976762123

Abstract

Cyanobacterial metabolism relies on the activity of many enzymes and other proteins containing metal-rich cofactors that are absent in non-photosynthetic organisms. Most of those micronutrients play key roles in or are associated to photosystems, respiratory electron-transport chains and many enzymes involved in nitrogen metabolism. Since metal homeostasis is crucial for the ecological success of cyanobacteria, trace metal bio-uptake is strictly regulated by a number of metal-sensor proteins and regulatory proteins that often also contain metals. This chapter discusses functional studies undertaken to date from a genomic point of view, as well as the main structural and mechanistic insights into the major families of metalloregulators in cyanobacteria. Reverse genetics, transcriptomics and other assays used for the identification of metal-regulated genes reveal interesting connections between metabolic networks and interactivity between major regulons. These data provide a better understanding of cyanobacterial physiology including maintenance of metal homeostasis, strategies to deal with different stresses and the basis of cyanotoxicity.

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I. INTRODUCTION

Transition metals are essential components of all living cells. They act as main cofactors for oxidation-reduction reactions in electron transfer chains, in hydrolytic and acid-base chemistry and are key structural elements that stabilize protein fold. Cyanobacterial metabolism relies on the activity of many enzymes and other proteins that contain metal-rich cofactors that are absent in non-photosynthetic organisms. Most of these micronutrients play key roles in or are associated to photosystems, for example manganese in thylacoidal water-splitting oxygen-evolving complex, magnesium in chlorophyll and copper in thylakoidal plastocyanin. Besides, biological nitrogen fixation needs a large amount of metals, such as the nitrogenase complex with molybdenum (or vanadium) and iron. For these reasons, cyanobacterial metal requirements are higher than in other prokaryotes. During their evolution, cells develop a broad network of metalloregulatory proteins involved in transport, metal trafficking and metal homeostasis and resistance to deficiency, as well as efficient cross-talk with other regulatory networks. In fact, the effectiveness of these systems confers on organisms an important adaptive advantage, which is very clear in the case of iron metabolism (Straus 1994). In many cases, the ability of a microorganism to capture and incorporate metals determines its ecological success. In fact, cyanobacteria developed during evolution very efficient mechanisms for maintaining metal homeostasis.

Metalloregulation in cyanobacteria is mainly carried out by metal-sensor proteins and regulatory proteins containing metals. In recent years, their characterization has contributed significantly to providing important insights into how bacteria handle metals and harmonize many aspects of their metabolism. The high sensitivity and selectivity of metalloregulatory

proteins has been explained on a molecular basis by solving the crystal structures of representatives from the main families involved in this important function (Cook et al., 1998; Pohl et al., 2003)

II. GENERAL FEATURES OF METALLOREGULATORS

Metal sensor proteins are usually allosteric proteins whose reversible interaction with the regulatory metal drives conformational changes that affect DNA-binding. They exhibit metal-binding and DNA-binding domains and, in some cases, they possess an additional structural metal (Dian et al., 2011; Lucarelli et al., 2011). Binding to DNA occurs as one or two dimers. In some cases, metal-sensors with different metal specificities may interact (Fleischhacker et al., 2011; Reyes-Caballero et al., 2011).

Residues involved in metal binding are highly conserved. Some metal-sensor proteins can also be involved in other functions apart from transcriptional regulation. This potential multifunctionality of metalloregulators is a challenging issue for future investigation aimed at better understanding of cell physiology.

III. MAIN FAMILIES OF METALLOREGULATORS IN CYANOBACTERIA

A. THE FUR (FERRIC UPTAKE REGULATOR) SUPERFAMILY

Fur proteins are among the most ubiquitous regulators in prokaryotic organisms. Initially identified as iron-sensing repressors in heterotrophic bacteria, it is now known that Fur proteins constitute a superfamily of regulators involved in the control of a large number of genes involved in general metabolism, electron transport, virulence and defense against different stresses. It is assumed that Fur works as a classical repressor using Fe^{2+} as a

cofactor to negatively regulate expression of their target genes. Moreover, in heterotrophic organisms Fur proteins not only respond to the availability of different divalent metals such as Fe^{2+}, Zn^{2+}or Mn^{2+} but are also involved in the control of the peroxide-stress response (Staggs et al.) and heme availability (Irr) (Lee and Helmann, 2007).

1. Structural features of Fur proteins

Although Fur proteins have a wide diversity of metal selectivity and biological functions, they share a similar architecture that serves to better understand the activation and metal discrimination mechanism of this group of proteins. The first crystal structure for a Fur homologue showed a basic folding with two well-defined domains: an N-terminal DNA binding domain and a C-terminal dimerization domain (Pohl et al., 2003). The DNA-binding domain of Fur from *Pseudomonas aeruginosa* was composed of four helices followed by a two-stranded antiparallel β-sheet displaying a winged helix-turn-helix motif (Fig. 1). The dimerization domain of each Fur monomer consisted of two structural elements that were involved in forming the functional protein dimer, an α/β-domain and a long α-helix covered by three antiparallel β-strands from the first element. Although this main folding has been confirmed by X-ray crystallography of other members of the Fur family of metalloregulators, such as *Helicobacter pylori* Fur (Dian et al., 2011), *Streptomyces coleicolor* Nur (An et al., 2009), *Mycobacterium tuberculosis* FurB/Zur (Lucarelli et al., 2007) or *Bacillus subtilis* PerR (Jacquamet et al., 2009), there are structural differences that may be responsible for some exceptions in the regulatory capacity observed in this protein family (Dian et al., 2011; Hernández et al., 2002). <Figure 1 near here>

No crystal structure is available for cyanobacterial Fur homologues, though their main folding is probably shared with other Fur proteins. In fact, FurA from *Anabaena* PCC 7120 shows around 40% sequence similarity with *Pseudomonas* Fur and has similar helical content, as

demonstrated by FTIR and far-UV CD (Hernández et al., 2005). A three-dimensional model for the FurA monomer from *Anabaena* PCC 7120 has been obtained by homology modelling based on its similarity with *P. aeruginosa* Fur, although the lack of strong sequence identity at the dimerization interface between *P. aeruginosa* Fur and FurA precluded dimer modelling (Hernández et al., 2005). Homology modelling has also been used to build the 3D structure of the complex between the PerR-like regulator Slr1738 from *Synechocystis* PCC 6803 and its target DNA (Garcin et al., 2012).

a. *Metal binding site.* Fur proteins show two potential metal binding motifs rich in histidines and cysteines; a conserved $HHXHX_2CX_2C$ and another, less conserved, carboxyl-terminal motif CX_2C (Fig. 2). The structure of Fur from *P. aeruginosa* exhibits two metal binding sites (Pohl et al., 2003). Site 1, placed in the dimerization domain, comprises the side chain of residues His^{86}, Asp^{88}, Glu^{107} and His^{124} and a water molecule resulting in a distorted octahedral geometry. Site 2 connects the DNA binding domain with the dimerization domain including the side chain of residues His^{32}, Glu^{80}, His^{89} and Glu^{100} with a tetrahedral geometry (Fig. 1). The role of sites 1 (regulatory) and 2 (structural) for *Pseudomonas* Fur is subject to controversy. Some authors propose that Fur from *P. aeruginosa* detect Fe^{2+} through the site initially described to accommodate the structural Zn^{2+} (site 2) , but according to biochemical analyses it lacks structural Zn^{2+}. Site 1 could be a metal binding site of low affinity without biological significance (Lee and Helmann, 2007).

The presence of a structural Zn^{2+} binding site has been found in most Fur proteins crystallised to date (Dian et al., 2011; Jacquamet et al., 2009; Lucarelli et al., 2007). This structural site usually represents a regular tetrahedral coordination of four cysteine residues belonging to two CX_2C motifs. However, the presence of such motifs does not seem to ensure the binding of structural zinc. In fact, Cys4-Zn appears not to be essential for maintaining the DNA competent conformation and hence for the DNA-binding activity of Nur (An et al., 2009).

This also seems to be the case of recombinant cyanobacterial FurA. Despite having two pairs of CX_2C (Cys^{101}, Cys^{104}, Cys^{141} and Cys^{144}), metal analysis and electrospray ionization MS evidenced that neither zinc nor other metals are present in this Fur homologue (Hernández et al., 2002). However, these motifs are probably important in the cyanobacterial FurA regulatory mechanism since Cys^{101} and Cys^{141} correspond to residues Cys^{93} and Cys^{133} present in the dimerization domain in *V. cholerae* Fur. They are connected by a disulphide bond which plays an analogous role to that of the salt bridge between Asp^{94} and Arg^{131} that stabilizes the β3-β5 antiparallel β-sheet in each subunit of the *Pseudomonas* Fur dimer (Dian et al., 2011), and Cys^{141} has been found to be involved in heme coordination by cyanobacterial FurA (Pellicer et al 2012). <Figure 2 near here>

The metal binding site that binds the regulatory metal seems to be conserved in all Fur and Fur-like proteins, although it shows some variability in its coordination depending on the Fur homologue and the metal. However, it always involves a histidine residue from the loop between α2 and α3 in the DNA-binding domain (Fig. 1). This histidine is also conserved in most cyanobacterial FurA and FurC proteins (Fig. 2). In FurA from *Anabaena* PCC 7120, it corresponds to His^{39} that according to a monomer three-dimensional model belongs to a buried core formed by polar residues His^{39}, His^{85}, His^{96}, His^{98}, Glu^{87} and Glu^{109} (Hernández et al., 2005). This histidine is noticeably absent in cyanobacterial FurB/Zur orthologues (López-Gomollón et al., 2009 and Fig. 2).

2. DNA-binding sites

Under conditions of iron abundance, iron-bound Fur dimers bind to target promoters in their Fur boxes, also called iron boxes. Fur-binding sequences were originally regarded as 19-bp inverted repeats with the consensus (GATAATGATAATCATTATC), and reinterpreted as arrays of NATA/TAT heptamers (Escolar et al., 1999). Binding of FurA from *Anabaena* to

their DNA targets is enhanced in the presence of divalent metals and reducing conditions (Hernández et al., 2006a). Footprinting assays using *furA* and flavodoxin promoters led to the identification of the first experimentally defined binding sites for a cyanobacterial FurA protein. These Fur-boxes consist of arrays of A/T rich sequences that show a faint homology with the *Escherichia coli* consensus sequence. Further experimental work confirmed a rather low sequence specificity for this regulator, suggesting that, in addition to proper A/T arrays, DNA conformation might be an important factor in FurA target recognition and binding (González et al., 2011). These features are somewhat different from the DNA-binding properties found for the other cyanobacterial Fur paralogues (Hernández et al., 2004a, López-Gomollón et al., 2009; Napolitano et al., 2012).

3. Occurrence and functions of Fur paralogues in cyanobacteria

The isolation of a *fur* gene in *Synechococcus* PCC 7942 through an *E. coli*-based *in vivo* repression assay was the first evidence of the existence of a Fur protein in cyanobacteria (Ghassemian and Straus, 1996). Deletion of *fur* resulted in meridiploids that showed iron-deficiency symptoms in iron-replete medium, as well as partial de-repression of flavodoxin and of hydroxamate siderophores. Advances in whole genome sequencing of diverse cyanobacterial strains allow identification of this essential *fur* gene as Synpcc 7942_0987. Moreover, searching for ORFs exhibiting the His-rich motif characteristic of Fur proteins across the 39 cyanobacterial genomes available in the cyanobase (http://genome.kazusa.or.jp/cyanobase) (Nakamura et al., 1998) reveals the presence of several Fur paralogues in all the strains sequenced to date. Although three Fur-like proteins are commonly found in most cyanobacteria, some *Prochlorococcus* strains, such as SS120 or MED4, harboring the smallest cyanobacterial genomes contain only two *fur* genes, in contrast with the five ferric-uptake regulator paralogues that have been annotated in

Synechococcus elongates. This scenario suggests that this family of transcriptional regulators presents a functional specialization in cyanobacteria, though microarray-based analysis has also evidenced redundant functions and cross-talking between Fur proteins.

a. *FurA and their orthologues*. Biochemical and genetic studies aimed at discovering the functions of the three members of the family identified in *Anabaena* (*Nostoc*) PCC 7120, namely FurA, FurB and FurC (Hernández et al., 2004a), showed that FurA, the *all1691* gene product, is the most abundant of the three proteins under standard culture conditions. FurA is a master regulator acting as a hub that connects iron homeostasis, oxidative stress defense and other relevant metabolic pathways (Fillat 2010). In *Anabaena*, FurA seems both to act as the ferric uptake regulator and to perform the PerR functions. Unlike the other two Fur paralogues, FurA is an essential protein under standard culture conditions (González et al., 2010; Hernández et al., 2006b). *Anabaena* mutants overexpressing FurA have lower iron content than the WT strain and an iron-deficient phenotype (González et al., 2010; Hernández et al., 2010). Because of the tight connection between iron metabolism and oxidative stress, the fact that the same protein may respond to both signals would allow a more efficient coordination between iron uptake and storage and the redox status of the cell. Since the current working model for FurA is based on the repression of target genes using Fe^{2+} as co-repressor, a dual-sensing mechanism could rely on iron oxidation produced by oxidative damage that, in turn, would lead to the dissociation of Fur from the DNA, thus allowing the transcription of genes involved in ROS quenching (Fig. 3). Single-molecule assays show the tendency of FurA to form trimers and higher aggregates via disulphide bridges in the presence of H_2O_2 (Lostao et al., 2010), suggesting that oxidative injury could irreversibly produce non-functional dimers, activating the concerted response to oxidative stress.

Similarly to *Anabaena PCC 7120*, the genome of *Synechocystis* PCC 6803 contains three *fur* paralogues. The high homology of *sll0567* with *furA* from *Anabaena* and the impossibility of obtaining fully segregated *sll0567* deletion mutants (Nakamura et al., 1999) indicate that both *all1691* and *sll0567* genes are orthologues.

b. *PerR and the oxidative stress response.* Inactivation of the *Synechocystis* PCC 6803 *slr1738* gene and functional genomics of the resulting mutant (Li et al., 2004; Singh et al., 2004) clearly point to this gene as a *perR* ortholog that is involved in the control of the peroxide stress response in this unicellular cyanobacteria. A comparison of differentially regulated genes of the peroxide stimulon in *Synechocystis* WT and in the Δ*perR* mutant strain allowed to define a rather small PerR regulon (Table 1) that also includes some genes related to iron homeostasis, such as *idiA* and *mrgA*. These results also showed that most genes responding to oxidative stress are PerR independent, indicating that Slr1738/PerR is not the main regulator of the concerted response to peroxide stress (Li et al., 2004). In *Anabaena* PCC 7120, neither FurB nor FurC seem to function as PerR orthologues. <Table 1 near here>

c. *FurB from Anabaena: a moonlighting protein?* It has been suggested that FurB, the product of *all2473,* may protect cells against oxidative stress, most likely by direct protection of the cyanobacterial nucleoid, in a similar way to a Dps protein (López-Gomollón et al., 2009). This hypothesis is based on the fact that expression of the gene is strongly induced under oxidative challenge and its overexpression in *E. coli* increases its tolerance to H_2O_2 and paraquat. In addition, FurB presents a very high isoelectric point (pI 8.7), similar to histones, and protects DNA *in vitro* from ROS and DNaseI damage. In a recent study (Napolitano et al., 2012), FurB has been identified as a Zur regulator in *Anabaena* PCC 7120. Unlike most FurA modulated genes that exhibit two or more iron-boxes, specific binding of

FurB to DNA occurs in the single 7-1-7 consensus motif (TGATAATNATTATCA). FurB is dispensable under standard culture conditions and is involved in the control of at least 23 genes, 17 of them organized in 6 operons. FurB-controlled genes fall into 4 different categories, namely paralogues of zinc metalloproteins, putative metallochaperones, components of ABC transporters and outer membrane proteins (Table 2).

These data indicate that FurB/Zur is a main component, together with SmtB proteins (Huckle et al., 1993; Thelwell et al., 1998), in the response and adaptation of cyanobacteria to Zn^{2+} deficiency and suggests that FurB might be a moonlighting protein, playing a dual role in *Anabaena.* <Table 2 near here>

d. *FurC: a potential regulator of regulators.* A third Fur paralogue, FurC (Alr0957), has been identified and purified in *Anabaena* PCC7120 (Hernández et al., 2004a). The FurC basal expression level is lower than those of FurA and FurB and exhibits a significant gap in homology with those proteins. As other members of this family, FurC is able to dimerize, although the dimer is only detected under oxidizing conditions. Our hypothesis is that FurC can form heterodimers through its C-terminus with other Fur family members, modifying their affinity for DNA. Residues involved in C-terminal domain dimerization are quite conserved in FurC, allowing the potential formation of heterodimers. Reverse genetics studies are expected to rule out that FurC can have another role, so far unknown, including target promoters not yet identified.

4. Deciphering the FurA regulon

The potentially essential role of FurA in *Anabaena* sp. physiology poses the challenge of deciphering its regulon. Comparative global analyses of transcriptomes and proteomes for *fur* deletion mutants and their parental wild-type strains have been traditionally used to

characterize Fur regulons in several heterotrophic bacteria (Baichoo et al., 2002; Gao et al., 2008; McHugh et al., 2003; Wan et al., 2004). However, gene knockout can not be used to define function when silencing implies death. In such cases, alternative approaches such as overexpression (Olmedo-Verd et al., 2005; Wu et al., 2004) or selectively regulating gene expression (Callahan and Buikema, 2001; Zhang et al., 2000) often overcome this limitation and can be used to discern functions, unravel regulation mechanisms or identify direct targets within regulatory networks.

The first studies discerning the FurA regulon showed that this metalloregulator specifically bound *in vitro* to A/T-rich sequences of its own promoter, while the absence of both divalent metal ions and/or reducing conditions as well as the presence of heme severely impaired its affinity for DNA (Hernández et al., 2006a; Hernández et al., 2004b). Surprisingly, the expression of FurA appeared strongly enhanced in proheterocysts and mature heterocysts, and this up-regulation seemed to be mediated by NtcA, a master regulator of nitrogen metabolism that triggers heterocyst differentiation and nitrogen fixation in diazotrophic cyanobacteria (López-Gomollón et al., 2007b). These findings represented the first evidence of FurA involvement in nitrogen metabolism and led to further definition of a cross-talk between FurA and NtcA, identifying overlapping genes in both regulons (López-Gomollón et al., 2007a).

More recently, overexpression has been successfully used as an alternative method to gain new insights into the FurA regulatory function. Overexpression of FurA in *Anabaena* sp. PCC7120 induced changes in the transcriptional pattern of a variety of genes, leading to alterations in photoautotrophic growth, filament integrity, cell morphology, ultrastructure, photosynthetic function and defense against oxidative stress (González et al., 2010). Although some of the effects observed under a FurA overexpression phenotype could result from an aberrant response unrelated to the normal function of the protein, the combination of

phenotypic studies with both transcriptional and proteomic profile variations in conjunction with FurA-DNA interaction analyses allowed more than twenty new direct targets of this transcriptional regulator to be identified (González et al., 2010; González et al., 2011).

Of the three different ferric uptake regulator homologues described in *Anabaena* sp. (Hernández et al., 2004a), FurA is the master regulator of iron homeostasis, controlling the expression of iron uptake and the storage machinery in response to iron availability (González A., et al., unpublished observations). However, the same protein appears to have a direct regulatory role in the transcription of several genes involved in oxidative stress defenses and redox signaling, modulating the expression of at least two peroxiredoxins (González et al., 2011), thioredoxin (López-Gomollón et al., 2007a), thioredoxin-reductase (González et al., 2011) and DpsA-homologues (Hernández et al., 2007). Since DNA-binding activity of FurA is critically dependent on reducing conditions (Hernández et al., 2006a) and its expression is slightly induced under oxidative stress (López-Gomollón et al., 2009), FurA could also act as an oxidative stress-responsive regulator, similar to other members of the Fur family like PerR of gram-positive bacteria (Herbig and Helmann, 2001; Ricci et al., 2002)

Overall, the variety of FurA regulated genes described so far (Table 3), including siderophore outer membrane transporters, bacterial actins, photosystem II reaction centre proteins, CO_2 concentrating mechanism proteins and peroxiredoxins, provides evidence that FurA functions as a global transcriptional regulator in *Anabaena* sp., supporting its role in major cyanobacterial processes. <Table 3 near here>

5. Genetic regulation of cyanobacterial Fur proteins

Most Fur proteins studied to date show moderate autoregulation. FurA from *Anabaena* binds to its own promoter with an estimated Kd of 0.49$\pm$1 nM, the presence of Mn^{2+} and a reducing environment being the optimal conditions for *in vitro* FurA-P_{furA} interaction (Hernández et

al., 2006a). *In vivo* assays aimed at understanding the relationship between the regulation and the functions of this master protein unveil a rather complex model. A slight increase in the expression of FurA has been detected under iron limitation (Hernández et al., 2002). Since iron deprivation leads to oxidative stress, the increase in FurA expression could be explained as a response to the rise in the level of ROS detected in the cell under iron deficiency (Latifi et al., 2005). This hypothesis is consistent with the observation that oxidants trigger *furA* transcription (López-Gomollón et al., 2009). This increase in FurA expression might be used by the cyanobacteria to down-regulate iron-uptake in order to arrest catalysis of the Fenton reaction. Alternatively, a non-regulatory role directly involving FurA in ROS-quenching has been proposed. Work intended to establish a plausible mechanism of FurA acting as a redox protein based on its two CXXC redox motifs is underway (Botello L. et al, manuscript in preparation).

Northern blot analysis of *furA* under nitrogen step-down in *Anabaena* PCC 7120 and the *ntcA* deletion mutant evidenced that the nitrogen status modulates FurA expression and that NtcA is involved in this process (López-Gomollón et al., 2007b). In order to know whether the increase in *furA* transcription was a general response in the cyanobacterial filament, constructs of the promoters from each *fur* paralogue leading the expression of GFP were used to identify a strong induction of *furA* in heterocysts.

The identification of an antisense RNA that covers the complete *furA* gene and is co-transcribed with the cell wall-binding protein Alr1690 adds another mechanism for dynamic, fine-tuning *furA* regulation at the post-transcriptional stage (Hernández et al., 2006b). Insertional inactivation of the *alr1690-α-furA* dicistronic message produces smaller cells exhibiting a 2.5-fold increase in FurA expression and 62% of iron-content with respect to *Anabaena* WT. Δ*alr1690-α-furA* cells display a reduced number of contorted thylakoids, as well as alterations in the photosynthetic apparatus, leading to lower photosynthetic

performance indexes (Hernández et al., 2010). These results indicate that the expression of the *α-furA-alr1690* message is required for the maintenance of a proper thylakoid arrangement, efficient regulation of iron uptake and optimal yield of the photosynthetic machinery.

The occurrence of anti-*fur* RNAs has been found in other cyanobacterial strains, namely *Microcystis aeruginosa* PCC 7806 and *Synechocystis* sp. PCC 6803, showing rather different gene contexts between them (Sevilla et al., 2011). In the case of *Microcystis*, the anti-*fur* RNA spans the whole Ma*fur* CDS and part of the flanking *dnaJ* and *sufE* sequences, while Syα-*fur* RNA covers only part of the coding sequence of the *fur* orthologue *sll0567*. It has been reported that in heterotrophic bacteria, Fur can indirectly activate several genes by repressing trans-acting, small antisense RNAs, such as RyhB in *E. coli* or the functional homologs PrrF in *Pseudomonas* and NrrF in *Neisseria* (Metruccio et al., 2009; Wilderman et al., 2004). However, α-*furA* is the first antisense RNA reported to modulate a Fur protein. The question of whether cyanobacterial Fur proteins can also repress small nc-RNAs will require further work addressing functional transcriptomics of the many encoded regulatory RNAs found in cyanobacteria (Georg and Hess, 2011).

At the posttranslational level, the DNA-binding ability of FurA is enhanced by the presence of FurC in contrast to the inhibition observed when FurA is complexed with heme (Hernández et al., 2004a; Hernández et al., 2004b). The estimated K_d= 0.4±0.1 μM for the FurA-heme interaction strongly suggests that the binding takes place in vivo as a regulatory mechanism, likely acting as a heme-sensor protein (Pellicer et al., 2012). In summary, the regulatory model for FurA from *Anabaena* can be presented as a complex balance of several signals that influence the final concentration of this protein along the three steps of the genetic flow of information.

Concerning other FurA paralogues, in vitro assays indicate that FurB and FurC might be regulated by FurA, since the latter binds to their promoters. RT-PCR assays confirm the influence of FurA on *furB* transcription that clearly decreases in the FurA overexpressing mutant (González et al., 2010). The influence of several nutritional and environmental factors on *furB* and *furC* expression was investigated by RT-PCR and using GFP constructs driven by their promoters (López-Gomollón et al., 2009). Among the conditions tested, neither osmotic stress induced by sucrose nor salt stress affected FurB or FurC expression. However, oxidative challenge induced by H_2O_2 enhanced the expression of both genes.

Like FurA, FurB exhibits the CP heme-regulatory motif, and binding to this cofactor impairs its interaction with DNA. *In vitro* assays show that FurB binds to its own promoter (Hernández et al., 2004a). This interaction is stronger in the absence of divalent metals and it is destroyed in the presence of Zn^{2+}. Reducing conditions managed by the presence of DTT positively affected FurB-DNA interaction. Further work involving *in vivo* studies should be done in order to address the role of Zn^{2+} in FurB autoregulation.

6. Metabolic and regulatory networks involving Fur proteins

Among the *fur* paralogues identified in cyanobacteria, *furA* and their orthologues seem to be the most important for the cell, since all attempts to fully inactivate the *furA* genes from several cyanobacteria have been unsuccessful under standard growth conditions (Ghassemian and Straus, 1996; Hernández et al., 2006b; Michel et al., 2001). Therefore, it is not surprising that FurA seems to be involved directly or indirectly in the modulation of genes participating in several metabolic pathways, including nitrogen metabolism, transcription, photosynthesis and respiration and, of course, iron uptake and oxidative stress, among others.

a. *Iron homeostasis and the oxidative stress response.* The relationship between iron homeostasis and oxidative stress has been extensively investigated (Cornelis et al., 2011; Faulkner and Helmann, 2011; Shcolnick et al., 2009). This connection is even tighter in cyanobacteria, whose need for iron is about ten times greater than that of heterotrophic bacteria, and whose photosynthetic and respiratory electron transport chains are particularly sensitive to spare ROS generated by the Mehler reaction (Shcolnick and Keren, 2006). In fact, several members of the FurA regulon (Table 3) participate in oxidative stress defense, while some Slr1738 (Staggs et al.) targets in *Synechocystis,* such as *isiA, idiA* and *mrgA* are metal-regulated genes (Li et al., 2004). Previous research on the role of IdiA and MrgA show that these proteins play a pivotal role in the coordination of iron homeostasis and the oxidative stress response (Michel and Pistorius, 2004). Moreover, phenotypic analysis of *Synechococcus* mutants lacking IscA and SufA also point to these proteins as important players in the concerted modulation of iron homeostasis and the sensing of redox stress, expanding cyanobacterial strategies to deal with adverse conditions (Balasubramanian et al., 2006). Noticeable among these is the response of *Synechocystis* to Cd stress consisting of an integrated reprogramming of the metabolism under the control of the Fur member Slr1738 (PerR). Under Cd stress, metal homeostasis and high-light tolerance are disturbed, as well as the functionality of the *suf* machinery (Houot et al., 2007). Since Cd regulates the Zn controlled genes *znuA* and *ziaA*, these authors suggest that this pollutant might be transported via Zn-transport systems. Considering that *znuA* has been identified as a member of the FurB/Zur regulon in *Anabaena* (Napolitano et al., 2012), these results suggest that PerR and the Zur ortolog in *Synechocystis* may operate as common elements of a regulatory network controlling the stress (likely oxidative stress) generated by Cd. In addition, the control of *ziaA* by SmtB proteins (see Fig. 6) highlights a potential functional interaction between Fur and SmtB regulators.

b. *Role of FurA in the modulation of nitrogen metabolism.* The iron pool in nitrogen-fixing cyanobacteria must fulfil the nitrogenase complex requirements. This metalloenzyme contains three different types of Fe–S clusters (Burgess and Lowe, 1996), including the iron-molybdenum cofactor (FeMo-co) at the active site, which contains 7 atoms of iron. As expected, nitrogen fixation in *Anabaena* decreases under low iron conditions (Sandmann et al., 1990). At the molecular level, transcription of the *nifHDK* operon, encoding nitrogenase, and excision of the 11 kb DNA fragment required for its activation take place in iron-starved *Anabaena*, even though cells grew in the presence of combined nitrogen (Razquin et al., 1994). Besides, several iron-responsive genes in cyanobacteria, such as *nblA*, *petH*, *pkn41* and *pkn42*, among others, are also modulated by NtcA (Cheng et al., 2006; Luque et al., 2001; Napolitano et al., 2012; Valladares et al., 1999), the global regulator of nitrogen control. Cross-talk between FurA and NtcA produces a significant overlapping between the regulatory networks controlled by those regulators, involving genes that belong to different functional categories (López-Gomollón et al., 2007a). All these results provide strong evidence for the link between iron and nitrogen metabolism in cyanobacteria that will also be affected by the redox status of the cell.

c. *FurA and carbon metabolism.* Carbon fixation in cyanobacteria relies on a proper assembly of holoproteins associated with/constituting photosystems, essential for the production of enough reducing power and ATP needed for a good photosynthetic performance. In *E. coli* the cyclic AMP receptor protein (CRP) regulates expression of the carbon regulon in response to carbon availability (Zhang et al., 2005). In cyanobacteria, the CRP regulons are highly diversified and CRPs have been lost in some lineages (Xu and Su, 2009). However, in the strains where this regulator has been preserved, though CRPs seem to

regulate different sets of genes, they are always involved in the modulation of photosynthetic pathways. Several genes encoding components of PSI and PSII from *Anabaena* PCC 7120, as well as *ccmK*, coding for a CO_2 concentrating mechanism protein, belong to the FurA regulon (González et al., 2010; González et al., 2011). As has been reported for *E. coli*, defining a potential functional interaction between FurA and Crp remains an interesting problem that will lead to a better understanding of how cyanobacteria allow integration of signals for iron and carbon availability.

d. *Modulation of cyanotoxicity.* Certain cyanobacterial species can produce a broad range of bioactive secondary metabolites potentially toxic to eukaryotic organisms, called cyanotoxins (Carmichael et al., 2001). Like many bacterial toxins, some cyanotoxins are products of modular peptide synthetases and polyketide synthses, as is the case of microcystins, nodularins, cylindrospermopsins or anatoxins-a. The cyclic heptapeptide microcystin is the most commonly found and one of the most hazardous classes of cyanotoxin. Microcystins are synthesized in a mixed polyketide synthase/nonribosomal peptide synthetase system called microcystin synthetase. The microcystin synthetase complex in *M. aeruginosa* PCC 7806 is encoded by the *mcy* operon (Tillett et al., 2000). FurA from *M. aeruginosa* recognizes and binds *mcy* promoter regions (Martin-Luna et al., 2006), suggesting a transcriptional control by this global regulator. Moreover, iron deficiency induces *mcyD* expression, correlating with higher levels of microcystin in cells (Sevilla et al., 2008). Fur and NtcA, the global nitrate regulator, balance iron, carbon and nitrogen metabolism resulting in a fine control of the expression of the microcystin gene cluster (Kuniyoshi et al., 2011).

A very similar control occurs in heterotrophic bacteria, where Fur regulates peptide synthetase systems involved in the synthesis of peptidic siderophores such as enterobactin and vibriobactin, among others (Crosa and Walsh, 2002). These non-ribosomal peptide

synthetases are similar to the enzymes involved in the synthesis of microcystins. Many of these toxins are siderophores, and even though the ecostrategy or physiological meaning of microcystin production is unknown, several observations suggest a link between microcystin production and iron metabolism. During iron-depletion, toxic strains of *Microcystis* maintained cell vitality much longer than non-toxic strains (Lyck et al., 1996). Moreover, the rate of iron uptake in toxic strains was higher than non-toxic strains (Utkilen and Gjolme, 1995). This suggests that microcystin production may be a FurA-controlled physiological response to iron deficiency.

In summary, these connections strongly suggest that Fur proteins play a central role in the adaptation of cyanobacteria to different environmental and nutritional stresses.

B. REGULATION OF IRON-SULFUR CLUSTER ASSEMBLY

Iron-sulfur clusters display versatile functions including stabilization of protein structure, gene regulation, environmental sensing and radical generation (Johnson, 1998). Because of their sensitivity to cellular redox status, iron-sulfur clusters are considered as molecular switches for gene regulation at both the transcriptional and translational levels (Kiley and Beinert, 2003). In cyanobacteria, a variety of enzymes crucial for the organization of fully functional photosystems, contain these clusters. Such iron-sulfur proteins also include some components of the respiratory electron transport complexes and enzymes involved in the central metabolism.

1. The iron sulfurcluster (isc) system

Cyanobacteria contain two main iron-sulfur assembly systems denoted *suf* (sulfur utilization factor) and *isc* (iron sulfur cluster) (Takahashi and Tokumoto, 2002; Wollenberg et al., 2003). Biosynthesis and assembly of iron-sulfur proteins is a highly regulated process. In *E. coli*, the

isc operon is under the transcriptional control of the IscR repressor, which is encoded as part of the *iscRSUA* locus and autoregulates its own expression as well as that of *iscSUA* (Schwartz et al., 2000). IscR is a member of the Rrf2 family (PF02082) of transcriptional regulators and contains a winged helix-turn-helix DNA binding domain. The active form of IscR presents an unstable [2Fe-2S] cluster that is coordinated by three conserved cysteines and a glutamic residue. Under unfavorable conditions, IscR loses its cluster and becomes inactive in the apo form, allowing the full expression of the *isc* machinery. Thus, the regulatory role of IscR relies upon its iron-sulfur cluster and senses redox changes in the cell for the optimal assembly of Fe-S clusters. Cyanobacterial *isc* genes are scattered throughout the genome, and some are present in multiple copies. IscR homologues identified in cyanobacterial genomes display high sequence similarity at the N-terminus in the helix-loop-helix, DNA-binding region. However, the homology at the C-terminus is relatively low. Cyanobacterial IscR homologues lack the 16-17 amino acids present at the C-terminus in IscR proteins from other microorganisms. Furthermore, the cysteine residues conserved in heterotrophic bacteria are missing in cyanobacterial IscR homologues, making the presence of an iron-sulfur cluster unlikely. Therefore, it has been proposed that the cyanobacterial IscR homologues might sense changes in iron status via interactions with other sensor proteins containing iron-sulfur clusters, such as IscA and SufA (Wu, 2008).

2. The sulfur utilization factor (suf) system

The *suf* system in cyanobacteria is considerably more important than the *isc* system. Results from reverse genetics studies performed on *Synechocystis* PCC6803 indicate that many genes belonging to the *suf* regulon are essential in cyanobacteria. Furthermore, the *suf* system, but not the *isc* system, is found in the chloroplasts of higher plants, suggesting that photosynthetic organisms may rely primarily on the *suf* system for assembling iron-sulfur clusters for electron transfer cofactors (Balasubramanian et al., 2006). Most Suf proteins are encoded by the

sufBCDS operon that is highly conserved in cyanobacterial genomes and negatively regulated by the *sufR* gene encoded by the complementary strand (Wang et al., 2004). SufR belongs to the DeoR family of helix-loop-helix proteins that contain an N-terminal DNA-binding domain and four highly conserved cysteine residues near the C-terminus. Active SufR is also an iron-sulfur protein whose binding affinity depends on the presence and redox state of its [4Fe-$4S^{2+1+}$] clusters (Shen et al., 2007). However, SufR does not present similar structural or function-related motifs to other redox-sensing regulators such as FNR, SoxR and IscR, that also possess Fe-S clusters as sensors. Footprinting and biochemical assays show that both apo and holo-SufR exist as dimers and the active form binds to two distinct sequences with different affinities. The fact that the operator sequences contain two perfect inverted repeats (CAAC-N_6-GTTG and TAAAACAAC-N_6-GTTGTTTTA) separated by 26 bp and the finding of SufR tetramers has led to propose that DNA bending might be involved in SufR regulation (Shen et al., 2007).

Furthermore, reverse genetics analysis of *Synechococcus* PCC 7002 highlighted the role of SufA and IscA in the modulation of Fe-S cluster homeostasis (Balasubramanian et al., 2006). These studies also showed that Nfu is essential for the Fe-S scaffold in cyanobacteria. Noticeably, Nfu is homologous to the C-terminus of NifU, a key protein for assembly of the Fe/S cluster in *Azotobacter vinelandii* (Fu et al., 1994).

C. MANGANESE HOMEOSTASIS IN CYANOBACTERIA: THE ManR AND RfrA REGULATORS

Manganese is particularly important in oxygenic photosynthetic organisms, playing a critical role in forming a cluster of four atoms on the donor side of photosystem II (PSII), which participates in catalyzing the water-splitting reaction (Barber, 2008b). The assembly of Mn^{2+}

ions to form the catalytically active Mn_4-Ca cluster of the oxygen-evolving complex of the PSII reaction center is a light-driven process termed photoactivation, which occurs during *de novo* formation of PSII as well as during the frequent repair of PSII in response to photoinhibition (Aro et al., 2005; Barber, 2008a).

In *Synechocystis* sp., Mn^{2+} limitation induces changes in the activity and organization of both photosystems, resulting in a reduction of photochemical activity of PSII as is made evident by lower oxygen evolution rates, lower maximal photosynthesis yield of PSII values, and faster plastoquinone reoxidation rates. On the other hand, Mn^{2+} deficit leads to loss of PSI activity as a result of loss of PSI core proteins and Mn^{2+} limitation-dependent dissociation of PSI trimers into monomers (Salomon and Keren, 2011). Thus, since Mn^{2+} is essential to the function of PSII, and even the state of cellular Mn^{2+} availability influences the rate of photochemical activities of both photosystems, there is clearly an intricate genetic network for controlling Mn^{2+} homeostasis in cyanobacteria (Chandler et al., 2003; Ogawa et al., 2002; Yamaguchi et al., 2002).

Mn^{2+} is accumulated in high concentrations in the cytoplasm of prokaryotes by high-affinity uptake systems. In *Synechocystis* sp., Mn^{2+} acquisition takes place through several transport systems. The best known is MntABC, an ABC-type permease that mediates high-affinity transport under starvation conditions (Bartsevich and Pakrasi, 1995). A second high-affinity transporter acting under Mn-sufficient conditions and a low-affinity transporter indirectly observed by transport kinetics have been reported, but they remain to be characterised (Bartsevich and Pakrasi, 1996). Mn^{2+} uptake appears to be dependent on active photosynthesis, leading to accumulation in the cyanobacterial envelope layer. The Mn^{2+} outer membrane pool is used as a reservoir for intracellular Mn^{2+}, which is kept constant at approximately 10^6 atoms per cell of which a large fraction is associated with PSII (Keren et al., 2002; Salomon and Keren, 2011).

Transcription of the *mntABC* operon in *Synechocystis* sp. occurs under Mn^{2+} starvation conditions (nM levels of Mn^{2+}), but not in a Mn-sufficient environment (μM Mn^{2+}). Such an inducible high-affinity Mn^{2+}-transport mechanism is controlled via a two-component signal transduction pathway that negatively regulates the expression of the *mntABC* operon (Ogawa et al., 2002; Yamaguchi et al., 2002). This two-component system, also described in *Anabaena* sp. PCC 7120 (Huang and Wu, 2004a; Huang and Wu, 2004b), includes a membrane-bound histidine kinase, ManS, which senses the extracellular concentration on Mn^{2+} ions and activates a transcriptional response regulator, ManR, which specifically binds to the promoter region of *mntABC* to repress the expression of the ABC-type transporter encoded by this operon. Under Mn^{2+} starvation conditions, ManS does not generate a signal, resulting in inactivation of ManR and subsequent expression of the *mntABC* operon.
ManS contains a histidine kinase domain in the C-terminal region that includes a phosphorylatable His residue, and two membrane-spanning domains in the N-terminal region. It has been proposed that the region between these two membrane spanning domains is located in the periplasmic space and perceives the extracellular concentration of Mn^{2+} ions by providing ligands to Mn^{2+} (Yamaguchi et al., 2002). The transcriptional response regulator ManR belongs to the OmpR/PhoB subfamily (Martinez-Hackert and Stock, 1997) and contains two functional domains, an N-terminal phosphorylation domain which includes a phosphorylatable Asp residue that receives the signal of ManS, and a C-terminal DNA-binding domain. Punctual mutation of either, the His-205 residue of the *manS* gene or the Asp-52 residue of the *manR* gene, induces the expression of the *mntABC* operon, suggesting that both residues are essential for the transduction of Mn^{2+} signals. Even though unphosphorylated ManR is bound to the promoter regions of mntABC *in vitro*, transcription seems to be repressed only by its phosphorylated form (Yamaguchi et al., 2002)

In *Anabaena* sp. PCC 7120, the ManS/ManR two-component system modulates the expression of genes *all3575*, *all3574* and *alr3576* that encode the homologous proteins of MntABC from *Synechocystis* sp. (Huang and Wu, 2004b). Besides, this two component Mn^{2+}-sensing system controls the expression of the natural resistance-associated macrophage protein (Nramp) homologous MntH (Huang and Wu, 2004a), another type of bacterial Mn^{2+} transporter that is widespread throughout diverse groups of eubacteria (Jakubovics and Jenkinson, 2001).

The transcriptional metallorepressor ManR is specifically bound to the *Anabaena* sp. *mntH* promoter through a DNA sequence of 19 bp composed of two direct repeats in the form of (T/A)ATGA(G/A)A(A/G) separated by 3 bp, which appears highly conserved in the promoter regions of genes encoding MntABC and MntH homologues of several cyanobacteria (Huang and Wu, 2004a). Since this two direct repeats arrangement is typical of DNA recognition motifs from OmpR/PhoB response regulators (Okamura et al., 2000), the conservative consensus sequence (T/A)ATGA(G/A)A(A/G) recognized by ManR appears as a novel regulatory DNA motif in cyanobacteria. The binding of ManR to its target promoters occurs through the C-terminal helix-turn-helix (HTH) domain, which contains at least three highly conservative amino acids residues among the OmpR/PhoB subfamily regulators that are essential for DNA binding activity (Huang et al., 2006). *In vitro* analyses suggest that two ManR molecules cooperatively bind to the DNA recognition sequences at the same time, and this cooperativity appears to be mediated by protein-induced DNA deformation since no protein-protein intermolecular interactions occurred between ManR monomers *in vitro* (Huang and Wu, 2005). Although MntH transporters of Mn^{2+} have been described in several bacterial groups, the regulation of its expression by the two-component signal transduction system ManS/ManR has only been found in cyanobacteria (Huang and Wu, 2004a).

Another Mn-uptake regulation mechanism described in *Synechocystis* 6803, different from the more thoroughly characterized ManS/ManR two-component signal transduction system, involves the RfrA regulator (Chandler et al., 2003). This protein modulates a second high-affinity Mn transport system which acts under Mn-sufficient conditions, but the mode of action of RfrA remains unknown. RfrA has no sequence or structural similarities to previously described bacterial manganese-regulated transcription factors, and it does not have any known DNA-binding domain. Hence, it is more plausible that RfrA regulates the second Mn^{2+} transporter through a mechanism other than transcriptional control, such as reversible protein modifications at posttranslational level. The regulator contains a conservative repeated-five residues (RFR) domain in the N-terminal, which define a 16-member family in *Synechocystis* sp. PCC 6803. Despite the fact that RFR domains seem to be relatively abundant in other bacterial genomes and especially in photosynthetic organisms, the RFR genes have no defined function (Bateman et al., 1998). Thus, RfrA becomes the first member of this family of proteins to be linked to a physiological process (Chandler et al., 2003). Further experiments are required to discern the exact mode of RfrA regulation in Mn-uptake in cyanobacteria.

D. THE ArsR/SmtB FAMILY OF METAL-SENSOR PROTEINS

The SmtB/ArsR proteins function as transcriptional repressors sensing elevated concentrations of different metals not only in cyanobacteria but also in other prokaryotes. This family contains two subfamilies, one comprising SmtB and its orthologues, more divergent than the other subfamily which contains ArsR and its closely related proteins. Similarly, all family members usually control the expression of a metallothionein to

sequester metal ions in the cytosol, or an ATPase to export the metal into the periplasm. Conversely, both groups of proteins mainly differ in the metal binding site (Rensing, 2005). This protein family includes zinc sensors (SmtB in *Synechococcus* and ZiaR in *Synechocystis*), arsenic, antimony and bismuth (ArsR in *E. coli* and *Synechocystis*), cadmium, lead and zinc (AztR in *Anabaena* and CadC in *Staphylococcus aureus*), cadmium and lead (CmtR in *M. tuberculosis*), zinc and cobalt (CzrA in *S. aureus*), nickel and cobalt (NmtR and KmtR in *M. tuberculosis*) and copper, silver, zinc and cadmium (BxmR in *Oscillatoria*) (Osman and Cavet, 2010).

One founder member of this family, SmtB, was first cloned and characterized from *Synechococcus* PCC 7942 in 1993. This 122 aminoacid-protein functions as a Zn(II)-responsive repressor of a metallothionein involved in chelating zinc from cytosol (Huckle et al., 1993). A few years later, a SmtB orthologue was described in *Synechocystis* PCC 6803, named ZiaR. This regulates the transcription of an ATPase which exports zinc into the cytoplasm (Thelwell et al., 1998).

The other founder member, ArsR, was first described as an As(III)/Sb(III)-responsive repressor in *E. coli* (Wu and Rosen, 1991). In *Synechocystis* PCC 6803, an ArsR orthologue is involved in arsenic and antimony resistance, and it thus controls the expression of an operon containing an As(III)/Sb(III)-efflux pump, among other proteins (Lopez-Maury et al., 2003).

More recently, some other SmtB/ArsR proteins have been discovered in cyanobacteria. *Anabaena* PCC 7120 AztR also represses the transcription of a zinc efflux pump (Liu et al., 2005). A further SmtB/ArsR family member, AzuR, has been reported but not fully characterized (Liu et al., 2008). Notably, in *Oscillatoria brevis,* the Zn(II)-responsive regulator BxmR controls the expression of an ATPase and a metallothionein, but both products are encoded in two physically separate transcription units (Liu et al., 2004). These

metal homeostasis systems in *Oscillatoria* remind the zinc response machinery present in mammalian cells with MTF1, which also regulates a metallothionein and a Zn-efflux pump (Jackson et al., 2008).

1. Metal-binding sites of SmtB/ArsR family members.

The X-ray crystallographic structure of *Synechococcus* apo-SmtB at 2.2 Å resolution shows this regulator as an elongated dimer consisting of two monomers related by a two-fold axis of symmetry (Fig. 4). Each monomer contains five α-helices and two β-strands in an ααααββα fold. Two of the helices, α3 and α4 (αR), form the standard helix-turn-helix (HTH) motif present in many DNA-binding proteins. SmtB has strong structural similarities to other HTH transcriptional regulators such as CAP protein or DtxR (Cook et al., 1998). *<Figure 4 near here>*

Analysis of mercuric acetate soaked crystals suggest the presence of four putative Zn^{2+}-binding sites per dimer. It has been proposed that one of them is formed by a molecule of water and residues from each monomer near the α3 helices: Cys61, Asp64 and His97. The other Zn^{2+}-binding site might involve residues Asp104 and His106 from one monomer and His117 and Glu120 from the other monomer, bridging α5 helices (Cook et al., 1998). The proposed DNA recognition α-helix (αR) is highly conserved among the SmtB/ArsR family members and it confers a high degree of sequence identity (25-50%), allowing the generation of models of SmtB/ArsR repressors based on the *Synechococcus* SmtB crystal structure (Busenlehner et al., 2003).

Two characteristic metal binding sites have been described in SmtB/ArsR family members, the α3N and the α5 sites. It has been proposed that the α3N binding site senses larger, thiophilic metals such as Cd(II) or Pb(II) and contains the highly conserved ELCVCDL

sequence named the "metal binding box", whose cysteine pair may be important for metal recognition (Shi et al., 1994). This metal binding site is not regulatory in SmtB (VanZile et al., 2002b). However, substitution of both cysteine residues in *Synechocystis* ZiaR inhibited metal responses *in vivo* (Thelwell et al., 1998). Consistent with this, the second cysteine residue, Cys74, has been suggested to be a critical metal ligand in *Anabaena* AztR (Liu et al., 2005). In *Synechocystis* ArsR, its "metal binding box" EQCVCDL sequence also contains the pair of cysteine residues suggested to interact with arsenite. To our knowledge, no studies about putative metal binding sites in cyanobacterial ArsR orthologues have been published. The X-ray structure of apo-SmtB revealed that the α3 helix contains this metal binding motif; however, in this regulator, the last cysteine residue is naturally substituted by a glycine residue: ELCVGDL (Cook et al., 1998). Surprisingly, the remaining cysteine residue in the motif, Cys61, is not essential in SmtB for Zn^{2+}-sensing *in vivo* (Turner et al., 1996) Conversely, the α5 metal binding site is composed of ligands derived exclusively from the α5 helix (Fig. 5). Substitution of His105/His106 in SmtB or His116 in ZiaR by arginine residues in the α5 helix resulted in a loss of induction by zinc, suggesting that this metal site may be important for Zn^{2+}-sensing *in vivo* for both regulators (Thelwell et al., 1998; Turner et al., 1996). The α5 metal binding site probably resists distortion to accommodate larger metal ions, interacting preferably with smaller divalent ions such as Zn(II), Co(II) and Ni(II) (Pennella and Giedroc, 2005). *<Figure 5 near here>*

Anabaena AztR lacks the α5 metal binding site, and thus it utilizes the α3N site to sense not only small essential Zn(II) ions but also larger toxic Cd(II)/Pb(II) ions (Liu et al., 2005). The noteworthy structural plasticity of the α3N site has also been shown by its ability to allow direct binding of monovalent ions Cu(I)/Ag(I) in *Oscillatoria* BxmR (Liu et al., 2008). This adaptation of the metal binding site to different kinds of metals suggests an evolutionary

mechanism developed to confer increased resistance to other toxic heavy metals on certain cyanobacteria.

The sequence of ArsR orthologues does not usually contain the N-terminal region present in SmtB-like proteins. Thus, *E. coli* ArsR possesses three α3 cysteines in the α3 helix, which induce metal responsiveness due to the lack of the α5 site in this regulator. Similarly, *Synechocystis* ArsR does not possess ligands related to the α5 site either, so it presumably binds metal ions through the α3 cysteine site (Osman and Cavet, 2010). <Table 4 near here>

An overall comparison of the cyanobacterial SmtB/ArsR repressors reveals distinct metal binding properties which are summarized in Table 4. *Synechococcus* SmtB binds two zinc ions per dimer through the α5 site (VanZile et al., 2002b). *Synechocystis* ZiaR requires the binding of four zinc ions to both α3N and α5 sites to induce zinc metalloregulation *in vivo* (Thelwell et al., 1998). *Oscillatoria* BxmR also possesses both sites, using either α5 or α3N to bind zinc ions. However, this regulator effects more selective and effective zinc regulation by binding Zn(II) to the α5 site, while the α3N site is required for Cu(I), Ag(I) or Cd(II) sensing. BxmR binds four Cu(I) ions per dimer in α3N site, but only two Zn(II) or Cd(II) ions per dimer in either α5 or α3N sites (Osman and Cavet, 2010). *Anabaena* AztR only retains the two α3N sites per dimer, so it senses not only two Zn(II) ions but also two Cd(II) and Pb(II) ions per homodimer through this site (Liu et al., 2005). AzuR is the other metalloregulator in *Anabaena* that belongs to the SmtB/ArsR family. This has been proposed to be a zinc repressor more closely related to SmtB (Liu et al., 2008). Finally, *Synechocystis* ArsR is induced *in vivo* by As(III) and Sb(III), but not by As(V) (Lopez-Maury et al., 2003).

2. DNA-binding sites.

The SmtB/ArsR transcriptional repressors often regulate expression of divergently

transcribed genes, arranged in operons. These metalloregulators are specifically bound to their DNA operator/promoter (O/P) binding sites in the metal-free state.

Most of the O/P sequences in this family contain one imperfect 12-2-12 inverted repeat, generally overlapping or located near the transcriptional start site of the genes (Fig. 6). For instance, only one pair of contact sites are described for *Synechocystis* ZiaR or *Anabaena* AzrR within the *zia* and *azt* divergons, respectively, (Fig. 6A) (Liu et al., 2005; Thelwell et al., 1998). Nonetheless, in *Synechococcus,* the *smt* operon displays two of these repeats contacted by SmtB at the conserved TGA sequence: S2/S1 lies at the *smtA* transcriptional start site while S4/S3 lies between the *smtA* and *smtB* -10 sequences. At low concentrations, recombinant SmtB binds *in vitro* as a monomer either to S1 or S2; as the SmtB concentration increases, it binds as a dimer at both sites. At high SmtB concentrations, the metal-free regulator may form a homotetrameric complex. The formation of all the three DNA-SmtB complexes is inhibited by Zn^{2+} *in vitro* (Erbe et al., 1995). In *Oscillatoria,* there is one imperfect 12-2-12 inverted repeat in the region between *bxmR* and *bmtA*, and another one found in the promoter region of *bxa1* (Fig. 6B) (Liu et al., 2004). The *Synechocystis arsBHC* operon is repressed by the product of a physically separated gene, ArsR. The ArsR binding site within the *arsBHC* O/P region contains two 17-bp direct repeats of the sequence ATCAAGTTTTTTTGATG, each one consisting of two inverted repeats (Lopez-Maury et al., 2003). *<Figure 6 near here>*

Synechococcus mutants with an interrupted *smt* divergon are 5-fold sensitive to Zn^{2+} and show some reduction in tolerance to Cd^{2+} (Turner et al., 1993). The same behavior is observed in *Synechocystis zia* divergon mutants and both operons seem to be interchangeable. Restoration of metal resistance is achieved by the introduction of *zia* in the *Synechococcus smt* mutant (Thelwell et al., 1998).

3. Functions of SmtB proteins.

In the absence of zinc, *Synechococcus* SmtB is bound to a specific region of the *smt* operon, repressing the divergent transcription of both itself and the gene s*mtA* encoding the metallothionein SmtA (VanZile et al., 2002a). When metal availability increases, two zinc ions are bound to the α5 site in the homodimer triggering transcription until no zinc is left to bind to newly synthesized SmtB and repression is again effective. *Synechococcus smtA* mutants exhibited a five-fold reduction in zinc tolerance (Turner et al., 1993). SmtA scavenges excess zinc from adventitious sites sequestering it in a non-toxic form. However, overexpressing *smtA* mutants are viable, implying that SmtA does not remove zinc from advantageous sites (Turner et al., 1995). The release of zinc from SmtA has not yet been elucidated. Interaction with another protein or even SmtA degradation have been proposed as zinc release possibilities (Robinson et al., 2001). Additionally to zinc detoxification, another function in zinc accumulation has been suggested for the metallothionein SmtA. The DNA-primase gene, *dnaG*, is located adjacent to *smtA* in *Synechococcus* PCC 7942. It is not known whether SmtA can influence the zinc content of the predicted zinc-finger present in this DNA-primase (Robinson et al., 2001).

The *Synechocystis zia* operon is organized in a similar way to the *smt* region; however, ZiaR controls the transcription of a zinc-exporting ATPase ZiaA. In the absence of zinc, the operon is repressed but when metal availability increases, the expression of both the regulator ZiaR and the ATPase ZiaA triggers zinc efflux into the periplasm until levels are low enough to arrest *ziaA* transcription. In addition to zinc hypersensibility, reduced zinc export to the periplasm is observed in *ziaA* mutants (Thelwell et al., 1998).

Thus, two alternative ways of zinc detoxification are represented in these two cyanobacteria: metal sequestration in cytosol by a metallothionein or metal export to the periplasm by an ATPase. In *Oscillatoria* both systems are present, since BxmR metalloregulates not only the

expression of the metallothionein BmtA but also the transcription of the ATPase Bxa1. As soon as Zn(II) or Cd(II) are sensed by BxmR, the transporter Bxa1 is firstly induced in a rapid response to restore the intracellular metal homeostasis, while BmtA is transcribed relatively slowly as a long-term defense against metallotoxicity (Liu et al., 2004).

In *Anabaena*, the AztR regulator responds to zinc, cadmium and lead allowing the transcription of the ATPase AztA, which transports divalent ions from cytosol to periplasm (Liu et al., 2005). Surprisingly, the genome of this cyanobacterium also encodes the metallothionein BmtA but an associated SmtB/ArsR regulator has not yet been found (Blindauer, 2008). Notably, a second SmtB ortholog AzuR has been described in *Anabaena*, but it has not yet been well characterized either biochemically or functionally (Liu et al., 2008).

In *Synechocystis*, ArsR senses As(III) and Sb(III) ions and regulates the *arsBHC* operon involved in arsenic and antimony resistance. The *arsC* gene encodes a putative arsenate reductase, related to arsenate detoxification, probably involved in reduce As(V) to As(III). ArsB is a putative arsenite and antimonite exporter, while no function has been assigned to ArsH (Lopez-Maury et al., 2003).

4. Allosteric regulation and autoregulation.

Direct interaction of the metal ions in the regulatory sites of the SmtB/ArsR repressors negatively regulates the specific operator/promoter binding affinity *in vitro*. Negative allosteric regulation of DNA binding is a common characteristic of these family members (Liu et al., 2004).

The binding of two Zn^{2+} ions to the α5 site in the dimer interface of *Synechococcus* SmtB probably induces an overall compaction of the repressor from its conformation in the metal-free state. This conformational change in SmtB could disrupt the geometry necessary for the

interaction of the regulator with the binding sites (Kar et al., 1997). In *Synechocystis* ZiaR, binding of the metal to both α3N and α5 sites seems to be necessary, implying co-operativity between these sites (Thelwell et al., 1998). *Anabaena* AztR lacks the dimer interface metal binding residues (α5 site), thus metal binding to the helix-turn-helix is the proposed simple mechanism to induce DNA dissociation (Liu et al., 2005). *Oscillatoria* BxmR is dissociated from specific DNA *in vitro* upon the addition of both monovalent and divalent metal ions. Cu(I), Ag(I) and Cd(II) inhibit BxmR-*bxa1* O/P DNA binding in an equally effective way, using α3N as metal binding sites. Concerning Zn(II), this metal is capable of functioning through both metal sites, although binding to the α5 site results in a more effective negative allosteric regulation (Liu et al., 2008).

A mechanism of autoregulation has been described for cyanobacterial SmtB-like proteins. Since the SmtB orthologues are encoded in the divergently transcribed operons that are induced by themselves, their expression is also controlled by metal availability. This autoregulatory mechanism allows returning to the repression condition. However, in *Synechocystis,* the *arsR* gene is not autoregulated, since it is expressed constitutively at low level. It is worth noting that in the absence of ArsR, normal growth parameters in *Synechocystis* are observed, in spite of the constitutive expression of ArsB which results toxic for *E. coli* when it is overexpressed (Lopez-Maury et al., 2003).

E. THE MerR FAMILY OF PROTEINS

MerR proteins act as dimeric transcriptional activators that may directly interact with RNA polymerase to achieve a functional fit to DNA. A metal-induced DNA-conformational change distorts the operator structure, allowing RNA polymerase to initiate transcription from a sub-optimal promoter which has indeed become a potent one. MerR proteins are also capable of autoregulating their own expression (Brown et al., 2003). The MerR family includes sensors

of mercury (MerR in transposable elements Tn*21* or Tn*501*), zinc, cadmium and lead (ZntR in *E. coli*), lead (PbrR in *Ralstonia metallidurans*), copper, gold and silver (CueR in *E. coli*, GolS in *Salmonella*) or cobalt (CoaR in *Synechocystis*) (Osman and Cavet, 2010).

It is noteworthy that MerR proteins also act as weak repressors in the absence of the metal despite being activators. Both repression and activation processes occur while MerR proteins are bound at the same DNA region, between the -10 and -35 promoter elements. This promoter sequence presents an unusual structure as it is about 2 bp longer (19-20 bp) than canonical bacterial promoters. It impairs RNA polymerase from functioning until DNA bending and twisting is possible by metal-regulator interaction. A high degree of sequence similarity in the N-terminal DNA binding region is another important feature among members of the MerR family, containing a predicted helix-turn-helix motif followed by a long coiled-coil region. A small C-terminal domain confers metal selectivity (Brown et al., 2003).

In Gram-negative bacteria, MerR regulates expression of the *merTP(C/F)AD(E)* operon in response to mercury. This metal resistance operon encodes the putative transporters MerT, MerC, MerF or MerE, a periplasmic protein MerP, a mercuric reductase MerA and a putative repressor MerD. MerR proteins from these transposons are 144-aminoacid long with a much conserved N-terminal sequence and a variable C-terminal region. MerR family members from Gram-positive bacteria differ from those from Gram-negative ones; they share about 37% aminoacid identity. Nonetheless, all MerR proteins contain three conserved cysteine residues, suggested to be involved in Hg^{2+} coordination.

In cyanobacteria, a MerR orthologue has been identified by two different research groups as CoaR or CorR. *Synechocystis* PCC 6803 *coaR* (*corR*) is divergently transcribed from *coaT* (*corT*), encoding a putative Co^{2+} efflux pump. CoaR is a 370-aminoacid long protein with two different domains. The N-terminal domain aligns with MerR proteins, while the C-terminal region shows sequence similarity to precorrin isomerases, involved in the biosynthetic

pathway of cobalamin. This vitamin B_{12} contains four corrin rings coordinating a cobalt atom. Notably, a Cys-His-Cys C-terminal motif is involved in cobalt sensing. The *coaR-coaT* intergenic region contains a 20 bp spacer with the AAACCTTGCATT-N_6-AATGTTAAGGTTT inverted repeat sequence. CoaR binds to this DNA region, and it responds to Co^{2+} and to Zn^{2+} to a lesser extent. There are two proposed activation models. The first, described by García-Domínguez et al., suggests that Co^{2+} binds the corrinoid ring and this complex interacts with CoaR to activate it. Conversely, Rutherford et al. propose that the metal and the corrinoid ring bind to different domains in CoaR (Garcia-Dominguez et al., 2000; Rutherford et al., 1999).

F. THE NICKEL-SENSOR PROTEINS

Another important family of metal sensor proteins is made up of NikR orthologues. In general, NikR proteins act as repressors when bound to DNA in the presence of the co-repressor metal nickel. However, nickel-bound *H. pylori* NikR can also function as a DNA activator. NikR proteins repress the transcription of the *nik* operon, encoding the nickel uptake transporter NikABCDE (Osman and Cavet, 2010). Neither NikR orthologues nor Ni(II)-dependent SmtB/ArsB family members have been identified in cyanobacteria to date.

1. Nickel-sensing systems in cyanobacteria.

Nickel sensing has best investigated in *Synechocystis* PCC 6803 where two kinds of mechanisms are involved, one controlled by a two-component system that detects periplasmic nickel while the other consists of a cytosolic nickel sensor.

Some years ago, García-Domínguez et al. discovered a metal-regulated cluster in *Synechocystis.* This includes the previously described zinc and cobalt response system ZiaR-ZiaA and CoaR-CoaT respectively (Fig. 7). Additionally, the nickel response *nrs* operon,

induced in the presence of Ni^{2+} and Co^{2+}, was also identified. This *nrsBACD* operon encodes a putative membrane-bound protein complex that exports Ni^{2+} by a cation/proton antiport (NrsA and NrsB), an unknown function protein (NrsC) and a putative permease belonging to the major facilitator superfamily (MSF) which contains a histidine-rich region involved in Ni^{2+} binding (NrsD) (Garcia-Dominguez et al., 2000). Subsequently, *nrsR* and *nrsS* genes were included in this metal-regulated cluster in *Synechocystis*. NrsR and NrsS constitute a two-component signaling system involved in Ni^{2+} sensing and in *nrsBACD* operon regulation.

<Figure 7 near here>

a. *Properties of the NrsR/ NrsS two-component system.* NrsS is a histidine kinase sensor with an N-terminal periplasmic domain that senses periplasmatic nickel. NrsR belongs to the PhoB/OmpR response regulator family with an N-terminal phosphorylation domain and a C-terminal DNA-binding region. It is suggested that the regulator NrsR binds two direct repeats GA(A/T)TTTCA separated by 3 bp in the intergenic region near the -10 box of the two operons, in a similar way to the founder member of its family PhoB. The proposed mechanism of action is that NrsS may sense the presence of nickel in the periplasm and transfer a phosphate to NrsR. Phosphorylated NrsR binds to the *nrsRS-nrsBACD* intergenic region inducing its expression (Lopez-Maury et al., 2002).

b. *The InrS repressor.* Recently, a new cytosolic-nickel sensor, InrS, has been described. This represses the expression of the permease NrsD, which exports nickel to the periplasm from a promoter in the *nrsC-nrsD* intergenic region. In *Synechocystis,* InrS possesses a histidine-rich motif in the N-terminal region, and it responds to nickel and cobalt but not to copper. It has been proposed that InrS binds two inversed repeats containing several G/C flanked by an A/T-rich sequence (Foster et al., 2012).

Overall, when Ni^{2+} accumulates in the periplasm of *Synechocystis*, the *nrsBACD* operon is expressed under the control of the NrsRS system, allowing nickel efflux across the outer membrane. Meanwhile, cytosolic Ni^{2+} is sensed by InrS inducing the expression of the NrsD permease that exports nickel from the cytosol.

G. METAL SENSORS AND NITROGEN METABOLISM

Nitrogen assimilation and biological nitrogen fixation require a large number of metalloproteins, and changes in the availability of transition metals pose a particular challenge to the supply of these critical nutrients (Glass et al., 2009). Nitrogen control in cyanobacteria is mediated by NtcA and the signal transduction P_{II} protein, but neither of them are metalloproteins. As mentioned previously, some key genes involved in nitrogen fixation and heterocyst development are coordinately modulated by NtcA and FurA, the latter also sensing iron availability.

Molybdenum is a key element in several enzymes involved in nitrogen assimilation and fixation, sulfur, and carbon metabolism. Nitrogenase requires Mo as part of its metal cofactor, and diazotrophic growth of *A. variabilis* has shown to be dependent on the presence of Mo or V, with little growth occurring in their absence (Herrero et al., 2001). In *Azotobacter*, Mo represses the synthesis of both V nitrogenase and nitrogenase-3, and in the absence of Mo, V represses the synthesis of nitrogenase-3 (Luque and Pau, 1991). Molybdenum metabolism and homeostasis are regulated by the molybdate-responsive transcription factor ModE. Orthologues of ModE are widespread amongst diverse prokaryotes but not ubiquitous, and DNA-binding motifs have been identified to be quite conserved (Studholme and Pau, 2003). Little is known about Mo-dependent transcriptional regulators in cyanobacteria. Putative *modE*-like genes have been described in several cyanobacterial genomes (Nakamura et al.,

1998), but little information is available in the current literature concerning the role of this regulator in cyanobacteria.

IV. CONCLUSIONS AND PERSPECTIVES

Metalloregulation in cyanobacteria is not restricted to ensuring optimal metal ion homeostasis. The coordination of metal homeostasis with the response to environmental stresses and central metabolic processes is often carried out by metalloproteins with regulatory functions. Although most of the main families involved in these tasks have been characterised in recent years, further work based on functional genomics and structural biology remains to be done. The identification of new players, such as potential metal-binding non-coding RNAs, will provide a more complete picture of the cyanobacterial metallome.

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TABLE 1

PerR regulated genes in Synechocystis PCC 6803 (Li et al., 2004)

Gene ID[b]	Synonym	Gene product description
slr1738	*perR*	Peroxide regulon repressor
sll1621	*ahpC*	Alkyl hydroperoxide reductase
sll1620		Unknown protein
slr1739	*psbW-like*	Unknown protein
ssl2667	*cnfU*	NifU-like C-terminal
sll0621	*dsb-like*	c-type cytochrome biogenesis
slr0513	*idiA*	Iron-deficiency induced protein A homologue
sll0247	*isiA*	Iron-stress inducible Chl-binding protein IsiA
sll1135		Unknown protein
sll1483		Fasciclin-like domain
slr1894	*mrgA*	Metal-regulated gene
slr1204	*htrA*	HtrA protease

TABLE 2
FurB/Zur regulated genes in Anabaena PCC 7120 (Napolitano et al., 2012)

Functional category[a]	Gene ID[b]	Synonym	Gene product description
ABC transport systems	*all0833*	*znuA*	Periplasmic solute binding protein
	all0832	*znuB*	ABC-transporter, ATP binding protein
	all0830	*znuC*	ATP-transporter permease protein
	alr3243		ABC-transporter, perimplasmic-binding protein
	alr4031		ABC-transporter, perimplasmic-binding protein (COG0614)
Outer membrane proteins	*alr3242*		TonB-dependent receptor (COG1629)
	alr4028-4029		TonB-dependent transporter (outer membrane)
	all3515		Putative outer-membrane protein
Paralogues of zinc metalloproteins	*all7621*	*aztR*	ArsR/SmtB-family transcriptional regulator
	all47121	*folE*	GTP-cyclohydrolase
	all4723	*thrS2*	Threonyl-tRNA synthetase
	all4725	*hemE*	Porphobilinogen synthase
Operons containing putative metallochaperones	*alr1197*		CobW-C superfamily
	all1198		Metallophosphoesterase COG0622
	alr1199		Metallo-dependent phospatase
	all1751		Putative metallochaperone COG0523
	all1750		WD40 repeat-containing protein
Glycosyl transferases	*alr2866*		Glycosyl transferase, family 2
Others	*alr3495*		Uncharacterized conserved protein COG1262
	all1474		CRISPR-associated RAMP protein, SSO1426 family COG1337
	alr4030		Putative ferredoxin (thiorredoxin fold) COG 3411
	all4722		P-loop GTPase (COG0523 family)
	all4724		Putative FAD-dependent oxidoreductase

TABLE 3

FurA regulated genes in Anabaena PCC 7120

Functional category	Gene ID[a]	Gene product description	Reference
Iron metabolism	*schT*	Siderophore outer membrane transporter	González et al, 2010
Oxidative stress defenses and	*dpsA*	Nutrient-stress induced DNA binding protein	Hernández et al, 2007
redox regulation	*gor*	Glutathione reductase	López-Gomollón et al, 2007a
	trxA	Thioredoxin	López-Gomollón et al, 2007a
	trxB	Thioredoxin-reductase	González et al, 2011
	all1541	Peroxiredoxin 2 family protein/glutaredoxin	González et al, 2011
	alr4641	Peroxiredoxin	González et al, 2011
Photosynthesis and respiration	*isiA*	Photosystem II chlorophyll α-binding protein	Leonhardt et al, 1994
	isiB	Flavodoxin	Bes at al, 2001
	rbcL	Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit	López-Gomollón et al, 2007a
	psaL	Photosystem I subunit XI	López-Gomollón et al, 2007a
	psbZ	Photosystem II 11 kDa protein	López-Gomollón et al, 2007a
	coxA	Cytochrome C oxidase subunit I	López-Gomollón et al, 2007a
	coxB	Cytochrome C oxidase subunit II	López-Gomollón et al, 2007a
	ndhF	NADH dehydrogenase subunit 5	López-Gomollón et al, 2007a
	petH	Ferredoxin-$NADP^+$ reductase	López-Gomollón et al, 2007a
	prk	Phosphoribulokinase	López-Gomollón et al, 2007a

	psbA	Photosystem II reaction center protein D1	González et al, 2010
	ccmM	CO_2 concentrating mechanism protein	González et al, 2011
Nitrogen metabolism	*ntcA*	Master transcriptional regulator of nitrogen metabolism	López-Gomollón et al, 2007a
	glnA	Glutamate-ammonia ligase	López-Gomollón et al, 2007a
	gltS	Ferredoxin-glutamate synthase	López-Gomollón et al, 2007a
	nifH	Nitrogenase iron protein	López-Gomollón et al, 2007a
	Abp1	DNA binding protein	González et al, 2011
Others	*furA*	Ferric uptake regulator	Bes at al, 2001
	furB	Ferric uptake regulator	Hernández et al, 2004a
	furC	Ferric uptake regulator	Hernández et al, 2004a
	sigC	RNA polymerase sigma-subunit	López-Gomollón et al, 2007a
	hanA	DNA binding protein HU	López-Gomollón et al, 2007a
	α-furA[b]	Antisense RNA	López-Gomollón et al, 2007a
	mreBCD	Operon encoding bacterial actins	González et al, 2010
	all3556	Succinate-semialdehyde dehydrogenase	González et al, 2011
	tldD	Putative modulator of DNA gyrase	González et al, 2011
	pmbA	Putative modulator of DNA gyrase	González et al, 2011
	orrA	Two-component response regulator	González et al, 2011
	thiC	Thiamin biosynthesis protein	González et al, 2011

[a]Gene identification according to the cyanobacteria genome database CyanoBase (http://genome.kazusa.or.jp/cyanobase)
[b]Gene identification according to Hernández et al, 2006b

TABLE 4
Summary and comparison of metal binding properties of cyanobacterial SmtB/ArsR proteins

Sensor	"Metal – binding box"	Metal	Metal site(s)	Ligands
***Synechococcus* SmtB**	ELCVGDL	Zn(II)	α5	Asp104 His106 His117 Glu120
***Synechocystis* ZiaR**	ELCVCDL	Zn(II)	α5 and α3N	α5: Asp114 His116 His127 Glu130 α3N: Cys71 and/or Cys73 Cys His
		Zn(II)	α5 and α3N	α5: Asp119 His121 His132 Glu135
***Oscillatoria* BxmR**	ELCVCDL	Cu(I), Ag(I), Cd(II)	α3N	α3N: Cys23 Cys31 Cys75 Cys77
***Anabaena* AztR**	ELCVCDL	Zn(II) Cd(II) Pb(II)	α3N	α3N: Cys21 Cys72 Cys74 His
***Anabaena* AzuR**	ELCVSDL	Zn(II)? Co(II)?	α5?	?
***Synechocystis* ArsR**	EQCVCDL	As(III) Sb(III)	α3	?

Figure legends (printed version, for figs in Black and White)

Figure 1. Three-dimensional model of *Pseudomonas aeruginosa* Fur generated by PISA Server (Krissinel and Henrick, 2007) and drawn using PyMOL (Delano, 2006). Several chains of amino acids discussed in the text are shown in darker grey.

Figure 2. Alignment of a representative subset of different members of the Fur family from cyanobacteria. A. 7120, *Anabaena* sp. PCC 7120; S. 6803, *Synechocystis* sp. PCC 6803; S. 7942, *Synechococcus* sp. PCC 7942. The conserved histidine in the N-terminal domain potentially involved in DNA-binding and the his-rich motif are boxed. Cysteine residues in the CXXC redox motifs are indicated with asterisks. Heme-regulatory CP motifs are indicated in grey boxes.

Figure 3. Proposed model for the influence of the intracellular iron concentration and the presence of reactive oxygen species (ROS) in FurA activity.

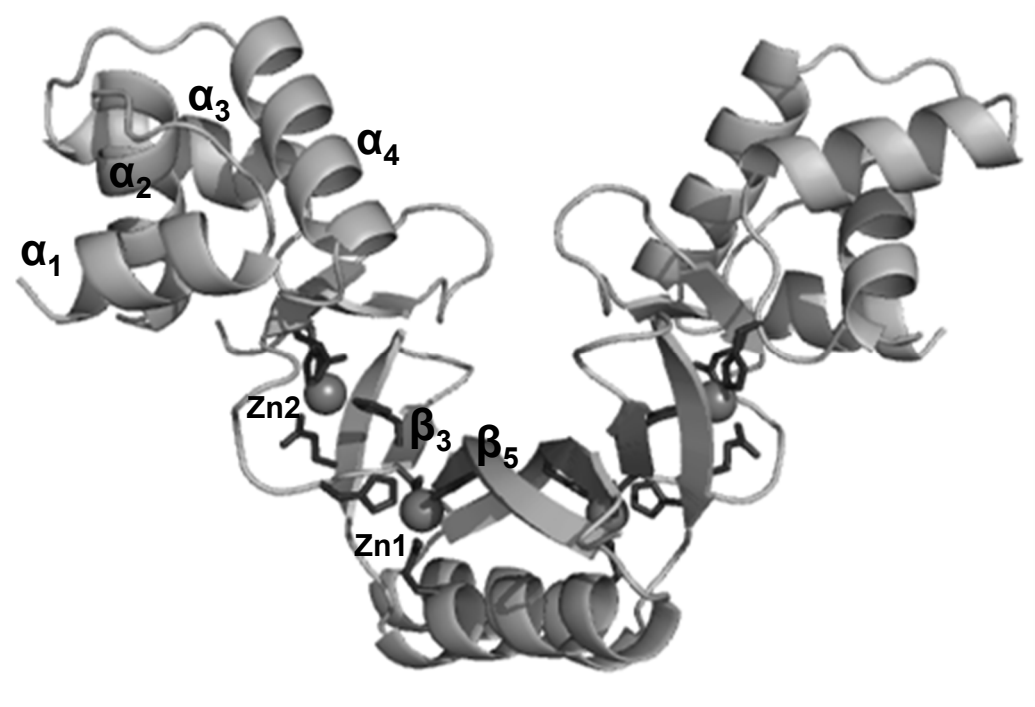
Figure 4. Ribbon diagram of the crystal structure of the apo-SmtB dimer. The structure was imported from UniProt KB (access No 1SMT) and drawn with PyMol program (ExPASy server). Each monomer shows an αααααββα fold (Cook et al., 1998). The α5, α3N and αR helices are labeled. Residues from 1 to 24 have been added manually in the figure.

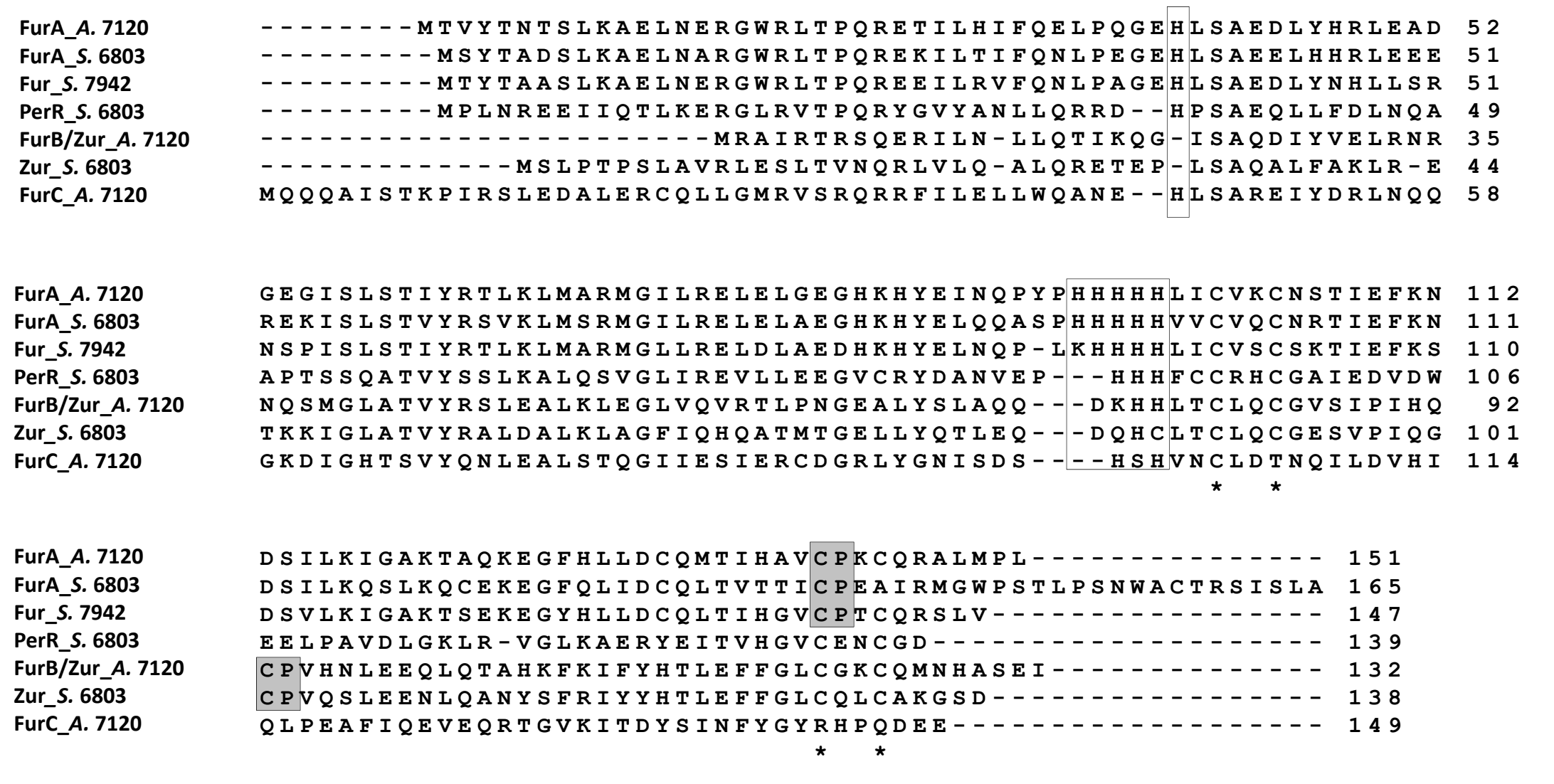
Figure 5. Sequence alignment of cyanobacterial SmtB/ArsR family metalloregulators generated using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The proposed "metal binding box" with the Cys-X-Cys motif is boxed and grey shaded. Residues known or predicted to be metal ligands in the α3N site are denoted with an asterisk, and

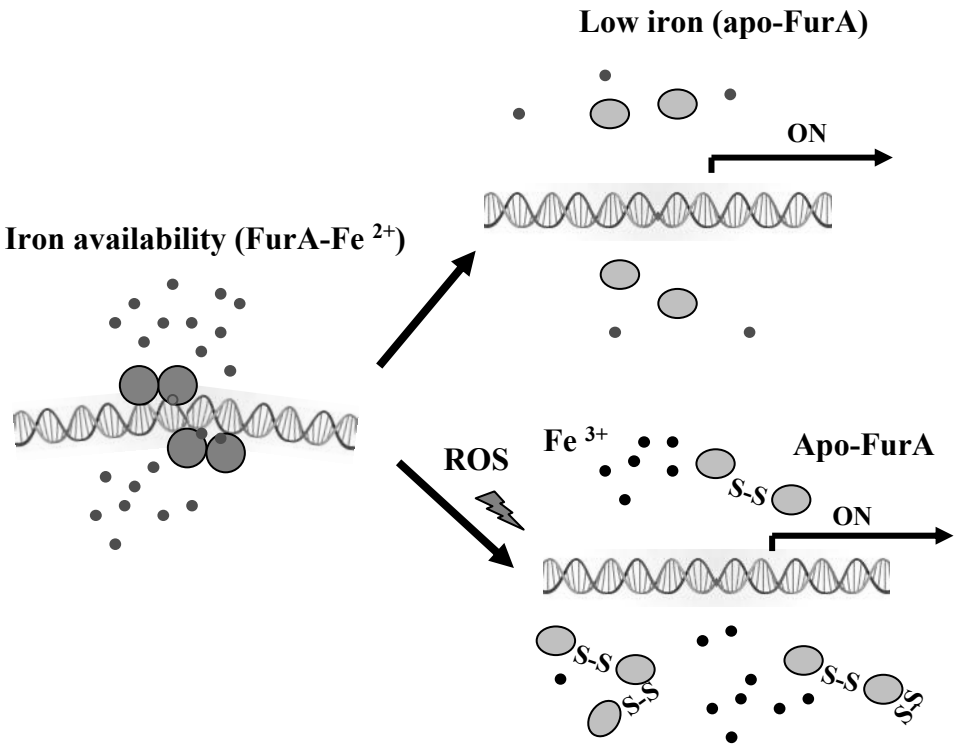
those in the α5 site are boxed. The secondary structure assignment is based on the studies of SmtB by Cook et al. (Cook et al., 1998).

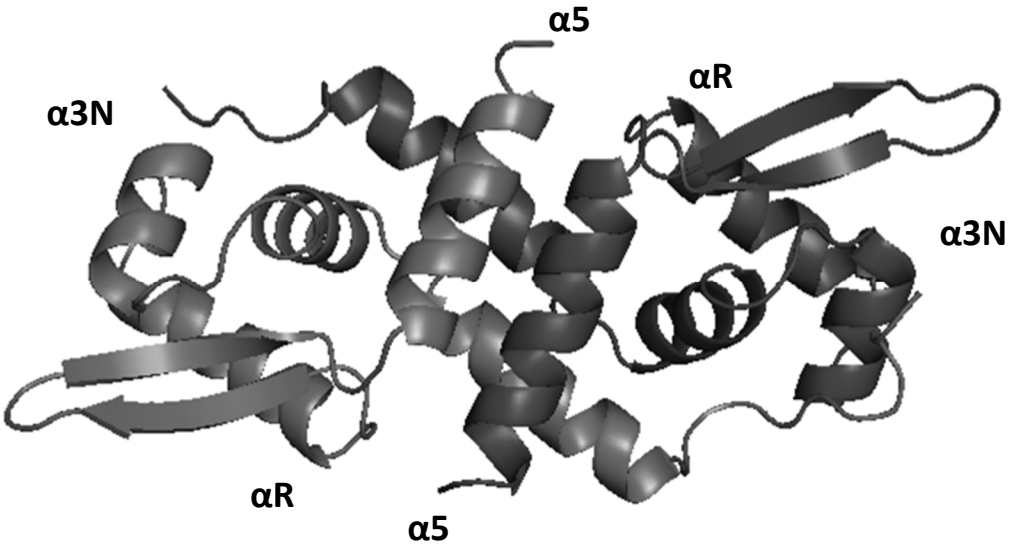
Figure 6. A. Organization of the operons encoding cyanobacterial metal-regulated SmtB repressors. Genes encoding metalloregulators (dotted arrows), ATPase genes (grey arrows) and genes encoding metallothioneins (white arrows) are represented. Black arrows indicate the imperfect inverted repeats where the SmtB regulators are bound. ([1](Erbe et al., 1995); [2](Thelwell et al., 1998); [3](Liu et al., 2004); [4](Liu et al., 2005)). B. Alignment of the DNA-binding sites containing the 12-2-12 inverted repeat sequences from the operons controlled by cyanobacterial SmtB metalloregulators. The conserved sequence TGA which is supposed to be in contact to the regulators is underlined.

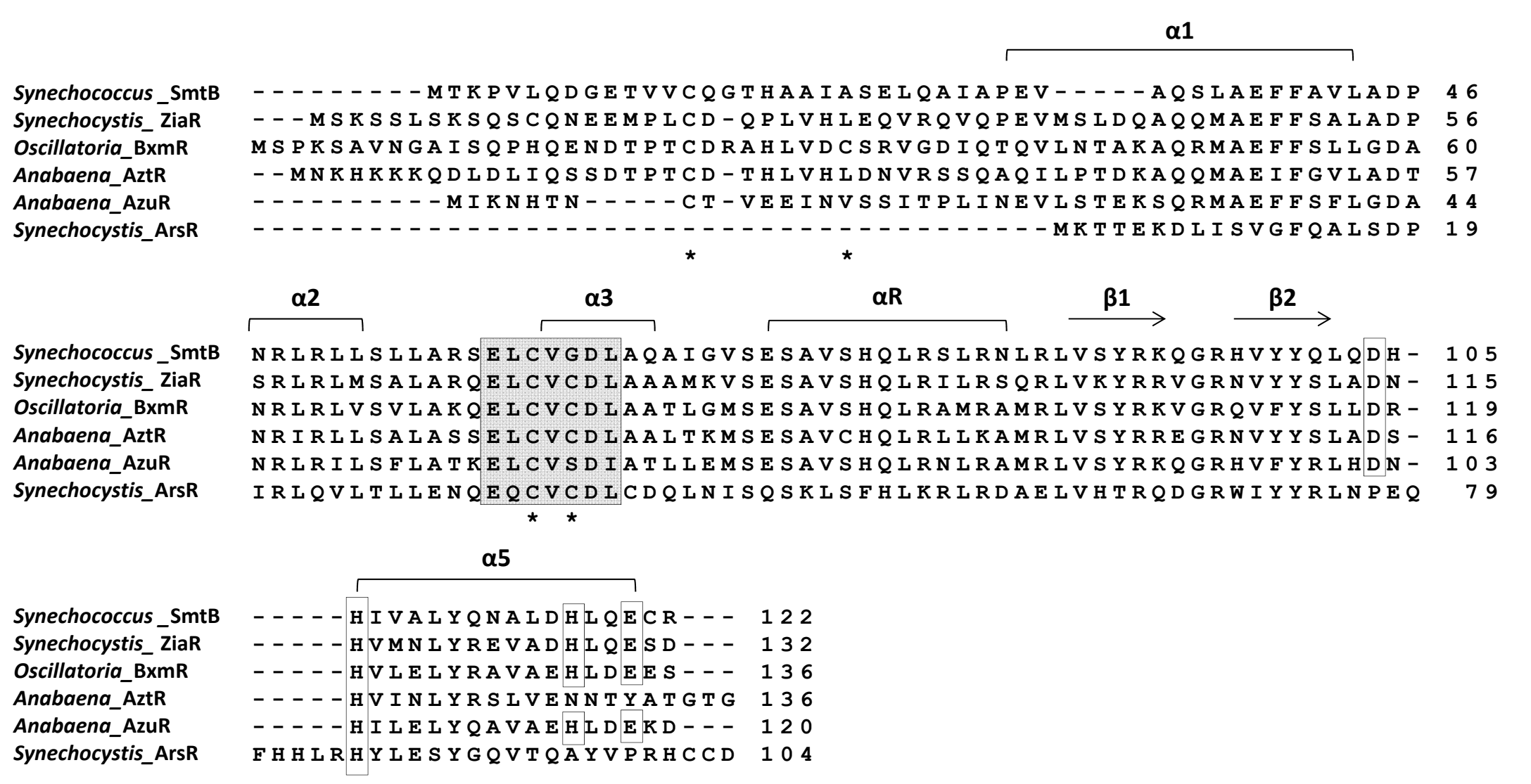
Figure 7. Genetic organization of the metal-regulated cluster in *Synechocystis*. The different metal-resistance operons and target DNA sequences for nickel resistance are indicated. Genes encoding metalloregulators (dotted arrows), ATPase genes (light grey arrows), genes encoding the two-component system NrsRS (dark grey arrows) and genes with unknown function (white arrows) represented. Direct or inverted repeat sequences present in the NrsR and InrS DNA-binding sites are denoted with arrows (Foster et al., 2012).

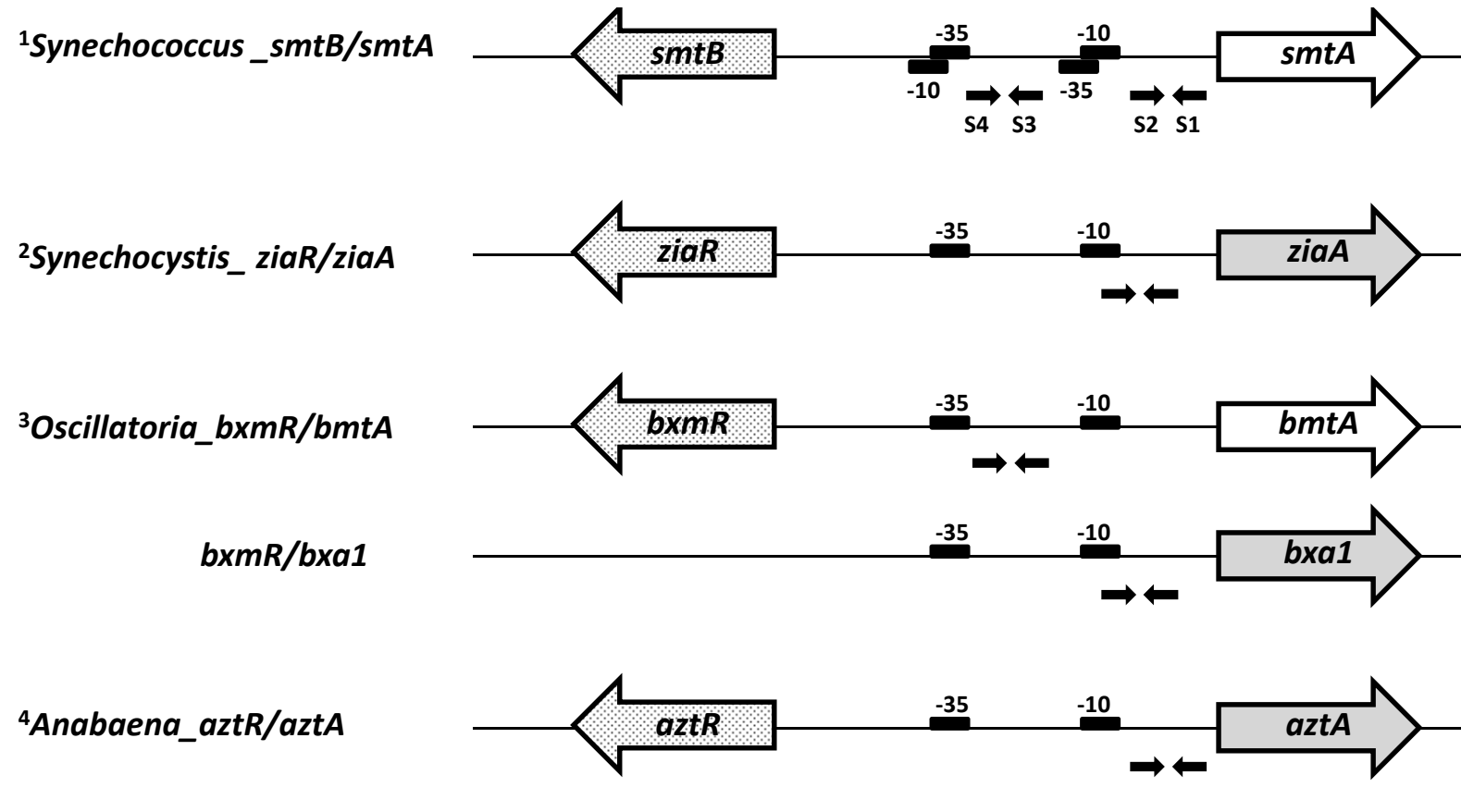

α3
α4
α2
α1
Zn2
β3
β5
Zn1

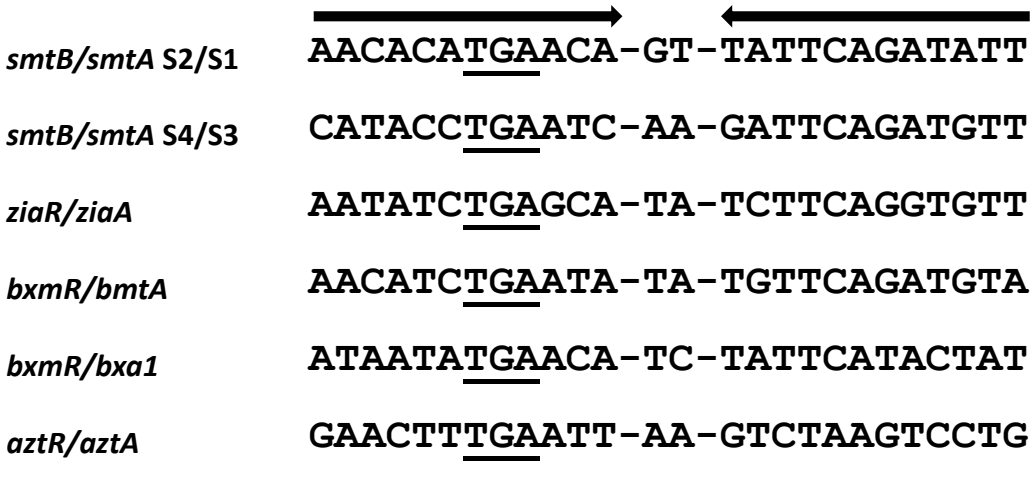

FurA_A. 7120 --------MTVYTNTSLKAELNERGWRLTPQRETILHIFQELPQGEHLSAEDLYHRLEAD 52
FurA_S. 6803 ---------MSYTADSLKAELNARGWRLTPQREKILTIFQNLPEGEHLSAEELHHRLEEE 51
Fur_S. 7942 ---------MTYTAASLKAELNERGWRLTPQREEILRVFQNLPAGEHLSAEDLYNHLLSR 51
PerR_S. 6803 ---------MPLNREEIIQTLKERGLRVTPQRYGVYANLLQRRD--HPSAEQLLFDLNQA 49
FurB/Zur_A. 7120 ----------------------MRAIRTRSQERILN-LLQTIKQG-ISAQDIYVELRNR 35
Zur_S. 6803 ----------MSLPTPSLAVRLESLTVNQRLVLQ-ALQRETEP-LSAQALFAKLR-E 44
FurC_A. 7120 MQQQAISTKPIRSLEDALERCQLLGMRVSRQRRFILELLWQANE--HLSAREIYDRLNQQ 58
FurA_A. 7120 GEGISLSTIYRTLKLMARMGILRELELGEGHKHYEINQPYPHHHHHLICVKCNSTIEFKN 112
FurA_S. 6803 REKISLSTVYRSVKLMSRMGILRELELAEGHKHYELQQASPHHHHHVVCVQCNRTIEFKN 111
Fur_S. 7942 NSPISLSTIYRTLKLMARMGLLRELDLAEDHKHYELNQP-LKHHHHLICVSCSKTIEFKS 110
PerR_S. 6803 APTSSQATVYSSLKALQSVGLIREVLLEEGVCRYDANVEP---HHHFCCRHCGAIEDVDW 106
FurB/Zur_A. 7120 NQSMGLATVYRSLEALKLEGLVQVRTLPNGEALYSLAQQ---DKHHLTCLQCGVSIPIHQ 92
Zur_S. 6803 TKKIGLATVYRALDALKLAGFIQHQATMTGELLYQTLEQ---DQHCLTCLQCGESVPIQG 101
FurC_A. 7120 GKDIGHTSVYQNLEALSTQGIIESIERCDGRLYGNISDS---HSHVNCLDTNQILDVHI 114
* *
FurA_A. 7120 DSILKIGAKTAQKEGFHLLDCQMTIHAVCPKCQRALMPL---------------- 151
FurA_S. 6803 DSILKQSLKQCEKEGFQLIDCQLTVTTICPEAIRMGWPSTLPSNWACTRSISLA 165
Fur_S. 7942 DSVLKIGAKTSEKEGYHLLDCQLTIHGVCPTCQRSLV---------------- 147
PerR_S. 6803 EELPAVDLGKLR-VGLKAERYEITVHGVCENCGD---------------- 139
FurB/Zur_A. 7120 CPVHNLEEQLQTAHKFKIFYHTLEFFGLCGKCQMNHASEI---------- 132
Zur_S. 6803 CPVQSLEENLQANYSFRIYYHTLEFFGLCQLCAKGSD---------- 138
FurC_A. 7120 QLPEAFIQEVEQRTGVKITDYSINFYGYRHPQDEE---------- 149
* *

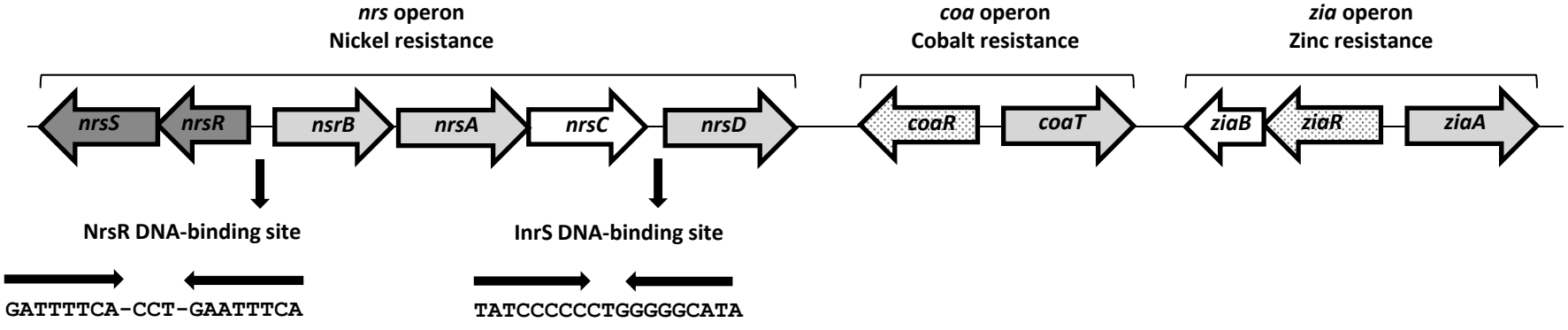

Low iron (apo-FurA)
ON
Iron availability (FurA-Fe 2+)
ROS
Fe 3+
Apo-FurA
S-S
ON
S-S
S-S
S-S
S-S


α5
αR
α3N
α3N
αR
α5


α1
Synechococcus _SmtB - - - - - - - - - MTKPVLQDGETVVCQGTHAAIASELQAIAPEV- - - - - AQSLAEFFAVLADP 46
Synechocystis_ ZiaR - - - MSKSSLSKSQSCQNEEMPLCD - QPLVHLEQVRQVQPEVMSLDQAQQMAEFFSALADP 56
Oscillatoria_BxmR MSPKSAVNGAISQPHQENDTPTCDRAHLVDCSRVGDIQTQVLNTAKAQRMAEFFSLLGDA 60
Anabaena_AztR - - MNKHKKKQDLDLIQSSDTPTCD - THLVHLDNVRSSQAQILPTDKAQQMAEIFGVLADT 57
Anabaena_AzuR - - - - - - - - - - MIKNHTN- - - - - CT - VEEINVSSITPLINEVLSTEKSQRMAEFFSFLGDA 44
Synechocystis_ArsR - MKTTEKDLISVGFQALSDP 19
*
*
α2
α3
αR
β1
β2
Synechococcus _SmtB NRLRLLSLLARSELCVGDLAQAIGVSESAVSHQLRSLRNLRLVSYRKQGRHVYYQLQDH- 105
Synechocystis_ ZiaR SRLRLMSALARQELCVCDLAAAMKVSESAVSHQLRILRSQRLVKYRRVGRNVYYSLADN- 115
Oscillatoria_BxmR NRLRLVSVLAKQELCVCDLAATLGMSESAVSHQLRAMRAMRLVSYRKVGRQVFYSLLDR- 119
Anabaena_AztR NRIRLLSALASSELCVCDLAALTKMSESAVCHQLRLLKAMRLVSYRREGRNVYYSLADS- 116
Anabaena_AzuR NRLRILSFLATKELCVSDIATLLEMSESAVSHQLRNLRAMRLVSYRKQGRHVFYRLHDN- 103
Synechocystis_ArsR IRLQVLTLLENQEQCVCDLCDQLNISQSKLSFHLKRLRDAELVHTRQDGRWIYYRLNPEQ 79
*
*
α5
Synechococcus _SmtB - - - - - HIVALYQNALDHLQECR - - - 122
Synechocystis_ ZiaR - - - - - HVMNLYREVADHLQESD - - - 132
Oscillatoria_BxmR - - - - - HVLELYRAVAEHLDEES - - - 136
Anabaena_AztR - - - - - HVINLYRSLVENNTYATGTG 136
Anabaena_AzuR - - - - - HILELYQAVAEHLDEKD - - - 120
Synechocystis_ArsR FHHLRHYLESYGQVTQAYVPRHCCD 104


[1]Synechococcus _smtB/smtA
smtB
-35
-10
-10
-35
smtA
S4
S3
S2
S1
[2]Synechocystis_ ziaR/ziaA
ziaR
-35
-10
ziaA
[3]Oscillatoria_bxmR/bmtA
bxmR
-35
-10
bmtA
bxmR/bxa1
-35
-10
bxa1
[4]Anabaena_aztR/aztA
aztR
-35
-10
aztA


smtB/smtA S2/S1
AACACATGAACA-GT-TATTCAGATATT
smtB/smtA S4/S3
CATACCTGAATC-AA-GATTCAGATGTT
ziaR/ziaA
AATATCTGAGCA-TA-TCTTCAGGTGTT
bxmR/bmtA
AACATCTGAATA-TA-TGTTCAGATGTA
bxmR/bxa1
ATAATATGAACA-TC-TATTCATACTAT
aztR/aztA
GAACTTTGAATT-AA-GTCTAAGTCCTG


nrs operon
Nickel resistance
coa operon
Cobalt resistance
zia operon
Zinc resistance
nrsS
nrsR
nsrB
nrsA
nrsC
nrsD
coaR
coaT
ziaB
ziaR
ziaA
NrsR DNA-binding site
InrS DNA-binding site
GATTTTCA-CCT-GAATTTCA
TATCCCCCCTGGGGGCATA