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REVIEW ARTICLE

Ferric Uptake Regulator (FUR) protein: properties and implications in cyanobacteria

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Abstract The Ferric uptake regulator (Fur) protein is a global iron regulator found in most prokaryotes. Although the Fur protein is involved in a variety of metabolic pathways, it is specifically known for the regulation of several iron responsive genes. It binds to the highly conserved sequences located in the upstream promoter region known as iron boxes, using ferrous ion as a co-repressor. Apart from that, the Fur protein is also directly/indirectly involved in a variety of other crucial physiological pathways. Hence, understanding the mechanism of action and the mechanistic pathways of iron regulation by Fur is necessary and important. The basic understanding of the functioning and properties of Fur protein along with its role, interaction and regulation at various levels in cyanobacteria has been discussed in detail.

Keywords Cyanobacteria · Fur protein · Fur box · Iron homeostasis

Introduction

Cyanobacteria are Gram-negative photosynthetic prokaryotes capable of fixing carbon dioxide with water as the reductant and oxygen as byproduct. Cyanobacteria are a highly diverse, widely distributed and ecologically important group of microorganisms (Abed and Garcia-Pichel 2001; Ferris 2005). They have been considered as major players in regulating carbon and nitrogen metabolism of soil and marine ecosystems. Cyanobacteria have a tremendous potential as nitrogenous biofertilizer and also produce amino acids, biological compounds and pharmaceutically important products. However, apart from their beneficial roles, they also have some harmful implications. Cyanobacteria form blooms in water reservoirs that are the primary source of toxins, named cyanotoxins, that have deleterious effects on human as well as animal health (de Figueiredo et al. 2004; Martin-Luna et al. 2006; Ferrao-Filho and Kozlowsky-Suzuki 2011; Deore and Bansal 2013). Iron availability represents one of the important factors controlling different metabolisms, including cyanotoxin production in cyanobacteria (Utkilen and Gjolme 1995; Martin-Luna et al. 2006). Although it is the fourth most abundant element in nature, iron is poorly soluble at physiological pH. In fact, while iron is easily available to microorganisms under anaerobic conditions, it is immediately converted to insoluble hydroxides when exposed to oxygen, which dramatically reduces its availability (Wang et al. 2009; Hayat et al. 2010).

Iron is considered to be one of the essential elements required for the growth and maintenance of cellular metabolism in a wide diversity of prokaryotes (Meyer 2000; Singh et al. 2008, 2010), the exception being members of the genus *Lactobacillus* (Archibald 1983). Iron availability is crucial for several important metabolic pathways such as photosynthesis, pigment synthesis, nitrogen fixation and metabolism (Straus 1994; Ferreira and Straus 1994; Kaushik et al. 2015). This is primarily because of the intricate association of iron as a cofactor of key metabolic enzymes (Rueter and Petersen 1987; Paerl et al. 2001). Accordingly, iron deficiency causes severe stress and abnormal changes in the growth and metabolism of microorganisms. However, not only the deficiency, but also the excess of iron results into deleterious effects on microbial metabolism, with the outcome being toxicity through the generation of reactive oxygen species (ROS) via the Fenton reaction (Andrews et al. 2003; Wang et al. 2011)

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and hydroxyl free radicals through the Haber-Weiss process (Bullen et al. 1978; Cox 1980; Cox et al. 1982). As a consequence, microorganisms have evolved complex metabolic pathways to regulate intracellular levels of iron for their survival, by tightly regulating iron uptake and storage systems.

Amongst all the molecular switches, the Fur (Ferric uptake regulator) protein is one of the most important regulators of iron homeostasis in prokaryotes. The extent of iron availability significantly influences the growth of cyanobacteria along with being an effective factor to the environment and human health, by regulating the production of cyanotoxins and carcinogens in the trophic chain (Lukac and Aegerter 1993; Boyd et al. 2000). Hence, understanding the mode of regulation and the mechanistic pathways of iron regulation by Fur in cyanobacterial system represents an important research field.

Fur protein: general properties

Ernst et al. (1978) isolated a mutant in *Salmonella typhimurium* for the first time while studying the mechanism of control of siderophore regulation. Hantke (1981) isolated the corresponding mutant in *Escherichia coli* strain K12 and named this mutant *fur* (ferric uptake regulator). Later, Wee et al. (1988) purified the Fur protein from *E. coli.* To date, more than 350 *fur* gene homologues have been purified and sequenced, with at least one putative *fur* regulatory sequence in several prokaryotes. With the increasing number of genomes that have been sequenced, it has been shown that many microorganisms have more than one putative Fur-like protein (Table 1).

Gram-positive and Gram-negative bacteria have been studied in different perspectives to understand iron stress and regulation (Braun and Hantke 1991; Heidrich et al. 1996; Bsat et al. 1998). All Fur proteins from different microorganisms form a family of proteins with different functions. The Fur family encompasses several subclasses, among which the Fur protein forms the major class. Apart from Fur, this family also consists of other regulators that perceive different signals to control the expression of a variety of genes (Table 2).

Fur proteins are global transcriptional regulators that were initially considered to be exclusively involved in iron homeostasis in most prokaryotes. They bind to unique sequences (Fur boxes) present in the upstream promoter region of the iron responsive genes, using ferrous iron as a co-regulator (Fillat 2014). However, it has now been well established that the mechanism of regulation by Fur involves a much higher level of complexity, as Fur controls several processes, such as nitrogen metabolism, photosynthesis and respiration, intermediary metabolism (Gonzalez et al. 2014), virulence factor production (Delany et al. 2004; Zhou et al. 2006; Gao et al. 2008), and is involved in the defense against different type of stresses (Touati 2000; Cornelis et al. 2011) (Fig. 1). Fur is also associated with the regulation of toxin-producing genes in prokaryotes (Martin-Luna et al. 2005, 2006). Fur mechanism of action and Fur-mediated regulation appear to be different, depending on the microorganism. Fur is capable of both positive as well as negative regulation of a variety of genes (Bagg and Neilands 1987; Pressler et al. 1988; Salinas et al. 1989; Litwin et al. 1992; Barton et al. 1996; Hernandez et al. 2004a, 2006b) (Table 3).

The Fur protein is in general a 17–21 kDa polypeptide (Bagg and Neilands 1987; Saito et al. 1991; Martin-Luna et al. 2005) that acts as an iron-dependent transcriptional regulator that regulates the expression of several iron responsive genes (Bagg and Neilands 1987; de Lorenzo et al. 1987; Escolar et al. 1997, 1998). When plenty of iron is available to microorganisms, Fur binds the Fe^{2+} ion and undergoes conformational changes. Fe-bound Fur dimerises and then binds to the iron boxes, inhibiting the transcription of all iron

Table 1 Bacterial and cyanobacterial species with more than one Fur homologue

Species	No. of Fur homologues	References
Escherichia coli	3	Pfam site at www.sanger.ac.uk
Bacillus subtilis	3	Pfam site at www.sanger.ac.uk
Mycobacterium smegmatis	3	Pfam site at www.sanger.ac.uk
Staphylococcus aureus	4	Pfam site at www.sanger.ac.uk
Brucella spp.	4	Pfam site at www.sanger.ac.uk
Thermoanaerobacter tengcongensis	5	Pfam site at www.sanger.ac.uk
Anabaena sp. PCC 7120	3	Cyanobase: www.Kazusa.or.jp
Anabaena variabilis ATCC 29413	3	Cyanobase: www.Kazusa.or.jp
Synechocystis sp. PCC 6803	2	Cyanobase: www.Kazusa.or.jp
Synechococcus elongatus PCC 6301	6	Cyanobase: www.Kazusa.or.jp
Synechococcus elongatus PCC 7942	5	Cyanobase: www.Kazusa.or.jp
Synechococcus sp. PCC 7002	2	Cyanobase: www.Kazusa.or.jp
Synechococcus sp. WH 7803	2	Cyanobase: www.Kazusa.or.jp

Table 2 Different Fur subclasses of the Fur family and their respective signals

Fur subclass	Signal	References
Fur (Ferric uptake regulator)	Fe^{2+}	Escolar et al. 1997
Zur (Zinc uptake regulator)	Zn^{2+}	Ahn et al. 2006
Mur (Manganese uptake regulator)	Mn^{2+}	Ahn et al. 2006
Nur (Nickel uptake regulator)	Ni^{2+}	Ahn et al. 2006
PerR (Peroxide response regulator)	O_2^{2-}	Bsat et al. 1998
Irr (Iron response regulator)	Fe^{2+}	Hamza et al. 1998
Sensor of acid tolerance and oxidative stress	Low ph and ROS	Hall and Foster. 1996
Sensor of toxin production in heterotrophic bacteria	Fe^{2+}	Somerville et al. 1999

responsive genes and operons (Fig. 2). However, when iron availability is scarce, Fe^{2+} ion is released from Fur, and the complex dissociates from target promoters, allowing the RNA polymerase to bind and initiate transcription (Klebba et al. 1982; Griggs et al. 1987; Griggs and Konisky 1989). Moreover, Fur is also known to autoregulate itself in the presence and absence of iron (Delany et al. 2001, 2002). Apart from its repressive effect, Fur also activates gene transcription by directly binding to promoter regions, although the actual mechanism for this Fur-mediated direct activation is not well understood. Several mechanisms were proposed to explain the above-mentioned process. Firstly, an activator may remove the Fur repressor from the binding sites and initiate the transcription of target genes (Browning and Busby 2004; Isabella et al. 2008; Nandal et al. 2010; Yu and Genco 2012). Secondly, Fur may recruit the RNA polymerase at the promoter of target genes, thereby enhancing transcription (Browning and Busby 2004), and thirdly, it was proposed that the binding of an activator may alter DNA morphology, making the promoter sequence free for the binding of RNA polymerase (Browning and Busby 2004; Teixido et al. 2011). In *Anabaena* sp. PCC 7120, FurA differentially activates the transcription of *psaK, amt4, hetC, alr1728, patA* and *asr1734*, depending on the presence of the metal co-regulator and a reducing environment (Gonzalez et al. 2014). Gonzalez and coworkers also demonstrated the dual role of Fur protein, i.e., as both activator and repressor in the same metabolism, as observed in the regulation of tetrapyrrole biosynthesis (Gonzalez et al. 2012).

In contrast to its iron-bound repression in several heterotrophic bacteria, Fur was also shown to repress the expression of iron responsive genes, even under iron deficient conditions (*apo*-regulation) (Bereswill et al. 2000; Ernst et al. 2005). Miles et al. (2010), through their plasmid complementation study, demonstrated an iron-bound and *apo*-Fur regulation in *Helicobacter pylori*. Fur also activates the *fur* gene itself (autoregulation) in *apo* form in *Vibrio vulnificus*, apart from its repressing activity (Lee et al. 2007). Iron may not have any role in either repression or activation by *apo*-Fur, but other metals are possibly involved in *apo*-Fur functions (Mills and Marletta 2005; Ducey et al. 2005; Sheikh and Taylor 2009). *apo*-Fur regulation in iron deficient conditions has not been detected in any of the cyanobacteria, to date.

Although *apo*-Fur regulation has not been identified in other species except *H. pylori*, certain genes in *E. coli, Pseudomonas aeruginosa* and *Vibrio cholerae* are known to be indirectly repressed (Litwin and Calderwood 1994; Wilderman et al. 2004; Masse et al. 2007). In these organisms, this regulation is mediated by Fur-regulated small RNAs (sRNA) (Masse and Gottesman 2002). Small RNAs generally pair with the ribosome binding site of the target mRNA at a specific 8–9 base pair sequence and decrease the stability of

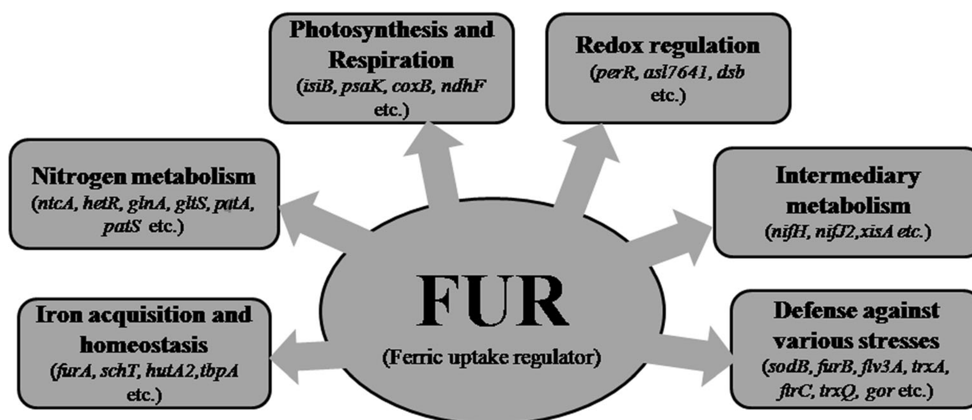

Fig. 1 Cellular processes and related target genes under Fur Regulation

Table 3 Mode of regulation of different Fur protein along with their targets

Fur homologues	Mode of regulation	Target	References
Escherichia coli Fur	Negative	All iron uptake systems	Pressler et al. 1988; Bagg and Neilands. 1987
Pseudomonas aeruginosa Fur	Negative	Many genes	Barton et al. 1996
Vibrio cholerae Fur	Negative	Various systems	Litwin et al. 1992
Vibrio anguillarum Fur	Negative Positive	Fat and ang systems RNAα expression	Salinas et al. 1989
Anabaena sp. PCC 7120 (FurA)	Negative	All iron responsive genes	Hernandez et al. 2004a; Gonzalez et al. 2012
	Positive	Expression of α-*furA* mRNA Expression of several gene regulating various functions, for example *psaK*, *amt4*, *hetC*, *patA*, *alr1278*, *asr1734* etc.	Hernandez et al. 2006b; Gonzalez et al. 2014

mRNA, which ultimately leads to a reduced translation. RyhB regulates the expression of *sodB, sdhCDAB, acnA, fumA* and ferritin gene in *E. coli* (Dubrac and Touati 2000; Masse and Gottesman 2002; Masse et al. 2003, 2005, 2007), just like *apo*-Fur regulation of *sodB* and *pfr* in the case of *H. pylori* (Ernst et al. 2005). Like RyhB in *E. coli*, several other sRNAs were also reported to be repressed by Fur, such as PrrF1 and PrrF2 in *P. aeruginosa* (Wilderman et al. 2004), RyhB in *V. cholerae* (Mey et al. 2005; Davis et al. 2005), FsrA in *Bacillus subtilis* (Gaballa et al. 2008) and NrrF in *Neisseria meningitidis* (Mellin et al. 2007, 2010; Yu and Genco 2012). Non-coding (antisense) RNA-based regulation of the *fur* gene has also been demonstrated in several cyanobacteria (Hernandez et al. 2006a; Martin-Luna et al. 2011). The mechanism of this regulation is described in detail in this review.

The Fur protein acts as a dimer (Coy and Neilands 1991; Neilands and Nakamura 1991; Michaud-Soret et al. 1997), and each monomer consists of two different domains (Coy and Neilands 1991; Stojiljkovic and Hantke 1995; Hernandez et al. 2002). The C terminus is involved in protein dimerization, while the N-terminus is responsible for the DNA binding ability of the Fur protein (Holm et al. 1994; Stojiljkovic and Hantke 1995; Hernandez et al. 2005). The Fur protein folds by two state mechanisms and 40 % of its structure is composed of α-helix (Hernandez et al. 2005). In contrast to the other Fur homologues, no zinc ion or zinc binding pockets have been experimentally detected in *Anabaena* sp. (Pohl et al. 2003). Fur binds to DNA on opposite faces of the helix as an overlapping dimer ("Overlapping dimer binding" model) (Lavrrar et al. 2003), as in the case of DtxR–DNA interaction in *Corynebacterium diptheriae* and other Gram-positive bacteria (White et al. 1998; Pohl et al. 1999). Isothermal titration calorimetry (ITC) experiments have also shown the presence of two metal binding sites in

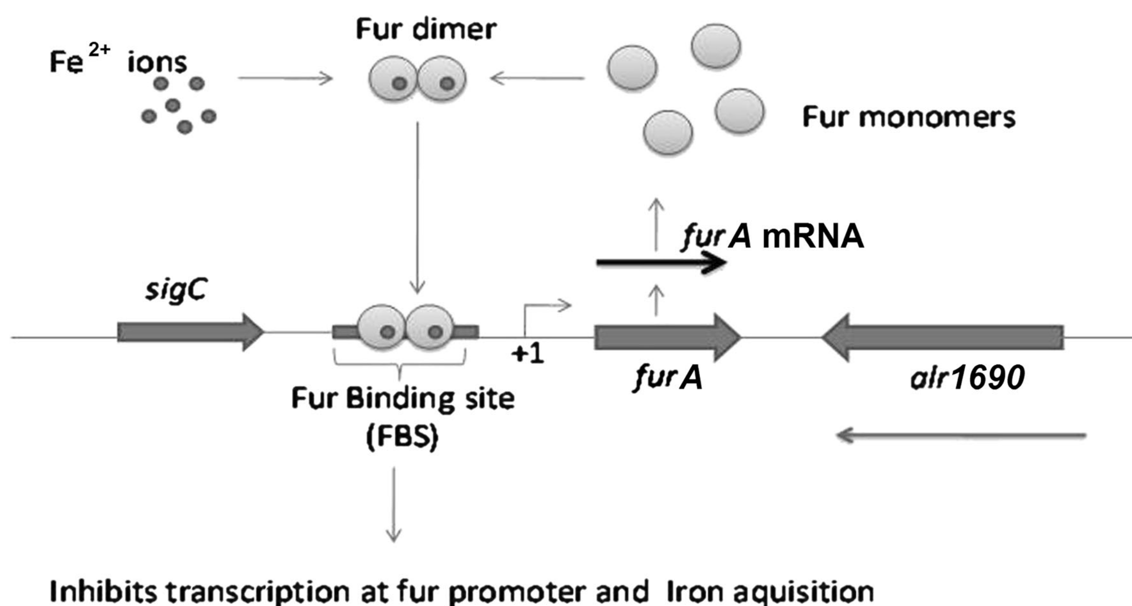

Fig. 2 Schematic representation of mechanism of action of Fur protein in cyanobacterium *Anabaena* sp. PCC 7120 (adopted from old www.bifi.es/research/protein_dna_inter/protein_dna_inter.php)

the FurA protein from *Anabaena* sp. PCC 7120, one for co-repressor binding and the other site having a structural role (Jacquamet et al. 2009; Althaus et al. 1999; Hernandez et al. 2002, 2004a).

Structure and properties of Fur binding sites (Fur Boxes)

Fur proteins were previously thought to recognize and bind to a highly conserved consensus of 19 bp inverted repeats (5 -GATAATGATAATCATTATC-3) found in the upstream promoter region of iron responsive genes in prokaryotes (Calderwood and Mekalanos 1988; Stojiljkovic et al. 1994) (Fig. 3). According to the recent hexamer model, this conserved consensus contains tandem repeats of three forward-reverse hexamers (5 -GATAAT-3) with a Fur recognition unit (5 - NAT(A/T)AT-3) (Escolar et al. 1998) (Fig. 3). Later, Baichoo and Helmann (2002) demonstrated that the Fur binding site is actually a 15 bp core region formed by a 7-1-7 heptamer motif. In the *Anabaena* sp. PCC 7120, Fur binding was observed at consensus sequences containing 7-1-7 inverted repeats (Napolitano et al. 2012). Fur boxes are generally characterized by the presence of an AT rich region. Several numbers of AT rich Fur boxes were also identified in different bacteria sharing 50–80 % of sequence similarity with the canonical consensus identified in *E. coli* (Baichoo et al. 2002; Sebastian et al. 2002; Thompson et al. 2002; Fillat 2014). The variations among Fur boxes were due to addition of sequences in the basal recognition sequence (GATTAT), which ultimately alters the affinity and polymerization of Fur homologues to the Fur boxes (Fillat 2014).

FurA boxes were also located within bidirectional promoters of genes involved in several metabolisms, for example *all0396-schT, all2624-alr2625, all2586-alr2587, alr2594-alr2595* involved in iron metabolism, *all0949-coxB, ccmK-ndhF* involved in photosynthesis and respiration, and *znuA-oprB, hupL3-xisC,* and *nifD3-xisA* involved in other important metabolisms in *Anabaena* sp. PCC 7120 (Gonzalez et al. 2014). These binding sites play a role in the simultaneous regulation of genes with overlapping promoters (Hunt et al. 1994; Christoffersen et al. 2001; Lavrrar et al. 2003). In *Anabaena* sp. PCC 7120, the *all2641–all2649* gene cluster regulating siderophore production and iron-siderophore transport systems (Jeanjean et al. 2008), contains multiple Fur binding sites (with different Fur binding affinities) located in various intergenic regions (one upstream and the other three downstream of the promoter region, respectively). These sequences sequentially modulate gene expression, depending on the iron status of the cell (Gonzalez et al. 2012). In silico analyses have also demonstrated the presence of FurA-binding sites in the promoter regions of *aphC* (encoding a putative photoreceptor of a two-component system), *cyaC* (encoding adenylate cyclase carrying two component sensorand regulatory domain) (Okamoto et al. 2004), *asr* (encoding bacteriorhodopsin) (Irieda et al. 2012) and *cyaD* (encoding adenylate cyclase) (Katayama and Ohmori 1997) in *Anabaena* sp. PCC 7120. These data suggested that Fur controls the expression of genes involved in the signal transduction cascade (Gonzalez et al. 2014). FurA binding site was also predicted in the upstream region of the promoter of the transposase-encoding gene *all4465* in *Anabaena* sp. PCC 7120 and FurA also regulates the transcription of *all4465* in metal and reducing condition dependent manners (Gonzalez et al. 2014). The transcription of *znuA, cyaD, aphC, alr0240* and *pbpH* is co-modulated by other transcriptional regulators along with FurA under iron limitation in *Anabaena* sp. PCC 7120 (Gonzalez et al. 2014). The *znuAB* operon, which encodes for the components of a high affinity zinc uptake system, is co-regulated by FurB/Zur in *Anabaena* sp. PCC 7120 (Napolitano et al. 2012). A Fur/YbtA regulatory network was also postulated in *Yersinia pestis*, where Fur and YbtA coregulate the functioning of the *ybt*A locus encoding a virulence dependent iron uptake system (Gao et al. 2008). In *Microcystis aeruginosa* PCC 7806, two putative Fur boxes

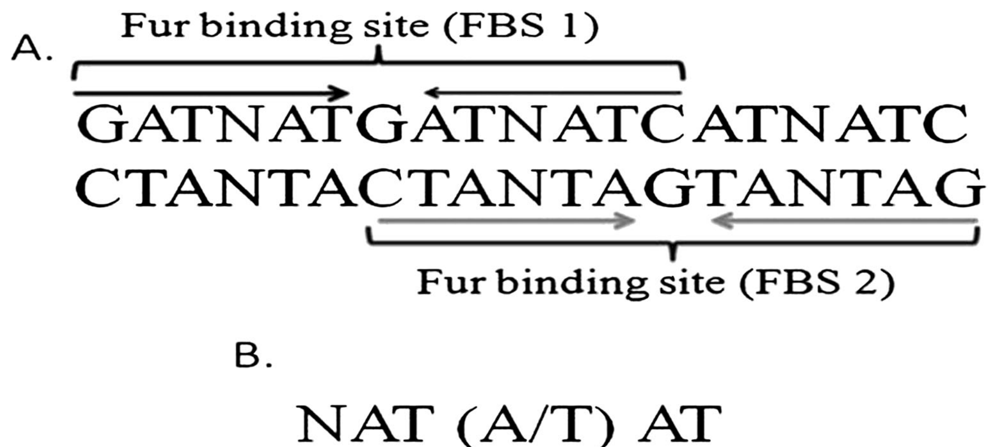

Fig. 3 19 bp consensus sequence containing palindromic sequence with two 9 bp inverted repeats as Fur binding site (FBS) (**a**), Fur recognition unit according to recent hexamer model (**b**) (Escolar et al. 1998)

were identified in the *mcyDA* bidirectional promoter (P_{mcyDA}), a 732 bp region between the *mcyA* and *mcyD* genes (Martin-Luna et al. 2006). *mcyE, mcyH, mcyG* and *mcyJ* genes also showed the presence of AT-rich DNA stretches in their promoter regions (Martin-Luna et al. 2006) having varied matching scores with the previously determined Fur consensus of other cyanobacteria (Hernandez et al. 2006b).

Fur homologues in cyanobacteria

Until now, Fur homologues in cyanobacteria have mostly been studied in *Synechococcus, Synechocystis, Microcystis* and *Anabaena* (Ghassemian and Straus 1996; Kaneko et al. 1996; Bes et al. 2001; Martin-Luna et al. 2005). Ghassemian and Straus (1996) isolated a Fur homologue from the cyanobacterium *Synechococcus* sp. PCC 7942 using an *E. coli*-based in vivo repression assay. DNA sequence analysis of the clones depicted the presence of an open reading frame (ORF) coding for a Fur protein that showed 36 % homology to *E. coli* Fur (Ghassemian and Straus 1996). The amino acid sequence of the Fur homologue from *Synechococcus* sp. PCC 7942 showed no convincing homology to the DtxR homologue, but showed significant similarity to all known Fur sequences (Ghassemian and Straus 1996). This Fur protein contained (147 amino acids) a conserved and a putative iron-binding domain of Fur repressors (HHXHXXCXXC). Inactivation of the Fur homologues in *Synechococcus* sp. PCC 7942 resulted in a disturbed iron homeostasis with severe iron stress symptoms, i.e., continuous flavodoxin expression and siderophore production, which suggested that the inactivation of the *fur* gene had lethal implications.

The whole genome sequencing of the nitrogen fixing filamentous cyanobacterium *Anabaena* sp. PCC 7120, allowed the identification of three open reading frames (*all1691, all2473 and alr0957*) encoding proteins with histidine rich regions sharing properties with the Fur protein family from other prokaryotes (Gonzalez et al. 2014; Hernandez et al. 2004a). Amongst these three ORFs, *all1691*, located between the *sigC* (group 2 sigma factor that responds to carbon: nitrogen imbalance) (Brahamsha and Haselkorn 1992) and *alr1690* gene (encoding cell wall binding protein with peptidoglycan-binding domain), encodes a putative FurA protein (17 kDa) (Fig. 4). The *all2473* and *alr0957* genes code for FurB (15.1 kDa) and FurC (17.3 kDa) paralogues, respectively (Lopez-Gomollon et al. 2009). The FurB protein has 132 amino acids, five cysteine residues and seven histidine moieties while the FurC protein has 149 amino acids, three cysteine residues and six histidine moieties (Hernandez et al. 2004a). The FurA and FurB proteins bind to the promoter region and regulate the expression of the three *fur*-homologue genes, whereas FurC regulates the DNA binding ability of FurA and FurB (Hernandez et al. 2004a) (Fig. 4). Apart from regulating the expression of the FurA protein at a transcriptional level, FurB also prevents DNA damage against reactive oxygen species (ROS) and increases the survival rate of the cyanobacterium *Anabaena* sp. PCC 7120 (Lopez-Gomollon et al. 2009). FurB expression increases drastically under the influence of ROS and protects DNA against the damage caused by ROS, by binding to unspecific sequences. As observed in several other cyanobacteria (Barnett et al. 2012) and heterotrophic bacteria (Patzer 2000; Lindsay and Foster 2001; Fuangthong and Helmann 2003; Maciag et al. 2007), FurB (or Zur) controls zinc homeostasis by binding to upstream promoter regions of target genes, such as those encoding putative metallochaperones (All4722, All1751), zinc metalloproteins (All4725/HemE, All4723/ThrS), components of plasma membrane ABC transport systems (ZnuABC) and several outer membrane proteins (Alr3242, Alr4028) in a zinc-dependent manner in the cyanobacterium *Anabaena* sp. PCC 7120 (Napolitano et al. 2012). Therefore, FurB has a dual role in *Anabaena* sp. PCC 7120, which depends on its concentration in the cell. When FurB concentration is low, it acts as a transcriptional regulator, while it provides protection against oxidative stress by binding unspecifically to the DNA when its concentration is high (Lopez-Gomollon et al. 2009; Sein-Echaluce et al. 2014). FurC has not been reported to regulate FurA paralogue directly, but it is known to affect the FurB binding to the upstream sequence of P_{fur} (Fur box) (Hernandez et al. 2004a). Unlike FurB, FurC is not involved in DNA protection against ROS, but it is thought to be involved in the regulation of photosynthetic proteins that act against oxidative stress (Lopez-Gomollon et al. 2009). Recently, microarray and qRT-PCR-based analyses depicted the increase in transcription of the *alr0957* (*furC*) gene in the presence of H_2O_2, and suggested that FurC is a PerR-like protein involved in regulating peroxide stress response, by sensing peroxide by metal catalyzed oxidation in cyanobacterium *Anabaena* sp. PCC 7120 (Yingping et al. 2014).

The complete sequence of the *fur* gene homologue from *M. aeruginosa* PCC 7806 has been identified using inverse PCR (Martin-Luna et al. 2005). The 296 bp sequence obtained showed a high level of identity to the *fur* gene homologues from other cyanobacteria. Analysis of the upstream sequence of the Fur homologue showed it to have iron boxes sharing 40–50 % similarity to those determined in the other cyanobacteria (Hernandez et al. 2005). The *mcy* operon contains several iron boxes in the promoter regions of various *mcy* genes that allow for control at different levels. In *M. aeruginosa* PCC 7806, Fur proteins are involved in the regulation of the expression of the microcystin gene cluster, which is ultimately influenced by iron availability (Martin-Luna et al. 2006). The Fur homologue from *M. aeruginosa* PCC 7806 is considered to be the sensor of iron availability and oxidative stress (Thompson et al. 2002; Martin-Luna et al. 2006). *M. aeruginosa* PCC 7806 Fur contains183 amino acids (21 kDa) and shares 81 % identity with Fur from

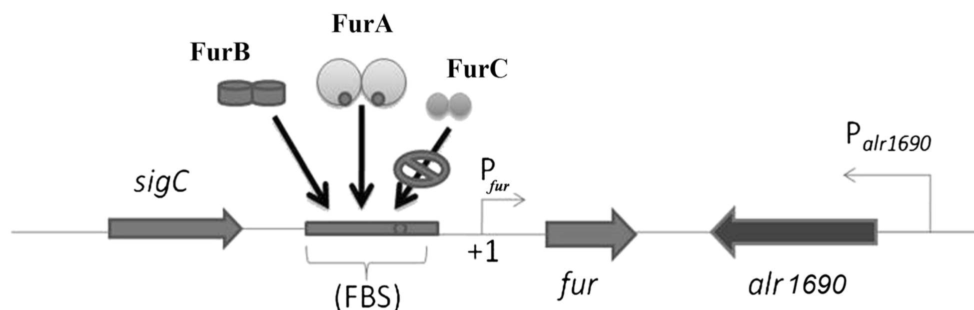

Fig. 4 Schematic representation of binding of three Fur homologues at Fur binding site (FBS) in *Anabaena* sp. PCC 7120 (adopted from old www.bifi.es/research/protein_dna_inter/protein_dna_inter.php)

Synechocystis PCC 6803 (*sll0567*) and 70 % with FurA from *Anabaena* sp. PCC 7120 (*all1691*) (Martin-Luna et al. 2005). The only difference lies in the C-terminal domain, which is larger when compared to other Fur homologues; however, the DNA and metal binding domains are highly conserved (Martin-Luna et al. 2005).

In the *Synechocystis* PCC 6803 genome, the *sll0567* gene (Kaneko et al. 1996) codes for a putative FurA protein, which revealed the highest degree of similarity to a *Synechococcus* sp. PCC 7942 Fur, and was involved in the repression of the *isiAB* operon under iron-repleted conditions (Ghassemian and Straus 1996). Mutations in *sll0567* resulted in iron-stress-like symptoms, and hence is considered essential for cellular function (Kunert et al. 2003). The protein encoded by the *sll0567* gene also possesses a specific motif (HHXHXXCXXC) involved in metal binding (Hennecke 1990), and is similar to the ones found in other heterotrophic bacteria and cyanobacteria (Ghassemian and Straus 1996; Bes et al. 2001). Kunert et al. (2003) identified a consensus Fur box in the promoter region of the *isiAB* operon of *Synechocystis* sp. PCC 6803, by using the on-line MEME motif discovery tool (Bailey and Elkan 1994). The proposed location of the Fur box in *Synechocystis* sp. PCC 6803 was found in the 3'-untranslated region of the *isiAB* promoter fragment fused to *gfp* in the reporter strain. Although Fur represses *isiAB* transcription under iron depleted conditions, its effect was only partial. A binding site for an unidentified activator was found to be located in the 5'-untranslated region of the *isiAB* operon, suggestive of a dual regulation of the *isiAB* operon in *Synechocystis* sp. PCC 6803. Similar regulation was also found in other organisms (Kammler et al. 1993; Michel et al. 1999).

Fur–DNA interaction

The affinity, stability and quality of Fur–DNA interaction in cyanobacteria can be influenced by several factors, such as the architecture of Fur boxes, the presence of divalent metal ions, the redox status of cysteine and histidine residues within the Fur protein, ionic interactions (Hernandez et al. 2002, 2006b) and the involvement of effectors like heme (Hernandez et al. 2004b; Pellicer et al. 2012). It was previously considered that the A and T bases at position 5 and 6 of the consensus hexamer played a major role in the Fur–DNA interaction (Escolar et al. 1998). Later, it was confirmed that it is in fact the architecture of the Fur binding site, which is essential for Fur binding (Lavrrar et al. 2003; Gonzalez et al. 2011). Moreover, it has been proposed that Fur can bind DNA only in the presence of Zn^{2+} ions (Bagg and Neilands 1987; Althaus et al. 1999; Bsat and Helmann 1999; Xiong et al. 2000; Zheleznova et al. 2000), but this has been further contradicted (Saito and Williams 1991; Bsat and Helmann 1999) since the presence of Zn^{2+} ions were not detected in an active recombinant FurA protein overexpressed in *Anabaena* sp. PCC 7119 (Hernandez et al. 2002) and in the Fur protein from *P. aeruginosa* (Lewin et al. 2002). Fur–DNA interaction in the *Anabaena* Fur is also influenced by the presence of Mn^{2+} ion and the redox status of the Fur protein (Hernandez et al. 2006b). Fur–DNA interaction attained maximum stability in the presence of Mn^{2+} ion. The presence of Mn^{2+} also influences the oligomerization of Fur monomers, suggesting the involvement of hydrophobic interactions in this process (Hernandez et al. 2002). Similarly, the redox status of cysteine residues present in the Fur protein also influences the Fur–DNA interaction and the oligomerization of Fur monomers (Ortiz de Orue Lucana and Schrempf 2000; Hernandez et al. 2002). In several prokaryotes, cysteine residues present in Fur proteins are known to be involved in metal binding and DNA recognition activities (Wee et al. 1988; Coy 1995; Althaus et al. 1999; Zou et al. 1999; Zheleznova et al. 2000). Reduced cysteine residues allow Fur monomers (inert) to reorganize into dimers or oligomers, which are the active forms of the Fur protein and interact with DNA sequences to regulate different functions. Similar effects of Mn^{2+} (metalloregulator) and redox status of cysteines on Fur–DNA interaction were also observed in the cyanobacterium *M. aeruginosa* PCC 7806 (Martin-Luna et al. 2006). In *M. aeruginosa* PCC 7806, Fur proteins differ at their C-terminal domains when compared to other Fur homologues. Seven redox active cysteine residues were detected in *M. aeruginosa* PCC 7806. Although the arrangement of the cysteine residues was the same as for the one identified in other cyanobacteria, only five of them were highly conserved (Martin-Luna et al. 2006). Cysteine residues identified in *M. aeruginosa* PCC 7806 are considered as a redox sensor and are also involved in metal binding and protein oligomerization (Ortiz de Orue Lucana and Schrempf 2000).

Fur from *Anabaena* has five cysteine residues, and three of them are located at the C-terminus domain. However, four of the five cysteine residues are arranged in two CXXC motifs, but only one cysteine is involved in DNA recognition and metal binding activities. FurA is the master regulator of iron homeostasis, and for its optimal binding to target DNA in vitro, it requires a reducing environment. Recently, a CXXC motif ($C_{101}XXC_{104}$) with a novel disulfide reductase activity was identified in the cyanobacterium *Anabaena* sp. PCC 7120 (Botello-Morte et al. 2014). Although FurA lacks Zn^{2+} ions responsible for the maintenance of the redox status of cell (Hernandez et al. 2002), the CXXC motif in *Anabaena* sp. PCC 7120 behaves as a redox rheostat (Botello-Morte et al. 2014). FurA senses the redox status of the cell by forming an intermolecular disulfide linkage when it is partially oxidized. The CXXC motif ($C_{101}XXC_{104}$) resembles sequences previously reported in the active sites of thioredoxins and thioredoxins-like proteins, which act as reducing agents, thereby reducing structurally important disulfide bonds in target proteins and maintaining redox homeostasis (Quan et al. 2007). This canonical sequence motif acting as a redox rheostat has also been reported in other proteins such as glutaredoxins (Florencio et al. 2006), peroxiredoxins (Latifi et al. 2009), Dsb proteins (Heras et al. 2007) and in the eukaryotic protein-disulfide isomerases (Sevier and Kaiser 2002). Cross-linking studies on FurA in *Anabaena* spp. suggest a role of ionic interactions in protein–protein interactions and protein oligomerization (Hernandez et al. 2006b), in line with the study of Pohl et al. (2003). The Fur dimer from *P. aeruginosa* consists of a large dimerization interphase state and salt bridges between arginine and aspartic acid residues (Pohl et al. 2003). This possibly indicates that, apart from the redox status, ionic strength also influences the affinity of Fur for its target DNA.

Fur–DNA interactions have also been influenced by the presence of heme (Hernandez et al. 2004b). The involvement of heme in regulating important bacterial metabolic pathways such as respiration and energy transfer is already well documented (Smith et al. 1996). In addition to its function as a gas-sensing compound (i.e., O_2, NO or CO), through its interaction with different heme-sensing proteins (Igarashi et al. 2011), heme also acts as a redox cofactor. Differential spectroscopy-based analyses have identified the presence of a Fur-heme complex in the cyanobacterium *Anabaena* sp. PCC 7120 (Hernandez et al. 2004b). Heme binds and inhibits the DNA binding ability of the Fur protein in vitro in a concentration dependent manner, which represses the expression of Fur-regulated genes (Hernandez et al. 2004b). Histidine residues present in Fur binding sites are responsible for the formation of Fur-heme complexes in vitro (Smith et al. 1996; Paoli et al. 2002), and this binding is critically affected by pH (Saito et al. 1991). Cysteine residues are also considered to be involved in Fur-heme complex formation. Based on site-directed mutagenesis and different spectral studies, it was observed that Cys141 located in the Cys-Pro (CP) motif (dipeptide Cys141-Pro142) at the C-terminal domain is an axial ligand of the Fe (III) heme (Pellicer et al. 2012). Except *E. coli*, which does not have any CP motif, the CP motif is also known for its heme sensing ability in other prokaryotes (Zhang and Guarente 1995; Ogawa et al. 2001). In *Anabaena* sp. PCC 7120, heme binds the Fur protein in an intricate, concentration-dependent and saturable manner at pH 8. In the case of the ferrous heme-FurA binding, Cys141 was not the ligand for heme binding, indicating that a redox-dependent ligand switch likely occurs in which the His residue becomes the ligand upon reduction of Cys141, by removal of thiol from heme iron (Pellicer et al. 2012). The binding of heme to FurA specifically affects the affinity of Fur protein (repressor) binding to the target sequence in vitro, even in the presence of Mn^{2+} ions (effector increases the affinity of Fur–DNA interaction) (Hernandez et al. 2004b). In *E. coli*, it was considered that the heme and Mn^{2+} moieties have similar or closely placed binding sites; hence, heme binding to the Fur protein blocks Mn^{2+} binding in vitro (Smith et al. 1996). Similar results were also observed in *Anabaena* sp. PCC 7120, where heme binding causes a conformational change in the metal binding site, which inhibits the affinity of Mn^{2+} to Fur protein in vitro (Hernandez et al. 2004b). The ability of the heme co-factor to impair the binding ability of other Fur homologues (FurB and FurC) was also studied in *Anabaena* sp. PCC 7120 (Lopez-Gomollon et al. 2009). Heme binding severely affects the Fur–DNA interaction in vitro, while a heme-protein complex was not detected in the case of the FurC homologue.

Fur and oxidative stress

The association of iron metabolism and oxidative stress is a well-established fact in prokaryotes (Andrews et al. 2003; Latifi et al. 2009; Wang et al. 2011). Fur is mainly associated with regulation of iron metabolism, but in several studies, it has been observed that the expression of the *fur* gene was drastically upregulated under oxidative stress environmental conditions (Lopez-Gomollon et al. 2009). Hence, an essential role of Fur in the defense against oxidative stress has also been hypothesized (Zheng et al. 1999; Thompson et al. 2002; Lopez-Gomollon et al. 2009). In *Anabaena* sp. PCC 7120, the redox status of the Fur cysteine residues seem to play a major role in combating oxidative stress (Gonzalez et al. 2011; Botello-Morte et al. 2014). In oxidative stress condition, FurA and FurB regulate the transcription at P_{furA} and P_{furB} in *Anabaena* sp. PCC 7120 (Hernandez et al. 2004a; Sein-Echaluce et al. 2014), suggesting a potential overlapping of FurA and FurB (Zur) promoters. In vivo and in vitro assays have demonstrated *prxA* and *dpsA* genes as the direct targets of FurB (Zur), and hence suggest that FurB plays an important role in connecting zinc homeostasis with oxidative stress protection in *Anabaena* sp. PCC 7120 (Sein-Echaluce et al. 2014). Semi-quantitative RT-PCR and EMSA analyses identified genes involved in oxidative stress

response, such as *sodA* (*all0070*), *prxA* (*alr4641*), *gct1* (*alr3183*)*, gct3* (*all2375*), and *dpsA* (*alr3808*), as putative targets for FurB (Zur) (Sein-Echaluce et al. 2014). In *Anabaena* sp. PCC 7120*, prxA* and *dpsA* contain multiple AT-rich regions that partially match with the FurB (Zur) consensus sequence described previously (Napolitano et al. 2012), and are modulated by additional regulatory factors (Hernandez et al. 2007; Yingping et al. 2014), as also reported in the case of heterotrophic bacteria (Kallifidas et al. 2009).

In *M. aeruginosa* PCC 7806, Fur could confer protection against oxidative stress by regulating the expression of the *mcy* genes, by binding to their bidirectional promoter (P_{mcyDA}) and further by modulating the production of microcystin (Martin-Luna et al. 2006). In iron starved conditions, Fur detaches from the bidirectional promoter of the *mcyDA* genes, which causes the production of microcystin. Microcystin acts as an intracellular iron chelator and increases the rate of iron uptake (Utkilen and Gjolme 1995) in the toxin-producing cyanobacterium *M. aeruginosa* PCC 7806, and thus it may manage survival in the oxidative stress (Martin-Luna et al. 2006).

Generally, the PerR protein is known for its action in combating oxidative stress conditions in prokaryotes; however, no PerR orthologue was found in *Anabaena* sp. PCC 7120. In this species, FurA plays a dual role by regulating the transcription of PerR-regulated genes as well as the one involved in iron homeostasis (Hernandez et al. 2004a, 2006a). The correlation between Fur and PerR regulators has already been established (Hahn et al. 2000; Horsburgh et al. 2001; Singh et al. 2003; Li et al. 2004). Recently, elevated transcription of the *alr0957* (*furC*) gene in the presence of H_2O_2 suggested that FurC is a PerR-like protein involved in regulating the peroxide stress response by sensing peroxide by metal catalyzed oxidation in *Anabaena* sp. PCC 7120 (Yingping et al. 2014).

Regulation of Fur in cyanobacteria

The complex regulation of *fur* has been extensively studied in several microorganisms. In cyanobacteria, the regulation of Fur occurs at three different levels i.e., transcriptional, post-transcriptional and post-translational (Botello-Morte et al. 2013). In *Anabaena* sp. PCC 7120, the *fur*A gene autoregulates its expression and has several putative Fur binding sites in its promoter region (Hernandez et al. 2004a). Under varying iron status, expression of *furA* is also influenced by other Fur paralogues, i.e., FurB and FurC (Hernandez et al. 2004a). Binding assays demonstrated the presence of binding sites for the FurB/Zur paralogue in P_{furA} , and hence suggested the direct regulation of the expression of *furA* at the transcriptional level (Hernandez et al. 2004a; Sein-Echaluce et al. 2014). The promoter region of *furA* does not contain binding sites for the FurC paralogue, but FurC has been shown to influence the binding affinity of both FurA and FurB/Zur paralogues on P_{furA}, by enhancing and inhibiting the FurA and FurB binding, respectively (Hernandez et al. 2004a). In the *Anabaena* sp. PCC 7120, northern blot and western blot analyses showed an increase in *furA* expression and a corresponding decrease in *alr1690-α-furA* expression in nitrogen fixing conditions. Nitrogen deprivation upregulates the level of *furA* mRNA and the corresponding proteins, specifically in pro-heterocysts and mature heterocysts (Lopez-Gomollon et al. 2006). Nitrogen status has no effect on the transcription level or on the expression of FurB and FurC paralogues. Lopez-Gomollón et al. (2007) also demonstrated an NtcA-based regulation of *furA* in heterocysts in the *Anabaena* sp. PCC 7120. Footprinting and EMSA assays depicted the presence of several putative NtcA binding sites on the upstream region of *furA* and on the dicistron *alr1690-α-furA* promoters. NtcA can either act as an activator or as a repressor, depending on the metabolic status of cells (Kolb et al. 1993; Herrero et al. 2001). Under nitrogen fixing conditions (Nitrogen starvation), NtcA binds to the upstream sequence of the *furA* promoter and enhances its expression in heterocysts. In contrast to in nitrogen limiting conditions, NtcA acts as a repressor and causes the down regulation of *furA* in vegetative cells in the presence of combined nitrogen. The presence of several NtcA binding sites in the upstream sequence of *furA* and *alr1690-α-furA* is decisive for this differential regulation depending upon the nitrogen status of cells. The identification of common elements overlapping the *ntcA* and *furA* regulon unravelled the presence of a possible

Table 4 Regulatory RNAs from different bacterial and cyanobacterial species

Species	Regulatory RNA	Length	Target	References
Escherichia coli	RyhB	90 nt	Iron using protein, free intracellular iron, adaptation to iron starvation	Masse and Gottesman 2002; Masse et al. 2005; Jacques et al. 2006
Pseudomonas aeruginosa	Prr1 and Prr2	110 nt	Iron using protein	Wilderman et al. 2004
Vibrio cholerae	RyhB	200 nt	Iron using protein, biofilm formation, chemotaxis	Mey et al. 2005; Davis et al. 2005
Vibrio anguillarum	RNAα	650 nt	Iron transport	Chen and Crosa 1996
Synechocystis sp.	IsiR	177 nt	IsiA Stablity	Duhring et al. 2006
Anabaena sp. PCC 7120	α-*furA*	2.2 kb	FurA translation	Hernandez et al. 2006b

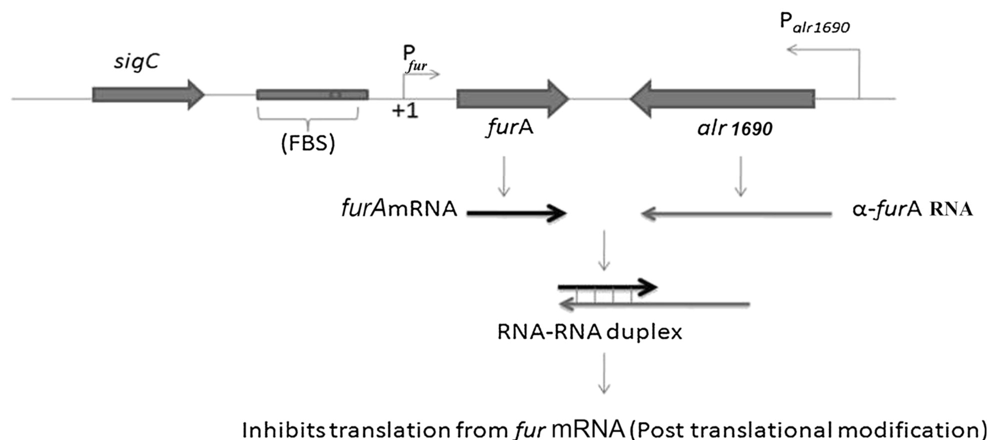

Fig. 5 Schematic representation of post-transcriptional regulation of *furA* by α-*fur*A antisense mRNA in cyanobacterium *Anabaena* sp. PCC 7120 (adopted from old www.bifi.es/research/protein_dna_inter/protein_dna_inter.php)

transcriptional regulatory network required for regulating iron homeostasis, redox control and nitrogen metabolism (Lopez-Gomollón et al. 2007).

The non-coding (antisense) RNA-based regulation of Fur was studied and is considered as an important regulatory system in several bacteria (Chen and Crosa 1996; Gottesman 2002; Wilderman et al. 2004; Davis et al. 2005; Masse et al. 2005; Duhring et al. 2006; Hernandez et al. 2006a; Jacques et al. 2006) (Table 4). Regulation of *fur* expression by α-*fur* antisense mRNA has also been observed in *Anabaena* sp. PCC 7120 and *M. aeruginosa* PCC 7806 (Hernandez et al. 2006a; Martin-Luna et al. 2011). Generally, these antisense RNAs are trans-encoded on the accessory elements such as plasmids or transposable elements and regulate the expression of several regulatory genes by forming a partial and imperfect RNA-RNA duplex (Hernandez et al. 2006a). The filamentous cyanobacterium *Anabaena* sp. PCC 7120 has a large dicistronic transcript containing the *alr1690* gene that possibly encodes a putative membrane protein Alr1690, and an antisense α-*furA* RNA, which is involved in the regulation of *furA* expression. An α-*furA* RNA covers the complete coding sequence of *furA* (Hernandez et al. 2006a) (Fig. 5). It has been hypothesized that α-*furA* RNA masks the ribosome binding site in the *furA* mRNA, and thus interferes with *furA* expression, which in turn alters the level of proteins belonging to the *furA* regulon. Mutants impaired in α-*furA-alr1690* dicistron resulted in an increased expression of *furA* and exhibited an iron-deficient phenotype (Hernandez et al. 2010). In *M. aeruginosa* PCC 7806, implication of α-*fur* RNA has been mainly observed as a consequence of oxidative stress and change in light conditions (Martin-Luna et al. 2011). The requirement of light and reduced environment has been essential for the expression of the Fur protein in the cyanobacterium *M. aeruginosa* PCC 7806. In the absence of either light or reduced environment, expression of the *fur* gene is inhibited due to expression of antisense α-*fur* RNA which binds to *fur* mRNA.

Regulation of the *fur* regulon has also been studied extensively at the post-translational level in prokaryotes. Binding of the heme complex to Fur proteins influences the affinity of Fur (repressor) for the Fur box in vitro (Hernandez et al. 2004b). In the *Anabaena* sp. PCC 7120, heme binding causes the conformational change in the ligand binding site, which inhibits the binding of Fur to the target sequences in vitro

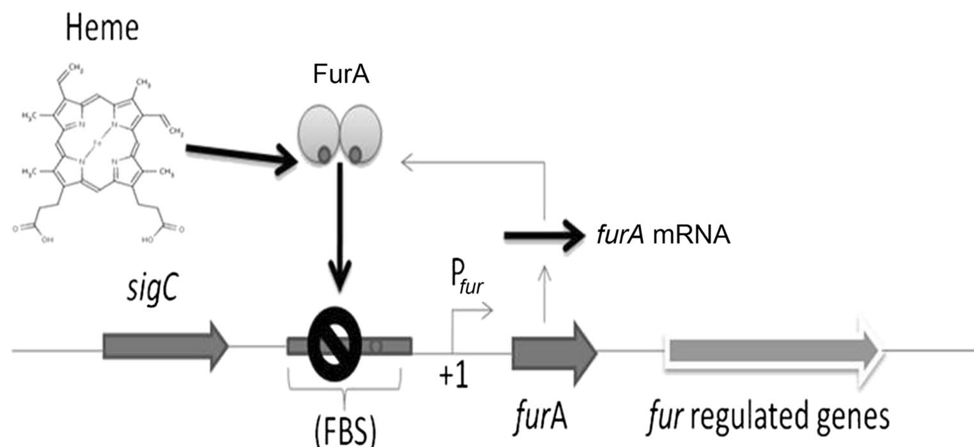

Fig. 6 Schematic representation of post-translational modifications of FurA in *Anabaena* sp. PCC 7120 (adopted from old www.bifi.es/research/protein_dna_inter/protein_dna_inter.php)

(Hernandez et al. 2004b) (Fig. 6). Krynicka and coworkers demonstrated the proteolysis-dependent regulation of abundance of Fur protein in *Synechocystis* sp. PCC 6803 (Krynicka et al. 2014). Two FtsH protease homologues, FtsH1 (encoded by *slr1390* gene) and FtsH3 (encoded by *slr1604* gene), together form a FtsH1/FtsH3 heterocomplex that is involved in the acclimation of cells to the iron deficiency (Krynicka et al. 2014). In *Synechocystis* sp. PCC 6803, it has recently been observed that the transcription of *isiA/isiB* operons and several other genes involved in iron homeostasis was also dependent on the level of the FtsH1/FtsH3 heterocomplex (Boehm et al. 2012). Under iron deficiency, the FtsH1/FtsH3 protease disturbed the equilibrium between DNA-bound and free Fur, by proteolytic degradation of detached Fur, and this would further induce the detachment of Fur from DNA (Krynicka et al. 2014).

Conclusion

In contrast to previous findings, the Fur protein in cyanobacteria controls a plethora of genes regulating different metabolisms other than iron homeostasis. To date, a large number of Fur paralogues have been identified and characterized in a wide range of prokaryotes. However, concerning cyanobacteria, many of the present studies associated with Fur protein were mainly focused on *Synechococcus* PCC 7942, *Synechocystis* sp. PCC 6803, *Anabaena* sp. PCC 7120 and *M. aeruginosa* PCC 7806. The Fur protein acts by binding to the fur boxes located upstream of promoter sequences using ferrous ion as a co-regulator. Fur–DNA interaction is influenced by several factors, such as the architecture of Fur boxes, the presence of divalent metal ions, the redox status of cysteine and histidine residues, ionic interactions and the involvement of effectors such as heme. In contrast to other prokaryotes, zinc showed no effect on Fur–DNA interaction in *Anabaena* sp. PCC 7120. The Fur protein acts as a redox sensor and protects cyanobacteria against oxidative stress, using the reductive property of its cysteine residues. Fur has also been demonstrated to be actively involved in the regulation of nitrogen fixation and cellular metabolism, by controlling the acquisition of iron. Regulation of the *fur* gene expression has been studied at different levels, i.e., transcriptional, posttranscriptional and post-translational levels, in cyanobacteria. In the *Anabaena* sp. PCC 7120, the increase in FurA expression and the corresponding decrease in *alr1690-α-furA* expression, specifically in heterocysts in nitrogen fixing conditions were related to the NtcA regulator. NtcA can either act as an activator or as a repressor, depending on the metabolic status of cells. Regulation of *fur* expression by *α-fur* antisense mRNA has also been observed in the *Anabaena* sp. PCC 7120 and *M. aeruginosa* PCC 7806. Thus, considering the importance of cyanobacteria as biofertilizers in the aquatic environment, deciphering the role and mechanism of action of the Fur protein in a wide variety of cyanobacteria is necessary.

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