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New insights into the role of Fur proteins: FurB (AlI2473) from *Anabaena* protects DNA and increases cell survival under oxidative stress

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The ferric uptake regulator (Fur) is a prokaryotic transcriptional regulator that controls a large number of genes mainly related to iron metabolism. Frequently, several Fur homologues with different physiological roles are found in the same organism. The genome of the filamentous cyanobacterium *Anabaena* (*Nostoc*) sp. PCC 7120 codes for three different *fur* genes. FurA is an essential protein involved in iron homeostasis that also modulates dinitrogen fixation. FurA interacts with heme, impairing its DNA-binding ability. To explore functional differences between Fur homologues in *Anabaena*, factors affecting their regulation, as well as some biochemical characteristics have been investigated. While incubation of FurB with heme severely hinders its ability to interact with DNA, binding of heme to FurC could not be detected. Oxidative stress enhances the transcription of the three *fur* genes, especially of *furB* and *furC*. In addition, overexpression of FurA and FurB in *E. coli* increases survival when the cells are challenged with hydrogen peroxide or methyl viologen, a superoxide anion-generating reagent. When present in saturating concentrations, FurB exhibits unspecific DNA-binding activity and protects DNA from cleavage produced by hydroxyl radicals or DNaseI. Based on these results, it can be suggested that whereas at low concentration FurB would act as a member of the Fur family, at saturating concentrations FurB protects DNA, showing a Dps (DNA protection during starvation)-like behaviour.

Keywords: ferric uptake regulator, heme, cyanobacteria, DNA protection, oxidative stress

Abbreviations: Fur, ferric uptake regulator; Dps, DNA protection during starvation; PSI, photosystem I; PerR, peroxide-sensing repressor; bp, base pair(s); DTT, 1,4-dithiothreitol; LB, Luria-Bertani; ORF, open reading frame; ROS, reactive oxygen species; IPTG, isopropyl- β -D-thiogalactopyranoside; EMSA, electrophoretic mobility shift assays; MV, methyl viologen (paraquat); SDS/PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

INTRODUCTION

Iron participates in numerous metabolic processes and is essential for most living organisms [1]. However, the low solubility of iron in oxygenated waters limits its bioavailability in aquatic environments. This fact, besides the close relationship between iron homeostasis and oxidative stress, make necessary a tight regulation of iron uptake and storage. The ferric uptake regulator (Fur) protein is one of the most important switches for the regulation of iron metabolism in prokaryotes. Under iron-rich conditions, a dimer of Fur binds to specific DNA sequences (“iron-boxes”) located in the promoters of iron responsive genes using Fe (II) as co-repressor and avoiding their transcription [2]. Apart from the main group of iron-dependent Fur proteins, the ability of some members of this family to sense other metal ions permits differentiate several subclasses, namely sensors of zinc (Zur) [3], manganese (Mur) [4] or nickel (Nur) [5]. Moreover, some of those Fur-like proteins respond to signals other than metal ions. In fact, using metal-catalyzed protein oxidation some of them regulate DNA-binding activity and thereby respond to the presence of peroxide (PerR) [6] or heme and oxygen (Irr) [7]. The crystal structures of Fur from *P. aeruginosa*, PerR from *B. subtilis* and Zur from *M.*

tuberculosis unravel that though each Fur subclass responds to different signals, the overall fold of the N-terminal DNA binding domain and the C-terminal dimerization domain is similar [8-10].

Linkage of Fur to the oxidative stress response has been demonstrated in several microorganisms, like *Staphylococcus aureus* [11] or *Mycobacterium tuberculosis* [12]. In *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803, many iron inducible genes are also influenced by short-term peroxide or methyl viologen stresses [13, 14]. Reactive oxygen species (ROS) are generated during aerobic growth, with the potential to damage almost all cellular components. The formation of these dangerous species is closely related to iron metabolism, since an excess of iron raises the production of ROS through the Fenton reaction [15]. Furthermore, in cyanobacteria iron starvation has shown to be a cause of oxidative stress [16]. Thus, it is not surprising that the cellular responses developed by the organisms to cope with iron starvation and oxidative damage frequently display common elements.

In the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120 (also known as *Nostoc* sp.) three different Fur proteins have been identified: FurA (*all1691*), FurB (*all2473*) and FurC (*alr0957*). *In vitro* assays suggest that these proteins might be cross-regulated [17]. FurA, as well as FurB, bind to the promoter sequences of the three *fur* genes, whereas FurC has been proposed to modulate the DNA-binding activity of FurA and FurB.

FurA is a constitutive and relatively abundant protein that seems to be essential in *Anabaena* sp. [18]. Northern blot analysis shows that under standard growth conditions, mRNAs of *furB* and *furC* are less abundant than *furA* transcripts (our unpublished results). Regulation of FurA takes place at both transcriptional and post-translational levels. Besides a moderate autoregulation, common among *fur* genes, transcription of *Anabaena furA* is modulated by the nitrogen regulator NtcA [19] and likely by FurB and FurC [17]. At the post-transcriptional level, an antisense mRNA interferes with the *furA* transcript [20]. Moreover, post-translational regulation of Fur operated by heme has been reported, since heme binds tightly to FurA impairing its DNA-binding activity [21, 22].

Even though gene regulation is a complex biological process that can happen at several levels, one of the most effective ways for a cell to cope with a change in its environment consists of altering its gene expression. In this work, we show that the expression of the three *fur* paralogues is strongly affected by oxidative stress. We have used an *in vivo* approach to demonstrate that, in *E. coli*, overexpression of FurA and FurB from an inducible promoter prior to oxidative challenge increases cell survival. Moreover, in a purified *in vitro* system, at saturating concentrations FurB binds to DNA in a sequence-independent fashion and protects DNA from cleavage produced by hydroxyl radicals or DNaseI. Those results unravel a new function for a Fur protein and suggest that in *Anabaena*, FurA and FurB connect iron regulation to oxidative stress defence.

EXPERIMENTAL PROCEDURES

Strains and culture conditions.

The present study was carried out with the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120 grown in BG-11 medium at 30°C [23]. RNAs used in RT-PCR assays were obtained from cultures grown until early-log phase ($OD_{600} = 0.4$) and then challenged with 250 μ M H₂O₂ or 1 mM methyl viologen for 0.5, 1, 2 and 4 hours. All the cultures were bubbled vigorously with filtered air.

Overexpression and purification of FurA, FurB and FurC.

Chromosomal DNA from *Anabaena* sp. was isolated as described previously [24] and used as template for amplification of the *furA* (*all1691*), *furB* (*all2473*) and *furC* (*alr0957*) genes. The PCR products were sequenced and cloned between *Nco*I and *Hind*III sites of pET 28a (+). These constructs were used to transform *E. coli* BL21(DE3) Gold cells to overexpress each Fur protein [17, 25].

Recombinant FurA was purified by chromatography through a heparin-sepharose 6 Fast-Flow column (Amersham-Pharmacia) [26]. Recombinant FurB was purified using a heparin-sepharose 6 Fast Flow column (Amersham-Pharmacia) followed by MonoS FPLC column (Pharmacia). Recombinant FurC was purified by heparin-sepharose 6 Fast Flow chromatography followed by MonoQ FPLC column (Pharmacia) as described before [17]. Fur proteins were quantified using the bicinchoninic acid method (BCA™ Protein Assay Reagent Kit, Pierce). FurA was also quantified spectrophotometrically

using an extinction coefficient of $13760 \text{ M}^{-1}\cdot\text{cm}^{-1}$ at 276 nm [26]. Quantification was confirmed by densitometric measures in gel using FurA as standard. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE) was performed as described by Laemmli [27] using 17% (w/v) polyacrylamide [30:0.8 (w/w) acrylamide/bisacrylamide] gels.

Heme-staining assay.

According to [28], a heme-staining procedure was applied to Fur proteins incubated with heme and separated by SDS/PAGE. Heme solution was prepared dissolving heme (hemin, SIGMA) in 0.1 N NaOH. The mixture was diluted in 1 mM sodium phosphate (pH 7.4) to approximately 9.6 M. Aliquots of 5 μM FurA, FurB and FurC were incubated with 1 M heme for 5 minutes. Since *Anabaena* FurA binds heme [21], this reaction was used as positive control of the assay. Lysozyme was used as negative control. Samples were analyzed by SDS/PAGE on a 17% (w/v) polyacrylamide gel. The procedure involves staining for heme, destaining and restaining with Coomassie Brilliant Blue for proteins.

Electrophoretic mobility shift assays (EMSA).

DNA fragments of 389-pb containing the upstream sequence of *furA* were obtained by PCR and further purified using the GFX DNA purification kit (Amersham Biosciences) as described previously [19]. Electrophoretic mobility shift assays were performed as described [25] in a final volume of 20 μl using a core binding buffer which contained 10 mM BisTris-HCl (pH 7.5), 40 mM KCl, 0.1 mg/ml BSA, 1 mM DTT, 5% (v/v) glycerol and 100 μM MnCl_2 . Samples were supplemented with heme at different concentrations. Complexes were separated using 4.8% polyacrylamide gels. Heme solution was prepared by dissolving heme in a (1:9 v/v) 0.1 N NaOH/ethanol solution and filtered through a 0.2 μm filter, as described [21]. Binding assays were carried out in the presence of a 228-bp control DNA fragment from the human *apoE* gene to demonstrate the specificity of the DNA-binding activity of FurB. Results were processed with a Gel Doc 2000 Image Analyser from Bio-Rad.

RT-PCR analysis.

Anabaena RNA was prepared as described [29], using 40 ml of cells containing 5 μg of chlorophyll $\text{a}\cdot\text{ml}^{-1}$. *Anabaena fur* genes were reverse transcribed using the Superscript II Reverse Transcriptase (Invitrogen) and the specific reverse oligonucleotides shown in table 1. The RT-PCR mixture contained 1 μg of RNA and 20 pmol of each primer in the annealing buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 150 mM KCl). The total reaction volume was 10 μl . Samples were incubated 10 min at 85°C to denature RNA followed by 60 min at 65°C to allow annealing of the primer to RNA. After that, 5 pmol of each deoxynucleoside triphosphate (dNTP), 0.2 μmol DTT and 200 U Superscript II Reverse Transcriptase (Invitrogen) were added into the RT-PCR buffer (50mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl_2 , 20 mM DTT) to a final volume of 20 μl . Samples were reverse transcribed at 47°C for 60 min followed by 15 min incubation at 75°C to inactivate the reverse transcriptase.

The products were amplified by PCR using 3 μl of each cDNA as template. The cycling conditions for the reactions were 3 min at 95°C, followed by 25 cycles of 93°C for 30 s, 50°C for 1 min and 72°C for 1 min, followed by extension at 72°C for 10 min. Positive control reactions using chromosomal *Anabaena* sp. DNA as template were run in parallel. PCR products were analyzed in 1% agarose gel.

In vitro DNA protection assay.

Protection of DNA from damage was performed using pUC19 plasmid DNA (100 ng) in 60 μl final volume of 10 mM BisTris-HCl (pH 7.5), 40 mM KCl, 0.1 mM MgCl_2 and 5% glycerol (v/v) incubated for 10 minutes with FurB (40 pmol or 80 pmol) or BSA (40 pmol or 80 pmol) as negative control. When indicated, 100 μM FeCl_3 , 400 μM EDTA, 150 μM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ or 100 mM H_2O_2 were added to the reaction mixtures and the reaction allowed to evolve for 20 minutes at room temperature. For protection against DNaseI-mediated cleavage, reactions were assembled as above and 10^{-5} units DNaseI- FPLC pure (Amersham Biosciences) added in a buffer containing 0.25 mM CaCl_2 , 0.25 mM MgCl_2 and 12.5% glycerol. Samples were incubated for 20 minutes at room temperature.

Each reaction mixture was divided in two 30 μ l-aliquots, one of them was used as control, while FurB and BSA were removed from the other fraction using a GFX DNA purification kit (Amersham Biosciences). Samples were resolved by electrophoresis in 0.8% agarose gel and visualized by ethidium bromide staining.

Exposure of E. coli to oxidative stress and disk diffusion assays.

Survival of *E. coli* cells overexpressing FurA, FurB or FurC was analyzed using methyl viologen (MV) or H₂O₂ disk diffusion assays by a modified version of the method described elsewhere [30]. *E. coli* BL21 (DE3) Gold bearing pET-28a (+) was used as negative control. Cells were grown in LB (Luria-Bertani) broth at 37°C with shaking. When OD₆₀₀ of the cultures reached a value of 0.6, isopropyl- β -D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM and the cultures were grown for another 2 hours. Cells from induced cultures (200 μ l) were spread onto LB agar containing 1 mM IPTG. Then, aliquots (2 μ l) of increasing concentrations of H₂O₂ (Panreac) or methyl viologen (SIGMA) were spotted on top of Whatman paper disks (0.5-cm diameter) that were placed over agar plates. Cells were allowed to grow for 24 h at 37 °C, before measuring the inhibition area around the paper disks.

RESULTS

Analysis of fur expression by RT-PCR.

Regulation of *fur* genes under different conditions (iron deficiency, nitrogen-fixing conditions, osmotic stress, saline stress and oxidative stress) has been investigated. When using semiquantitative RT-PCR in those studies, it should be considered the existence of an α -*furA* antisense RNA [31] that can interfere with the results of this assay. To avoid this problem, a specific cDNA for each *fur* gene has been prepared. Transcription of *furA* slightly increases under iron starvation as well as in nitrogen-fixing conditions, as reported before [19, 31]. However, these situations do not seem to affect the transcriptional levels of *furB* and *furC*. When the cultures were grown under osmotic stress (200 mM sucrose) or salt stress (100 mM NaCl) the amounts of *furA*, *furB* and *furC* mRNAs remain unchanged (data not shown). Under oxidative stress conditions, transcriptional levels of the three *fur* genes are affected, being the effect much stronger on *furB* and *furC* m-RNAs. In all cases, challenge with H₂O₂ induced a faster response than MV (Figure 1).

FurB protects DNA from cleavage produced by hydroxyl radicals or DNaseI.

The dramatic increase of *furB* expression under oxidative challenge, its high isoelectric point (pI 8.7) and the fact that at high concentrations FurB binds to DNA in a sequence-independent fashion (our unpublished results), led us to examine whether purified FurB can afford protection against DNA cleavage. Two situations were tested. On the one hand, DNA was challenged with hydroxyl radicals generated *via* iron-mediated Fenton reactions, on the other DNA was treated with DNaseI. In those experiments, the combination of Fe (III)/EDTA or Fe (II) and H₂O₂ or addition of DNaseI results in the introduction of single-strand breaks that changes supercoiled DNA mobility in agarose gels (figure 2, lanes 3, 6 and 9).

Addition of 40 pmol and 80 pmol of FurB to the reaction mixture containing DNA shows a progressive increase of FurB-DNA complexes, followed by formation of large aggregates that are retained in the wells (figure 2.A.I lanes 4, 5, 7, 8, 10 and 11). FurB binds to supercoiled or linear DNA, suggesting no apparent predilection for either topological form. Removal of FurB before electrophoresis permits to visualize the degree of damage suffered by DNA that was bound to FurB (figure 2.A.II lanes 4, 5, 7, 8, 10 y 11). It can be observed that the presence of increasing amounts of FurB results in a progressive decrease in DNA damage.

In order to check the specificity of the DNA protection by FurB, similar experiments were performed in the presence of FurA (not shown) or BSA (figure 2.B) instead of FurB. In those cases DNA is nicked by hydroxyl radicals or DNaseI despite the presence of FurA or BSA. Those results show that FurB binds to and protects DNA from cleavage produced by reactive oxygen species or by DNaseI.

FurB binds heme.

Free heme is a potential cause of oxidative stress inside the cell [32]. It damages lipids, proteins and DNA through the generation of ROS. In particular, redox-active iron released after degradation of heme plays an important role for heme toxicity [33]. In this way, heme detoxification constitutes a way of ensuring cell survival in the redox environment produced by heme. The ability of Fur and other proteins involved in iron homeostasis to bind heme has been reported previously [21, 22, 34]. In the case of *Anabaena* FurA, it has been proposed that heme acts as a potential regulator impairing its DNA-binding activity. With the aim to investigate whether FurB and FurC were able to bind heme, a heme-staining procedure was performed as described previously [21]. FurA, used as positive control, FurB, FurC and lysozyme, the latter as negative control, were incubated with heme and the complexes resolved by SDS/PAGE. As shown in figure 3.A, FurB binds heme whereas FurC does not seem to interact with it. This result is in good agreement with the existence of the CP (Cys-Pro) regulatory motif in the sequence of FurA and the CPV (Cys-Pro-Val) one in the sequence of FurB (supplementary material figure S1).

Influence of heme in the DNA-binding activity of FurB.

Protein-DNA interactions occur between transcription factors and DNA control sequences that lead to gene regulation. They are often the result of extracellular stimuli. The interaction of heme with FurB suggests the possibility that, as in the case of FurA, its DNA-binding activity could be affected. EMSA assays were carried out to assess the influence of heme in the interaction of FurB with the *furA* promoter. In the absence of heme, FurB binds to P_{*furA*}, as previously reported [17]. Addition of heme to the binding assay prior to the incubation with P_{*furA*} inhibits completely the interaction of FurB with DNA, as shown in figure 4.

FurA and FurB increase cell survival under oxidative stress in vivo.

In sight of the results shown to this point, we sought to investigate whether the Fur family is involved in protecting cells from oxidative damage *in vivo*. If that was the case, the presence of high amounts of Fur in the cells induced by H₂O₂ or methyl viologen should increase cell survival. Fur proteins from *Anabaena* were overexpressed in *E. coli* from the IPTG-inducible promoter T7 of pET-28a (+) [17]. Those *E. coli* cells were allowed to grow on LB-agar plates supplemented with IPTG and Whatman paper disks on top containing increasing concentrations of H₂O₂ or MV. Figure 5 shows that when the cells are challenged with H₂O₂, overexpression of FurA increases significantly cell survival, as shown by a smaller zone of growth inhibition. Cells overexpressing FurB are also less sensitive to H₂O₂ damage. In the MV assays, similar results were observed (figure 6). In both cases, the effect is more evident at high concentrations of H₂O₂ or MV. Overexpression of FurC seems not to influence bacterial survival in any condition tested.

DISCUSSION

The existence of different Fur homologues in distantly related organisms suggests an evolutionary specialization. Although all Fur proteins show common characteristics, there are substantial differences among the signals affecting their affinity for DNA, the sets of target genes or the elements that modulate their transcription. To get a better understanding of the roles of *fur* paralogues in *Anabaena*, we have investigated the regulation of those genes under different situations, as well as the behaviour of their protein products in face of agents that could induce DNA damage.

While osmotic and saline stresses did not affect the transcription of *fur* genes, oxidative challenge lead to increased levels of the three *fur* transcripts, particularly of *furB* and *furC*. This indicates that the raise of the transcription is not a general response to any stress and are consistent with previous reports linking iron-responsive gene expression and oxidative stress response [13, 14, 16, 35]. In many microorganisms, Fur proteins regulate the expression of genes involved in the concerted defence against oxidative stress, such as superoxide dismutase or catalase. In the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, the Fur-like Slr1738 plays a regulatory role in the induction of the potent antioxidant gene *sll1621(ahpC)* [14, 35]. Both genes are strongly induced by methyl viologen or peroxide treatment and are located contiguously in the genome.

In addition to ROS generated by the Fenton's reaction and by the adventitious delivery of electrons from the photosynthetic and respiratory pathways to oxygen, the potential of heme to catalyse free radical formation has to be considered. Histidine residues and sometimes cysteine-proline (CP) [36] or cysteine-proline-valine (CPV) [37] sequences, have been proposed to be involved in the interaction between heme and proteins. A common feature of the Fur family is the presence of a histidine-rich motif that is involved in metal binding. Moreover, FurA and FurB from *Anabaena* contain in their sequences CP or CPV motifs, respectively, located in their carboxyl ends (figure S1), which accounts for the ability to bind metal and dimerise [8, 38]. The CP or CPV sequences are thought to be regulatory elements for non-covalent binding of heme to proteins [37]. Several functions are proposed for these sequences. They could act, for instance, as a secondary heme binding sites or contribute to the complex formation by increasing the concentration of heme around them [21]. The presence of the CP motif in the sequence of FurA and CPV one in the sequence of FurB is in good agreement with the ability of these proteins to bind heme, while FurC, that lacks any of these signatures, does not interact with it.

In Rhizobiales, heme regulates the degradation of the Fur-related response regulator Irr, whose stability depends on the iron status [34]. In the case of FurA and FurB, degradation products are not detected after incubation with heme, suggesting that heme plays a different role in Irr than in Fur proteins.

Different mechanisms of redox control of gene expression have evolved to adapt rapidly to redox changes within bacteria. The regulator FixL utilizes heme as co-repressor to elicit a conformational change in response to oxygen [39]. Similarly, the interaction of heme with FurA and FurB could produce a conformational change providing a mechanism of post-translational regulation. This model is in good agreement with the fact that heme impairs the DNA binding activity of FurB and, as previously reported, of FurA [21]. Fur regulation seems to be a complex process which involves not only the existence of Fe(II) and reducing conditions [18]. The DNA-binding ability of Fur, and consequently, its functionality, is impaired also by the dimerisation rate and by heme binding.

The contexts of the *fur* homologues in *Anabaena* are rather different that those of the corresponding orthologues in *Synechocystis* sp. PCC 6803. While the peroxide-dependent *slr1738* is contiguous to *ahpC*, the orthologue to *furB*, *sll1937*, lies divergently next to *zntC*, that codes for a component of an ABC type zinc uptake transporter [40]. Analysis of the sequences flanking *furB* and *furC* in *Anabaena* (Cyanobase: <http://bacteria.kazusa.or.jp/cyano/>) shows that the ORFs contiguous to those *fur* genes encode hypothetical or unknown proteins. On the other hand, the closest homologues to *ahpC* and *zntC*, the *all1541* and *all0833* genes, are located next to other ORFs unrelated to the *fur* family.

Our results demonstrate that FurB protects DNA from cleavage produced by hydroxyl radicals or DNaseI. In addition, overexpression of FurA and FurB in *E. coli* increases survival when the cells are stressed with hydrogen peroxide or methyl viologen. Although overexpression of FurC does not seem to influence cell survival, it could be hypothesized that FurC might play a role in the regulation of cyanobacterial metabolic processes that are absent in *E. coli*, such as those related to photosynthesis. In fact, expression of *furC* is enhanced specifically by addition of methyl viologen, a superoxide anion-generating reagent. In photosynthetic organisms, the most important production of superoxide anion takes place in the photosynthetic electron transport chain, when molecular oxygen adventitiously oxidizes PSI [41]. Thus, FurC might be involved in the protection of the photosynthetic proteins from oxidative damage.

In the case of FurA, it might increase cell survival modulating a set of genes involved in the detoxification of ROS. Indeed, FurA recognises the regulatory regions of several key genes related to redox homeostasis, such as *gor* and a number of thioredoxins [42] suggesting that FurA could coordinate the cellular response to oxidative stress.

Overexpression of FurB possibly increases cell survival under oxidative challenge avoiding DNA damage by unspecific binding. The cationic isoelectric point of FurB (theoretical pI = 8.7), together with its high affinity to DNA, its ability to bind heme and its DNA-protective activity suggest that FurB could act as a Dps-like protein [43]. In addition to Dps, there are other bacterial, relatively non-specific DNA-binding proteins that exert different functions depending on the level of expression. H-NS is one of the most abundant chromatin-associated proteins [44]. At sub-saturating concentrations, H-NS exhibits a preferred DNA-binding site modulating transcription of the *proU* operon. However, when present in saturating concentrations, H-NS binds to DNA in a sequence-independent fashion.

This could be the case of *furB*, whose transcription is triggered by reactive oxygen species. Under those conditions, the level of FurB would be high enough to coat DNA unspecifically and prevent oxidative damage. To our knowledge, this is the first evidence that a Fur protein protects DNA from cleavage by direct binding.

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FIGURE LEGENDS

Figure 1. RT-PCR analysis of the *fur* family transcription under oxidative stress conditions. Expression of *furA*, *furB* and *furC* under oxidative stress conditions produced by 250 μ M H₂O₂ and 1 mM methyl viologen. Transcripts were analyzed at different times by RT-PCR. PCR reactions with the corresponding oligonucleotides using chromosomal *Anabaena* sp. DNA as template were carried out as positive controls.

Figure 2. *In vitro* DNA protection assay.

The introduction of a single-stranded break in the plasmid DNA converts it to a nicked form changing its mobility in an agarose gel. As the substrate was used 100 ng of pUC plasmid. When indicated, Fe (III)/EDTA or Fe (II) and H₂O₂ or DNaseI were added to the samples as denoted below the panels. FurB (40 pmol and 80 pmol) as is shown in panel A, and BSA (40 pmol and 80 pmol) as negative control, in panel B, were added to the samples. After incubation for 20 minutes at room temperature, the reactions were stopped and proteins were removed from half of each sample. DNA was visualized by electrophoresis in 0.8% agarose gel. N: nicked DNA, L: linear DNA, SC: supercoiled DNA.

Figure 3. Heme binding and staining assay.

(A) 5 μ M of each protein were incubated 5 minutes with heme. FurA (MW= 17.2 KDa) was used as positive control or the experiment. Lysozyme (MW= 14.3 KDa) was used as a control of non-specific binding of heme to protein. Samples were analyzed in SDS/PAGE and a specific heme-staining assay was performed [28]. (B) After that, proteins were detected with Coomassie blue staining. The sizes of the molecular mass markers are indicated on the left side. Note that the samples are loaded using a non-reducing loading buffer, thus several oligomeric forms of FurA, FurB and FurC could be detected.

Figure 4. Inhibition of *in vitro* DNA binding activity of FurB by heme.

Electrophoretic mobility shift assay of FurB to a 389-bp DNA fragment containing the *Anabaena* sp. *furA* promoter. A fragment of exon IV from the human *apoE* gene was used as non-specific competitor DNA. Lane 1: free DNA. Lane 2: 500 nM FurB. Lane 3: Reaction mixture in the presence of 1:5 FurB /heme concentration ratio (500 nM FurB and 2.5 μ M heme). Lane 4: Reaction mixture in the presence of 1:8 FurB /heme concentration ratio (500 nM FurB and 4 μ M heme).

Figure 5. H₂O₂ disk assay.

In the assay, LB-agar plates were inoculated from liquid cultures of *E. coli* strains overexpressing FurA, FurB and FurC in the presence of 1 mM IPTG. *E. coli* containing pET-28a(+) was used as negative control. Sensitivity to H₂O₂ was measured by the zone of growth inhibition (diameter). (A) Effect of H₂O₂ on growth of *E. coli* bearing (a) pET-28a(+) plasmid (negative control), (b) *furA* cloned into pET-28a(+), (c) *furB* cloned into pET-28a(+), (d) *furC* cloned into pET-28a(+). H₂O₂ concentration assayed: (1) 9.7 M, (2) 4.85 M, (3) 1.94 M, (4) 0.97 M and (5) 0.48 M. (B) The zone of inhibition at each concentration of H₂O₂ of negative control (black bars) and *furA* (light grey bars), *furB* (white bars), *furC* (dark grey bars) cloned into pET-28a(+) was measured five times, and the mean values were represented. The error bars are the standard errors of the mean value.

Figure 6. Methyl viologen disk assay.

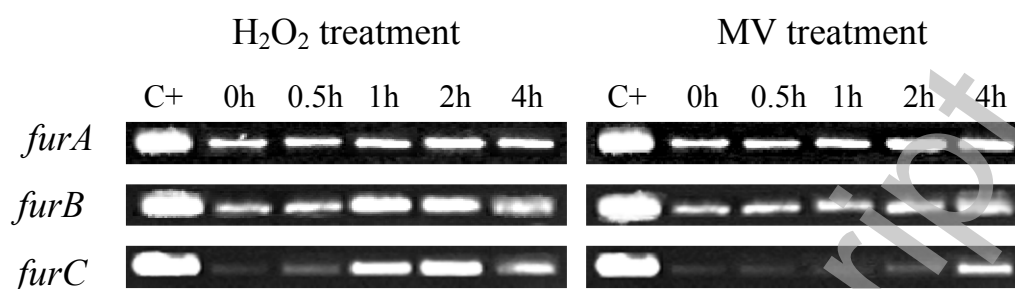
In the assay, LB-agar plates were inoculated from liquid cultures of *E. coli* strains overexpressing FurA, FurB and FurC in the presence of 1mM IPTG. Sensitivity to MV was measured by the zone of growth inhibition (diameter). (A) Effect of methyl viologen on growth of *E. coli* bearing (a) pET-28a(+) plasmid (negative control), (b) *furA* cloned into pET-28a(+), (c) *furB* cloned into pET-28a(+), (d) *furC* cloned into pET-28a(+). MV concentration assayed: (1) 1000 mM, (2) 500 mM, (3) 100 mM, (4) 75 mM, (5) 50 mM, (6) 25 mM and (7) 10 mM. (B) The zone of inhibition at each concentration of MV of negative control (black bars) and *furA* (light grey bars), *furB* (white bars), *furC* (dark grey bars) cloned into pET-28a(+) was measured five times, and the mean values were represented. The error bars are the standard errors of the mean value.

Table 1. Oligonucleotides used in this work

Primer	Sequence (5' - 3')	Description
FurN	ATTACCATGGCTGTCTACAC	RT-PCR of
FurC	CTTGTAAGCTTACCAGGTGC	<i>furA</i>
<i>all2473</i> -N	ATAGTCCATGGGAGCCATAC	RT-PCR of
<i>all2473</i> -C	CAGTAGTTTAAGCTTTTGACTA	<i>furB</i>
<i>alr0957</i> -N	GACATAACCATGGAGCAACAG	RT-PCR of
<i>alr0957</i> -C	CTACTCCAAGCTTTCTACTC	<i>furC</i>

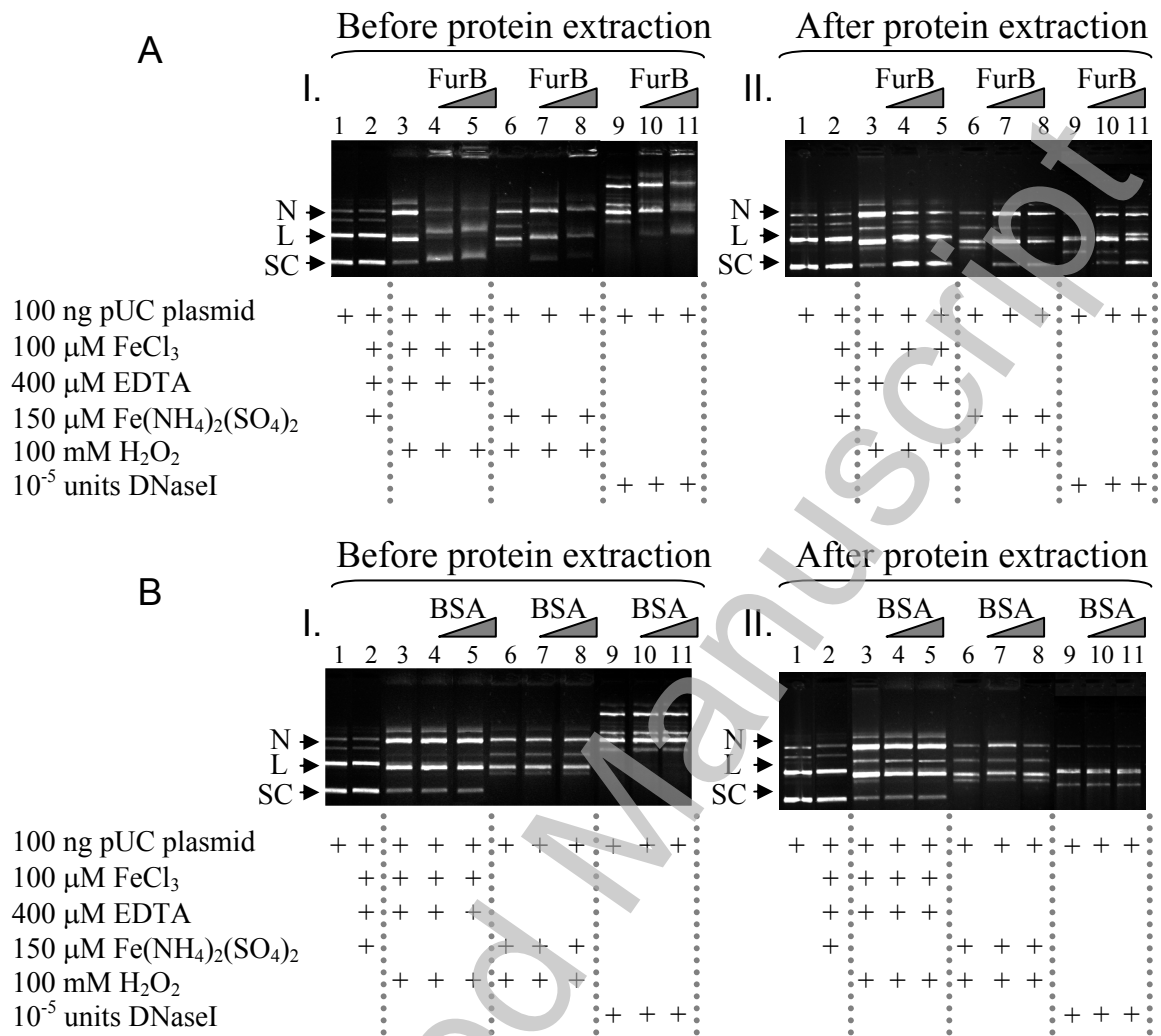
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Figure 1. RT-PCR analysis of *fur* family expression under oxidative stress conditions

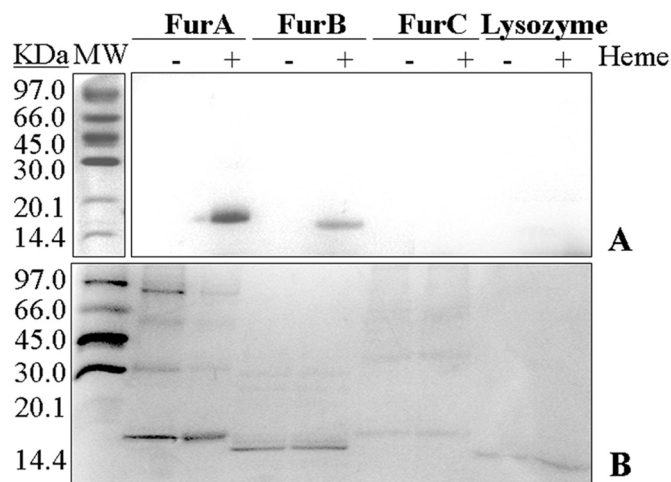
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Figure 2. *In vitro* DNA protection assay



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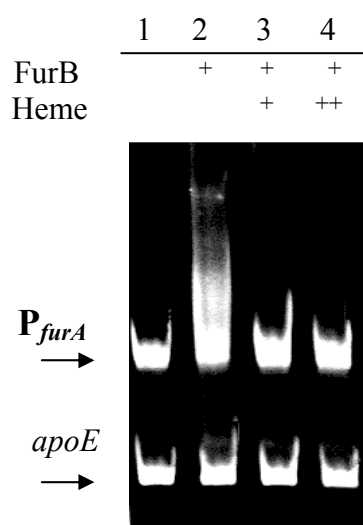
Figure 3. Heme binding and staining assay



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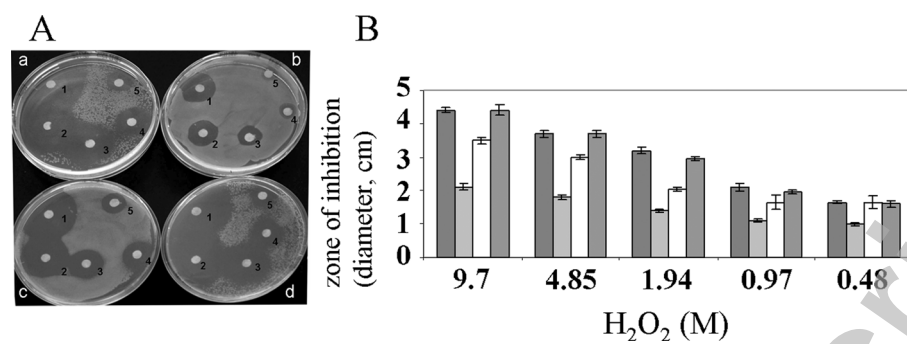
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Figure 4. Inhibition of *in vitro* DNA binding activity of FurB by heme



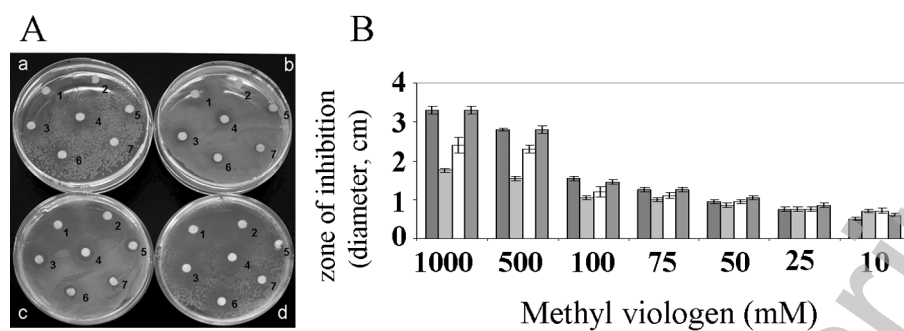
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Figure 5. H₂O₂ disk assay

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Figure 6. Methyl viologen disk assay



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