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Expression of *fur* and its antisense *α-fur* from *Microcystis aeruginosa* PCC7806 as response to light and oxidative stress

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ABSTRACT

Ferric uptake regulation (Fur) proteins are prokaryotic transcriptional regulators that integrate signaling of iron metabolism and oxidative stress responses with several environmental stresses. In photosynthetic organisms, Fur proteins regulate many genes involved in photosynthesis, nitrogen metabolism and other key processes. Also, Fur triggers the expression of virulence factors in many bacterial pathogens, and Fur from *Microcystis aeruginosa* has been shown to bind promoter regions of the microcystin synthesis gene cluster. In this work, we studied transcriptional responses of *fur* genes under different light intensities and oxidative stress. An antisense of *fur*, the *α-fur* RNA, plays an important role in regulating *fur* expression under oxidative stress, affecting levels of Fur protein in cells. Importantly, an active photosynthetic electron chain is required for the expression of the *fur* gene.

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Introduction

Ferric uptake regulation (Fur) proteins are global regulators present in a wide diversity of prokaryotes. Among many other functions, Fur regulators are mainly responsible for the maintenance of iron homeostasis, acting as a fine-tuned rheostat of genes involved in iron incorporation and storage. Moreover, the ability of iron to catalyze the formation of hydroxyl radicals links iron metabolism to oxidative stress responses, also modulated by some members of the Fur protein family (Andrews et al., 2003). Fur is also a key element in the cross-talk between iron and nitrogen metabolism regulatory networks, as described in *Anabaena* PCC 7120 (Lopez-Gomollon et al., 2007a).

It is widely accepted that Fur acts as a classical repressor that uses iron as a co-repressor and binds as a dimer to consensus DNA sequences (iron boxes) when there is sufficient iron in the environment. When iron becomes scarce in the cell, Fur is inactivated by the release of the iron, allowing transcription of Fur-controlled genes (Bagg and Neilands, 1987). In all systems that have been investigated thus far, Fur is an abundant constitutive protein. Regulation of the expression of Fur proteins is governed by different factors depending on the microorganism, and it usually takes place at several steps in the genetic information flow (Hernandez et al., 2004a; Lopez-Gomollon et al., 2007a; Vecerek et al., 2007). At the transcriptional level, among other mechanisms, a common type of Fur control is moderate autoregulation, which is not yet well understood. The presence of several iron boxes in the promoter regions of many *fur* genes allows sequential binding of the repressor providing different levels of control (Escolar et al., 1998). Usually, organisms have more than a single member of the Fur family, and cross recognition of their Fur boxes has been described in *Anabaena* PCC7120 (Hernandez et al., 2004b). Also, a measure of the binding affinity suggests a regulatory interaction of the Fur family members in this cyanobacterium (Hernandez et al., 2004a). In *Escherichia coli*, transcription of *fur* is activated by the general regulators of the oxidative stress response, OxyR and SoxRS (Zheng et al., 1999). In *B. subtilis*, PerR is an interactive regulator with Fur, where PerR represses *fur* by using Mn^{2+} (Fuangthong and Helmann, 2003).

Recently, it has been established that nitrogen metabolism and heterocyst development are strongly related to iron availability, and the expression of FurA in *Anabaena* sp. PCC7120 is modulated by NtcA, the master regulator in nitrogen metabolism (Lopez-Gomollon et al., 2007a). At the post-transcriptional level, a cis-acting antisense RNA *fur* (*α-fur*) has been described, modulating *furA* expression in *Anabaena* sp. PCC 7120 (Hernandez et al., 2006). *Anti-fur* RNAs have also been identified in *Microcystis aeruginosa* PCC7806 and *Synechocystis* PCC6803 (Martin-Luna, 2008; Sevilla et al., 2011). Moreover, post-translational modulation of *E. coli* Fur activity by different ligands has been reported. Tight binding of heme by Fur from *Anabaena* points to a physiological role for Fur-heme complexes (Hernandez et al., 2004a). Inhibition of Fur activity by nitric oxide also establishes a role of Fur in the regulation of *E. coli* responses to reactive nitrogen species (D'Autreaux et al., 2002).

Fur from *M. aeruginosa* sp. PCC7806 holds particular interest, as we have found that the regulator recognizes and binds several promoter regions of the *mcy* gene cluster, involved in microcystin

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synthesis (Martin-Luna et al., 2006). In this study, real time RT-PCR transcriptional analysis of *fur* from the toxic strain *M. aeruginosa* PCC7806 as a response to several stresses was performed.

Materials and methods

Strains and culture conditions

Microcystis aeruginosa strain PCC 7806 was grown in BG11 medium (Rippka, 1988) with 2 mM of $NaNO_3$ and 10 mM of $NaHCO_3$ as indicated by the Pasteur Institute. Cells were grown in batch conditions with continuous aeration at 25 °C. The cells were cultured using a light intensity of 10 μmol of photons $m^{-2}\,s^{-1}$, unless otherwise indicated. Light was measured using a Quantum Sensor photometer (Skye Instrument, SKP 200).

Electrophoresis and immunoblotting

Crude extracts were prepared by sonication of *Microcystis* cells followed by centrifugation to remove cell debris. Protein content was quantified using a bicinchoninic acid protein assay reagent (Pierce) and the proteins were separated on SDS/15% polyacrilamide gels. For immunoblotting, the proteins were electrophoretically transferred to PVDF (0.45 mm pore size transfer membrane from Waters), as described previously (Towbin et al., 1979). Rabbit polyclonal antibodies raised against *Anabaena* FurA proteins were used and blots were visualized and quantified as described previously (Hernandez et al., 2002). The blots presented are representative of at least 3 replicas.

Sampling and RNA isolation

Cyanobacterial cells were harvested from 25 mL of control and stress-treated cultures by centrifugation at 4000 rpm (Biofuge primo R Heraeus) for 3 min at 4 °C. Cell pellets were resuspended in 600 mL of 50 mM Tris–HCl pH 8, 100 mM EDTA and 130 mL of chloroform and then incubated for 3 min in ice in order to remove external RNases. The samples were then centrifuged at 13,000 rpm for 5 min al 4 °C and the cell pellets were frozen in liquid nitrogen until RNA extraction.

Total RNA was isolated using a “FastRNA Pro Blue kit” (Qbiogene, Inc.) in accordance with the manufacturer’s instructions and as previously described (Sevilla et al., 2008). RNA integrity was verified by agarose electrophoresis with ethidium bromide staining and its concentration was measured spectrophotometrically from optical densities at 260 nm. Furthermore, RNA purity was evaluated by OD_{260}/OD_{280} nm absorption ratio (>1.95).

Reverse transcription (cDNA synthesis)

Prior to RT-PCR, total RNA was treated with 40 units of DNase (Pharmacia) in a volume of 100 μl using a buffer containing 4 μl of 1 M Tris–HCl (pH 7.5), 0.6 μL 1 M of $MgCl_2$ in DEPC–H_2O. The sample was incubated at 37 °C for 45 min. The digestion was stopped by heating for 10 min at 65 °C. RNA integrity was checked on a 1% agarose gel, the concentration determined by measuring the absorbance at 260 nm and the purity assessed by the ratio $A_{260\,nm}/A_{280\,nm}$.

For reverse transcription, 2 μg of total RNA were mixed with 300 ng of random hexamer primers (Invitrogen Corp.) and diluted with the annealing buffer (10 mM Tris–HCl (pH 8), 1 mM EDTA, 150 mM KCl) to a final volume of 20 μL. The mixture was heated at 85 °C for 10 min and then incubated at 50 °C for 1 h. The sample was subsequently split into two aliquots of 10 μL. One was incubated with 1 μL of 10 μg/mL RNaseA free of DNase for 30 min at room temperature, in order to be used as control to verify by PCR the absence of DNA. Both samples (control and problem) were reverse transcribed with 200 U of SuperScript™ (GibcoBRL) in the presence of 2 μL of deoxyribonucleoside triphosphate mixture (2.5 μM each one), 2 μL of dithiothreitol (100 mM) and 4 μL of the 5x buffer provided by the manufacturer with the Reverse Transcriptase enzyme. Finally, the volume was adjusted to a final volume of 20 μL in DEPC-H_2O. The mixture was incubated at 47 °C for 1 h followed by a final 15 min heating step at 75 °C. The sequences of the specific primers used for the RT-PCR reactions are defined in Table 1.

Table 1
PCR primers and TaqMan MGB probes used for the real-time RT-PCR analysis and semiquantitative RT-PCR. MFurN and MFurC are the primers used for the *α-fur* expression analysis.

Primer designation	Primer sequences (5ĭ–3ĭ)
fur-N	GATGGGAATTTTGCGGGAGTTG
fur-C	GGATAGGGTTGATTGAGTTCGTAGT
fur MGB probe	ACCCTCGGCCAATTC
16S-N	TGCGTAGAGATTGGGAAGAACATC
16S-C	GCTTTCGTCCCTGAGTGTCA
16S MGB probe	CCAGTAGCACGCTTTC
R16S-F	CAAGTCGAACGGGAATCTTC
R16S-R	CTCAAGTACCGTCAGAACTTC
NdnaJ	CGGGGAATTTGGAGGATTTC
ATS3	GGGTTGATTGAGTTCGTAGTG
MFurN	GTCGATCGCCCATGGCTGCCTAC
MFurC	CAGTTGGGAATTCCCGCTTAGATG

Real-time PCR analysis of gene expression

Real-time RT-PCR was carried out using the ABI Prism 7000 HT Sequence Detection System. The reaction mixture consisted of 5 μL of reverse-transcribed cDNA, 10 μL of TaqMan Universal PCR Master Mix, 1 μ L of unlabeled PCR primers and TaqMan MGB probes (Table 1, FAM dye-labeled) from the Assay-on-Demand Service (all reagents from Applied Biosystems, Foster City, California, USA) and 4 μL of sterile milliQ-H_2O. All samples were run in triplicate. The PCR program was one cycle of denaturation at 95 °C for 10 min followed by 50 cycles at 95 °C for 15 s and 60 °C for 60 s. Sequence Detector Software (SDS) (Applied Biosystems) was used for data analysis. A threshold cycle (Ct) value was determined from each amplification plot.

The relative expression ratio or fold change of the target gene was calculated based on its real-time efficiency (*E*) and the difference between the mean Ct of the sample in the problem condition and the mean Ct of the control sample, and normalized for the expression of 16S RNA used as control gene (Pfaffl, 2001). The equation used was (Pfaffl, 2001):

$$\text{ratio} = \frac{E_{\text{target gene}} \cdot \exp(\Delta \text{Ct}_{\text{target gene}} \cdot (\text{control-problem}))}{E_{\text{ref gene}} \cdot \exp(\Delta \text{Ct}_{\text{ref gene}} \cdot (\text{control-problem}))}.$$

Triplicate samples were measured and blotted as fold increase between stressed and control samples. Each analyzed gene was assessed previously by the slope of the calibration curve, the Ct values on the *Y* axis and the logarithm of the equivalent amount of total RNA on the axis ($E = 10^{[-1/\text{slope}]}$) in accordance with (Pfaffl, 2001). Expression of the target gene was normalized by endogenous reference gene 16S rRNA (AF139299).

RT-PCR in agarose gels

The total RNA (1 μg) was heated at 85 °C for 10 min and used as a template for the first strand cDNA synthesis. Residual DNA in RNA preparations was eliminated by digestion with RNase-free DNase I (Roche). The absence of DNA was checked by PCR. Reverse transcription was carried out using SuperScript retrotranscriptase (Invitrogen) in a 20 μl reaction volume containing 150 ng of random

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