Identification of a Ferric uptake regulator from *Microcystis aeruginosa* PCC7806

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Abstract

Ferric uptake regulator (Fur) proteins are widely recognized as repressors that in many prokaryotes regulate a large number of genes involved in iron homeostasis and oxidative stress response. In our study, we were able to identify the complete sequence of the *fur* gene from *Microcystis aeruginosa* using inverse-polymerase chain reaction. DNA sequence analysis confirmed the presence of a 183 amino-acid open reading frame that showed high identity with Fur proteins reported for cyanobacteria. The recombinant Fur protein has been purified and electrophoretic mobility shift assays shown to be active. Mn$^{2+}$ and dithiothreitol enable Fur to bind to its promoter, with dithiothreitol being more potent. The expression of Fur in *Microcystis* was induced about twofold in iron-deficient conditions.

Introduction

*Microcystis aeruginosa* is one of the strains of cyanobacteria that causes toxic blooms worldwide. It produces the hepatotoxic heptapeptide microcystin, which inhibits eukaryotic protein phosphatases PP2A y PP1. The evaluation of environmental factors that control the proliferation of cyanobacteria in combination with studies of the processes that regulate toxin biosynthesis could contribute to the implementation of management strategies to avoid undesirable blooms and toxin production.

Iron availability and its influence on phytoplankton growth have been an important focus of discussion for oceanographers. After IronExII (Behrenfeld et al., 1996; Coale et al., 1996; Kirchman, 1996) it was shown that iron availability limits rates of cell division, as well as abundance and production of phytoplankton of the equatorial Pacific and possibly in other ‘high nutrient, low chlorophyll’ regions (Frost, 1996; Boyd et al., 2000, 2004). Although iron is one of the most abundant elements in nature, its deficiency is a constant source of stress in many biological systems because it is not always freely available in its soluble form (Lewin, 1984). Paradoxically, its uptake and incorporation must be tightly regulated, as iron catalyses the formation of reactive oxygen species through the Fenton reaction. In fact, cyanobacteria have developed very efficient mechanisms to maintain iron homeostasis. Iron limitation induces the expression of several proteins to overcome this problem, such as proteins involved in the synthesis of siderophores or a new chlorophyll-binding protein, and causes the replacement of proteins containing iron cofactors with noniron proteins, such as flavodoxin (Strauss, 1994). Success of a species would depend on the correct functioning and precise control of all the genes involved in the response to iron deficiency. One of the key factors for this response in prokaryotes is a DNA-binding protein called Ferric uptake regulator (Fur). Fur acts as a classical repressor. The current model explains that when complexed to ferrous ions, a dimer of Fur binds to a specific DNA sequence (known as Fur-box) located in iron-responsive gene promoters (de Lorenzo et al., 1988; Coy & Neilands, 1991). Even though Fur is an iron-dependent repressor, the
interest of Fur is not only limited to its participation as a modulator of genes related to iron homeostasis. The relevance of Fur in the oxidative stress response has been widely described (Nunoshiba et al., 1999) and its participation to overcome acidic stress situations has also been proposed (Hall & Foster, 1996). Moreover, Fur has been demonstrated to be an essential element in triggering the expression of virulence factors in heterotrophic infectious bacteria (Escolar et al., 1999). Fur regulates peptide synthetase systems involved in the synthesis of peptidic siderophores such as enterobactin, vibriobactin, etc (Crosa & Walsh, 2002). These nonribosomal peptide synthetases are similar to the enzymes involved in the synthesis of microcystins, one of the most ubiquitous cyanotoxins. The linkage between iron metabolism and microcystins has been widely discussed, with contradictory results (Lukac & Aegerter, 1993; Utkilen et al., 1995; Lyck et al., 1996). However, several observations support the conclusion that toxic strains overcome iron deficiency much better than nontoxic ones (Utkilen & Gjolme, 1995) and, moreover, toxic strains exhibit greater ability of iron uptake than nontoxic ones (Utkilen & Gjolme, 1995).

Ferric uptake regulator is a master regulator in most prokaryotes that controls a large number of genes, including those implicated in iron homeostasis, oxidative stress responses and central metabolism. In order to study the role of Fur in the toxic strain M. aeruginosa PCC7806, a fur homologue has been cloned and overexpressed. The recombinant product was purified and its activity tested using its own promoter. The affinity of Fur for the DNA was tested in a reducing environment and in the presence of metal, for the purpose of investigating the optimal interaction of Fur with its target sequences.

Materials and methods

Cloning procedures

Microcystis aeruginosa PCC7806 was provided by the Pasteur Culture Collection and growth in BG11 media with 2 mM of NaNO₃ and 10 mM of NaHCO₃ (Rippka et al., 1979). Chromosomal DNA from M. aeruginosa was obtained according to Neilan et al. (1995), and used as template for PCR amplification.

Oligonucleotides designed according to the N-terminal of Anabaena sp. PCC7120 furA gene and the histidine-rich motif of the Fur family of proteins were used for PCR amplification. The reaction mixture (50 μL) contained 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.6 μM of each sense and antisense primer, 200 μM of each dNTP, 1.5 mM MgCl₂ and 2.5 U of Taq DNA polymerase (GIBCO-BRL, Gaithersburg, MD). Before amplification, tubes were incubated at 95 °C for 4 min to ensure that the template DNA was completely denatured. Thermal cycling was performed in a Perkin-Elmer Cetus (Wellesley, MA) thermal cycler, with 30 cycles of denaturing (94 °C for 30 s), annealing (46 °C for 1 min) and extending (72 °C for 1 min), plus a final extension step at 72 °C that lasted for 10 min. A fragment of 296 bp was amplified and sequenced, showing a high degree of identity with the Anabaena PCC7120 furA gene. DNA sequences and protein alignments were performed using Blastp 2.2.6 at the NCBI GenBank online service. Inverse polymerase chain reaction (PCR) was performed to determine the flanking regions of this sequence. Restriction fragments were obtained using approximately 0.3 μg of source DNA treated with 10 U of HindIII and XbaI. Digested DNAs were separated by electrophoresis through a 0.8% (weight in volume) agarose gel. Restriction fragments of HindIII and XbaI were long enough to contain the fur gene. For circularization, 30 ng of HindIII and XbaI restriction fragments were diluted to a concentration of 1.2 ng μL⁻¹ in the ligation mixture. The ligation reaction was initiated by the addition of 2 U of T4 DNA ligase (Fermentas, Hanover, MD) and the reaction was allowed to proceed for 21 h at 15 °C.

PCR was carried out with different quantities of circularized DNA: 1.2 and 2.4 ng, in the presence of 0.6 μM of the two primers homologous to the ends of the core sequence, that is, the 296 bp fragment amplified previously, but with opposite orientations. First, amplification tubes were incubated at 95 °C for 3 min to ensure that the template DNA was completely denatured. The amplification consisted of 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 52 °C for 1 min and extension with Taq DNA polymerase at 72 °C for 2 min to favour the extension of long fragments. The final extension step was carried out at 72 °C and lasted for 10 min (Perkin-Elmer Cetus). Several PCR products were sequenced, and one of them contained the putative fur gene and flanking regions upstream and downstream of the gene. DNA sequences and protein alignments were performed using ClustalW Multiple Alignment tool on the BioEdit Sequence Alignment Editor v. 5.0.9.

Microcystis fur gene was amplified by means of the PCR using a PTC-100 (Programmable Thermal Controller) from MJ Research Inc., Waltham, MA. Synthetic oligonucleotides were used to generate double-stranded DNA with restriction enzyme sites for cloning. A forward primer 5'-GTGCGATC GCCCATGCGCTGCTAC-3' encoding an Neol restriction site and the reverse primer 5'-CAGTTGGGAAATTCGCT TAGATG-3' encoding an EcoRI restriction site were designed on the basis of the putative fur gene sequence obtained by inverse PCR. Total chromosomal DNA from M. aeruginosa PCC7806 served as a template for PCR amplification. Amplification was carried out in a reaction mixture (50 μL) formed by 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.6 μM of each sense and antisense primer, 200 μM of...
each dNTP, 1.5 mM MgCl₂ and 2.5 U of Taq DNA polymerase (GIBCO-BRL). Samples were incubated at 95 °C for 3 min, prior to thermal cycling performed during 30 cycles of denaturing (30 s at 93 °C), annealing (58 °C for 1 min) and extending (72 °C for 1 min), plus a final extension step at 72 °C for 10 min (Perkin-Elmer Cetus).

**Overexpression of the recombinant Fur protein from Microcystis aeruginosa PCC7806**

The single product obtained by PCR was purified using GFX™ PCR-DNA band purification kit (Amersham-Pharmacia, Little Chalfont, UK) and digested with the restriction enzymes NcoI and EcoRI. The band containing the corresponding fur gene was purified again to remove the digestion mix and cloned into the NcoI- and EcoRI-cloning sites of the expression vector pET-28a (+) (Novagen, San Diego, CA), using T4 DNA ligase (Takara, Shiga, Japan). *Escherichia coli* JM 109 cells (Promega, Madison, WI) were transformed with the ligation product. Automatic DNA sequencing was performed to confirm the sequence.

In order to overexpress the recombinant Fur protein, *E. coli* BL21-Gold DE3 cells (Stratagene, La Jolla, CA) were transformed with the construct pET-28a (+)/fur and colonies were grown in Luria–Bertani plates containing kanamycin (30 μg mL⁻¹). Several colonies containing the pET-28a (+)/fur plasmid were grown overnight in Luria–Bertani medium supplemented with 30 μg mL⁻¹ of kanamycin. Then, a culture aliquot was diluted 70 times in the same culture medium. After 3 h of growth at 37 °C, with absorbance at 600 nm around 0.7–0.9, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM to induce the expression. In control cultures, IPTG was omitted. Cells were allowed to grow at 37 °C for 4 h. The level of expression was monitored evaluating total protein content by electrophoresis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and further analysis with a Gel Doc 2000 image analyser from Bio-Rad.

**Purification of the Fur protein**

The Fur protein was purified by previously described procedures (Hernandez et al., 2002). The initial step consisted of nucleic acid digestion in crude extracts using 100 U mL⁻¹ DNasel (Sigma, St. Louis, MO). After 35 min of incubation at 4 °C, the extract was clarified by centrifugation for 1 h at 14000 r.p.m. Two chromatographic steps, Heparin-Sepharose 6 Fast Flow (Amersham-Pharmacia) (10 cm × 1.5 cm) and a Zn-iminodiacetate column (Chelating Sepharose Fast Flow from Amersham-Pharmacia) (1.8 cm × 20 cm), were used. *Microcystis* Fur eluted between 0.3 and 1 M imidazol in the metal affinity column, and the pure protein was in the last part of the gradient. After dialysis with 50 mM Tris-HCl, pH 8, containing 10% glycerol and 0.5 M glycine, it was concentrated and stored at −20 °C.

**Analytical methods**

Total protein was quantified using the bicinchoninic acid method (BCA™ Protein Assay Reagent Kit from Pierce, Rockford, IL). The extinction coefficient used for *Microcystis* Fur was 22 781 M⁻¹ cm⁻¹. This value was calculated based on the method of Gill & von Hippel (1989). Also, quantification of *M. aeruginosa* Fur was confirmed by densitometric measurements in gel, using purified FurA from *Anabaena* sp. PCC7120 as standard.

**SDS-PAGE and western blotting**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using 15% (weight in weight) polyacrylamide gels. Immunoblotting was performed using antibodies against FurA from *Anabaena* sp. PCC7120 (Hernandez et al., 2002). Samples were electrophoresed in a 15% SDS-PAGE gel and electroblotted to an IMMOBILON™ P (filter type PVDF, pore size 0.45 μm) from Millipore (Billerica, MA), at 20 V for 40 min. Immunodetection was performed according to Towbin et al. (1979). N-terminal analysis was performed at the sequencing service of the Centro de Investigaciones Biológicas (Madrid, Spain).

**Gel mobility shift assays**

The mobility shift assays were carried out as described in Hernandez et al. (2002) with a modified binding buffer, which contained 40 mM Tris, pH 7.5, 40 mM KCl, 0.1 mg mL⁻¹ bovine serum albumin, 2 mM MgCl₂ and 5% glycerol. 10 mM dithiothreitol (DTT) and 100 μM Mn²⁺ were used as indicated in the experiments. Mn²⁺ was used instead of ferrous iron because it readily oxidizes to Fe³⁺ in air. The DNA fragments that used electrophoretic mobility shift assays (EMSAs) were obtained by PCR (Bes et al., 2001). A fragment of 387 bp located upstream of the fur gene was amplified with the primers *pfurC* 5'-CAGACATAAGCGATCGACTC-3' and *pfurN* 5'-CTCTTGAAGGATAGTGCACC-3'. This fragment was used in the bindings assays as the promoter region of *Microcystis* fur gene. To demonstrate the specificity of the DNA-binding activity of the proteins to the promoters, reactions were carried out in the presence of an equal concentration of a 224 bp fragment of nonspecific competitor DNA (part of the fourth exon of human apoE gene). Each EMSA was performed four or five times, and the gels shown in the figures are representative of the obtained results. The binding was evaluated by the estimation of remaining unbound DNA in each sample, compared with the band measured for free DNA, taken as 100% (Gel Doc 2000 Image Analyser from BioRad).
Fig. 1. Total nucleotide sequence of the *fur* gene from *Microcystis aeruginosa* PCC7806 and the predicted protein sequence (accession number AY641528). Upstream nucleotide sequence is shown, and putative iron boxes are indicated in boxes. The DNA fragment used for binding experiments is also shown (*pfurN* and *pfurC*).
Results and discussion

The complete genome sequence of Microcystis aeruginosa PCC 7806 is not available yet at the databases, and for this reason we used the information about the nucleotide sequence from cyanobacterial Fur proteins available in the Cyanobase (www.kazusa.or.jp) to design the oligonucleotides. Two oligonucleotides located complementary to the beginning of furA from Anabaena sp. PCC7120 and to the histidine-rich motif of the putative fur-encoding region were synthesized. After amplification, a 296 bp band was obtained with sequence homology to the fur genes from other cyanobacteria. Inverse PCR allowed the identification of the complete sequence of the Microcystis aeruginosa fur gene. Figure 1 shows its nucleotide sequence as well as its upstream nucleotide sequence. The upstream sequences were analyzed using a consensus sequence previously determined for iron boxes present in the furA gene from Anabaena sp. PCC7120 (Hernandez et al., 2005). Possible iron boxes were identified in the sequence of the promoter region of the fur gene (Fig. 1), with about 40–50% of identity with those determined for the Anabaena fur and isiB genes (Hernandez et al., 2005). The presence of iron boxes suggests that the M. aeruginosa gene is autoregulated, as it was found in other cases (Hernandez et al., 2004). The complete sequence of this gene has been deposited in GenBank under accession code AY641528. The deduced amino acid sequence encoded by the fur gene from M. aeruginosa was compared with proteins of the Fur family, showing high identity with the sequence of Fur proteins from other cyanobacteria (Fig. 2). The protein shows 81% identity with Fur from Synechocystis PCC6803 (sll0567) and 70% with FurA from Anabaena sp. PCC7120 (all1691), homologues of Fur involved in response to oxidative stress and iron availability. The main differences between the proteins are located in the C-terminal domain, where the Fur from M. aeruginosa is extended compared with the others. The DNA-binding domain and the metal-binding domain were highly conserved. The Fur protein from Microcystis has 183 amino acids, with a deduced molecular weight of 21 014 Da, and contained more cysteine residues than usually observed. The cysteines are arranged similarly as in other cyanobacteria, and five of them are very conserved in the sequences (Fig. 2). They may function as redox sensor and be putatively involved in metal binding and oligomerization (Ortiz de Orue Lucana & Schrempf, 2000). The calculated isoelectric point is

Fig. 2. Alignment of the Microcystis aeruginosa PCC7806 Fur protein with some members of the Fur family from cyanobacteria. Identical amino acids are enclosed in boxes. The DNA binding helix-turn-helix motif and the conserved His-rich motif involved in metal binding are illustrated in the figure. The homologues with higher identity were Synechocystis sll0567 gene product, 81%, Trichodesmium erythraeum (accession number ZP00073336), 72%, Thermosynechococcus elongatus tll0048 gene product, 72%, and Anabaena (Nostoc) sp. PCC7120 all1691 gene product (FurA), 70%.
6.5, which is in the range of those described for other family members.

Among several expression systems that are available to overproduce recombinant proteins in *E. coli*, pET-28a(+) vector has proven to be one of the most efficient systems in our case. *Escherichia coli* BL21-Gold DE3 cells were transformed with the expression plasmid containing the fur gene from *Microcystis*. When the cells were induced with IPTG, a marked increase in the amount of Fur was observed in the crude extract by SDS-PAGE analysis (Fig. 3, lane 3 vs. lane 2). The apparent molecular weight was determined as 21 kDa, which is congruent with the value calculated from the deduced sequence of amino acids.

Purification of Fur family proteins is not an easy task, and the yield of the process is usually very low. The purification process of the recombinant Fur protein on 15% SDS-PAGE is shown in Fig. 3, lanes 3–6. A nucleic acid digestion results in some Fur lost by precipitation (lane 4 vs. lane 3), but it is a key step in order to achieve an optimal binding to the Heparin column. After the metal affinity column, the final purity of the protein was estimated to be over 95%. The N-terminal sequence analysis of the purified recombinant protein was performed and it was as expected (AAYTA...), considering the processing of the N-terminal methionine and the substitution of the first serine by an alanine because of the processing of the N-terminal methionine and the substitution of the first serine by an alanine because of the in silico prediction of the expression plasmid containing the fur gene from *Microcystis*. When the cells were induced with IPTG, a marked increase in the amount of Fur was observed in the crude extract by SDS-PAGE analysis (Fig. 3, lane 3 vs. lane 2). The apparent molecular weight was determined as 21 kDa, which is congruent with the value calculated from the deduced sequence of amino acids.

**Fig. 3.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the expression and purification of Fur from *Microcystis aeruginosa* PCC7806. A clone of *Escherichia coli* BL21-Gold (DE3) was transformed with pET28a(+) plasmid carrying the fur gene. Cells were induced with isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM, and samples were collected at different times. Electrophoresis was performed in a 15% SDS-PAGE. Lane 1, molecular mass markers; lane 2, soluble crude extract of cells without the expression plasmid; lane 3, soluble crude extract from transformed cells after induction with IPTG; lane 4, crude extract after nucleic acid elimination; lane 5, Fur-containing fractions after the heparin-Sepharose column; lane 6, Fur-containing fractions after the metal affinity column.

**Fig. 4.** Electrophoretical mobility shift assays of recombinant Fur from *Microcystis* showing that it is active and binds its own promoter. All the assays were performed using a fragment of exon IV from the human apoE gene as nonspecific competitor DNA. MnCl2 was 100 µM and dithiothreitol, 1 mM. The 387 bp promoter region fragment used (containing the putative iron boxes) is indicated in Fig. 1. Lane 1, free fur promoter; lanes 2–8, p-fur and 0.1, 0.2, 0.3, 0.5, 0.75, 1 and 1.5 µM Fur, respectively.

1). This fragment contains the three boxes determined in silico. Figure 4 shows the retardation of electrophoretic mobility obtained when different amounts of the Fur protein were incubated in the presence of Mn2+ and DTT, and in the presence of unspecific competitor DNA. Mn2+ was used instead ferrous iron because it readily oxidizes to Fe3+ in air. The purified Fur protein is active, showing binding activity to its own promoter region, suggesting autoregulation. Also, the obtained Fur binds to the described Fur-regulated promoters, such as isiB (flavodoxin gene) and furA from the cyanobacterium *Anabaena* sp. PCC7120 (data not shown) (Bes et al., 2001). It is generally accepted that Fur acts as a positive repressor that regulates transcription after interacting with its corepressor Fe2+. However, the DNA-binding behaviour of the different members of the Fur family may differ depending on the presence of metal and the redox state of the cysteines. To define the binding requirements of the *Microcystis* Fur protein we carried out EMSA in the presence or absence of Mn2+ and/or DTT. The *Microcystis* Fur binds its promoter in a DTT- and Mn2+-dependent manner, with DTT increasing the affinity significantly (Fig. 5a). In the absence of DTT, Fur binds DNA with lower affinity, with or without metal (Fig. 5a). Also, Fig. 5a shows that the presence of Mn2+ increases the binding slightly, indicating that this Fur family member could also be metal responsive. The presence of oxidants such as H2O2 diminishes the binding activity of Fur (Fig. 5b), and this activity is fully restored when DTT is added to the H2O2-treated protein (Fig. 5b). Fur from *Microcystis*, having seven cysteines, is highly responsive to the redox state of these residues, indicating a possible role as a sensor of oxidative stress.
Microcystis aeruginosa causes toxic blooms, which have been observed with increasing frequencies in the surface waters of the world. Iron plays a key role in phytoplankton composition and abundance (Behrenfeld et al., 1996; Coale et al., 1996; Kirchman, 1996), and Fur is one of the central regulators involved in iron homeostasis. Thus, the identification of a fur gene from this organism, in addition to the purification of the recombinant protein, may help to understand the mechanisms behind M. aeruginosa success and toxin production.

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