Fur regulates the expression of iron-stress genes in the cyanobacterium *Synechococcus* sp. strain PCC 7942

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A homologue of the 'ferric uptake regulation' gene (*fur*) was isolated from the cyanobacterium *Synechococcus* sp. strain PCC 7942 by an *Escherichia coli*-based 'in vivo repression assay'. The assay uses a reporter-gene construct containing the promoter region of the iron-regulated cyanobacterial gene *isiA*, fused to the coding region for chloramphenicol acetyltransferase. The isolated gene codes for a protein that has 41% sequence similarity (36% identity) to Fur from *E. coli* and contains the putative iron-binding motif found in the Fur proteins of purple bacteria. No significant similarity was found to the DxtR repressor that regulates the expression of toxin and siderophore production in Gram-positive bacteria. Insertional mutagenesis of the cloned cyanobacterial *fur* gene led to the creation of heteroallelic mutants that showed iron-deficiency symptoms in iron-replete medium, including the constitutive production of flavodoxin and of hydroxamate siderophores. Failure to eliminate wild-type copies of the *fur* gene from the polyploid genome of *Synechococcus* 7942 implies that in this cyanobacterium Fur may have essential functions in addition to the regulation of genes involved in iron scavenging or photosynthetic electron transport.

Keywords: Fur, iron regulation, cyanobacterium, *Synechococcus*

INTRODUCTION

Even though iron is the fourth most abundant element in the earth's crust, iron deficiency is a recurring nutrient stress condition for micro-organisms in both marine and fresh water oxic ecosystems because of the very low solubility of the ferric ion. In fact, iron deficiency has been hypothesized to be the cause of low phytoplanktonic biomass in pelagic environments, where iron concentrations are as low as 0.02 nM, while other nutrients such as nitrate and phosphate are abundant (Martin & Fitzwater, 1988). Recently, this hypothesis was supported by two large-scale oceanic experiments (Martin *et al.*, 1994; de Baar *et al.*, 1995). In one of these experiments, the addition of enough iron to increase the oceanic iron concentration south of the Galapagos Islands to only about 4 nM caused a fourfold increase in phytoplanktonic production. The largest contributors to biomass increase

were cyanobacteria of the *Synechococcus* group, red fluorescing picoplankton and autotrophic dinoflagellates (Martin *et al.*, 1994).

Since iron is an essential redox component for many critical cellular processes, including photosynthesis, respiration, nitrogen fixation, ribonucleotide synthesis and haem synthesis, iron deficiency has profound effects on the physiology of cyanobacteria and other micro-organisms. Molecular responses that enable eubacteria to survive periods of iron deficiency include the production of siderophore-based iron-scavenging systems, the replacement of ferredoxin by flavodoxin and, for many pathogenic bacteria, the production of toxins. The molecular switches for the regulation of iron-stress responses have been elaborated for Gram-negative purple bacteria (Braun & Hantke, 1991) and Gram-positive bacteria (Tao & Murphy, 1992). Each of these bacterial groups has a repressor protein that recognizes specific operator sequences: the Fur repressor functions in purple bacteria while the larger DtxR repressor controls iron genes in Gram-positive bacteria. Since all cyanobacterial iron-stress-induced genes appear to contain operator

Abbreviations: EDDA, ethylenediamine-di(o-hydroxyphenylacetic acid).

The GenBank accession number for the sequences reported in this work is L41065.

sequences related to the Fur-binding consensus sequence of purple bacteria (Straus, 1994), a method was devised to isolate the iron-stress repressor gene from *Synechococcus* sp. strain PCC 7942. This paper reports on the cloning and properties of the *fur* homologue from this unicellular cyanobacterium.

METHODS

Bacterial strains, plasmids and medium. Characteristics of *Escherichia coli* and *Synechococcus* sp. strains and plasmids used in this study are described in Table 1. *E. coli* JM109 *fur* mutants were created by the method of Hantke (1981) using hydroxylamine as the mutagenizing agent. *Synechococcus* 7942 was grown in BG11 medium liquid culture (Allen, 1968) at 29 °C. Iron-deficient growth medium BG11-CH pF16 was BG11 that did not contain EDTA, ferric ammonium citrate and citric

acid, but was supplemented with 2.3 µM FeCl₃. BG11 pF17 was BG11 without ferric ammonium citrate and citric acid, supplemented with 0.23 µM FeCl₃.

Isolation of cyanobacterial iron repressor gene in *E. coli*. PCR was used to amplify the promoter region of the iron-regulated, *Synechococcus* 7942 *isiA* gene (Laudenbach & Straus, 1988) from genomic DNA. The promoterless *cat* gene was amplified from pBC SK+ (Stratagene) using DNA primers to the translational start region and the transcriptional stop region of this gene. Both products were used to construct pAisiAPCATB through the manipulation of a series of intermediate plasmids listed in Table 1. pAisiAPCATB is a derivative of pACYC177 that contains an iron-regulated reporter-gene. Finally, pAisiAPCATB was transformed into *E. coli* JM109F. Transformants were resistant to both kanamycin (35 µg ml⁻¹) and chloramphenicol (22.5 µg ml⁻¹). A library of DNA fragments from *Synechococcus* 7942 was created by partially

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference*
Strains		
<i>E. coli</i>		
JM109	<i>recA1</i>	a
JM109F	JM109 with <i>fur</i> mutation	This study
H1680	<i>fhuA lac ser thr galK fhuF::λplacMu</i> (Neo ^r)	b
H1681	H1681 <i>fur-31 xbf::Tn10</i>	b
<i>Synechococcus</i>		
7942	<i>Synechococcus</i> sp. strain PCC 7942	—
Plasmids		
pBC SK(+)	Cloning vector with chloramphenicol resistance marker	Stratagene
pUC18, pUC19	Cloning vectors with ampicillin resistance markers and the pMB1 origin of replication	a
pR2C2	pUC19 containing the <i>isiA</i> gene	c
pUisiAP	<i>isiA</i> promoter cloned into the <i>HincII</i> site of pUC18	This study
pUCAT	<i>cat</i> coding region cloned into the <i>HincII</i> site of pUC18	This study
pUisiAP-CAT	pUisiAP with the coding region of <i>cat</i> fused to the <i>isiA</i> promoter	This study
pACYC177	Cloning vector with kanamycin and ampicillin resistance markers and the p15A origin of replication	d
pAisiAPCATA	The <i>isiA/cat</i> fusion construct from pUisiAP-CAT was excised using <i>PstI</i> and inserted into the <i>PstI</i> site of pACYC177	This study
pAisiAPCATB	pAisiAPCATA digested with <i>SaI</i> and <i>BstEII</i> , treated with T4 polymerase and then recircularized	This study
pBluescript SK(+)	Cloning vector with ampicillin resistance marker	Stratagene
pBSauFUR	pUC18 with PCC 7942 <i>fur</i> on a <i>Sau3AI</i> DNA fragment	This study
pBSΔ <i>SacI</i>	pBluescript SK(+) with the <i>SacI</i> site removed	This study
pBSFUR	pBSΔ <i>SacI</i> containing <i>fur</i> on a 0.9 kb <i>XbaI</i> – <i>AclI</i> fragment from pBSauFUR	This study
pBSFURCAT	pBSFUR with the complete <i>cat</i> gene inserted into the <i>SacI</i> site	This study
pXB7	An <i>E. coli</i> /PCC 7942 shuttle vector with the ampicillin resistance marker	e
pXB7-CAT	pXB7 with the complete <i>cat</i> gene inserted into the <i>SmaI</i> site	This study

* References: a, Yanisch-Perron *et al.* (1985); b, Hantke (1987); c, Laudénbach & Straus (1988); d, Chang & Cohen (1978); e, Lau & Straus (1985).

digesting *Synechococcus* 7942 DNA with *Sau3AI* and ligating 2–4 kb DNA fragments, that had been size-selected on low-melting-temperature agarose, into pBluescript. To screen for the cyanobacterial *fur* gene in this DNA library, *E. coli* JM109F containing pAisiAPCATB was transformed with the *Sau3AI* DNA library and transformants were grown on LB agar plates containing kanamycin and ampicillin. Individual colonies of these transformants were streaked onto LB agar containing kanamycin (35 µg ml⁻¹), ampicillin (60 µg ml⁻¹) and chloramphenicol (22.5 µg ml⁻¹), as well as plates with just kanamycin and ampicillin, to identify those transformants that had acquired chloramphenicol sensitivity as a result of transformation. Chloramphenicol-sensitive transformants were streaked onto iron-deficient solid medium [4.0 g tryptone l⁻¹, 2.5 g NaCl l⁻¹, 15 g agar l⁻¹, 20 µg ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDA) ml⁻¹, 35 µg kanamycin ml⁻¹, 60 µg ampicillin ml⁻¹, and 22.5 µg chloramphenicol ml⁻¹] to identify transformants that showed iron-regulated chloramphenicol resistance. A *Sau3AI* DNA fragment that conferred these properties was sequenced and the DNA fragment was then used as a probe to clone two independent *PstI* DNA clones of the putative *fur* gene for further sequencing.

β-Galactosidase assay. The method of Miller (1992) was used. For iron-replete conditions, LB growth medium was supplemented with 100 µM ferric ammonium citrate. For the iron-deficient conditions, LB medium was supplemented with 50 µg EDDA ml⁻¹. All growth media included the appropriate antibiotics.

Insertional inactivation of the *fur* locus. The *SacI* site of pBluescript was removed by digestion with *SacI* followed by digestion with T4 DNA polymerase in the absence of dNTPs to remove the 3'-OH overhangs. The *fur* gene was subcloned as an *AclI*-*XbaI* fragment into this vector. This clone was then digested with *SacI* and treated with T4 DNA polymerase. The resulting DNA was ligated to a 1.1 kb PCR fragment containing the complete *cat* gene. This construct was used to transform *Synechococcus* 7942 by the method of Golden (1988). Transformed cells were plated on BG11 plates supplemented with 7.5 µg chloramphenicol ml⁻¹. The resulting colonies were streaked onto BG11 plates supplemented with chloramphenicol. When these streaked cultures had grown up, they were inoculated into liquid BG11 liquid medium supplemented with 35 µg chloramphenicol ml⁻¹. Each liquid culture was then carried through four serial transfers to allow the chromosomes containing the insertionally inactivated form of the gene to segregate from those with the wild-type allele. Chromosomal DNA was extracted from 40 transformants and analysed for insertional inactivation of *fur* using Southern blots of genomic DNA.

Protein analysis. Immunoblot analysis was performed as described by Sambrook *et al.* (1989). Protein biosynthesis by *Synechococcus* 7942 was investigated by *in vivo* labelling of the cell with ³⁵SO₄²⁻. Mid-exponential cells were harvested from 50 ml of culture by centrifugation at 5000 *g* for 10 min and resuspended in fresh BG11 or BG11-CH pF16, which was prepared without the addition of sulfate. Cell suspensions were washed several times with these media by repeated centrifugations and then grown in the absence of sulfate for 3 h. Then Na₂³⁵SO₄ was added to a final concentration of 2 µCi ml⁻¹ (74 kBq ml⁻¹) and the cells were harvested by centrifugation 2.5 h later. The soluble protein fraction was isolated as reported elsewhere (Coleman & Grossman, 1984) and separated by SDS-PAGE on a 10–15% linear gradient of polyacrylamide.

Csáky test. Samples (3 ml) of mid-exponential cultures were centrifuged at 10000 *g* for 20 min. The supernatant was passed through a Gelman GA-8, 0.2 µm pore size filter; 2 ml of the

filtered supernatant was pipetted into a 15 ml screw-capped test tube and analysed for hydroxamate siderophores by the method of Csáky (1948).

RESULTS

Identification of *Synechococcus* 7942 DNA fragments capable of *in vivo* repression of the *isiA/cat* reporter construct

A *fur* homologue was isolated from *Synechococcus* 7942 using an *in vivo* repression assay in *E. coli*. This approach uses a reporter-gene construct that contains an iron-dependent down-regulated promoter from *Synechococcus* 7942 to screen for cyanobacterial DNA clones capable of inhibiting the expression of the reporter gene in an *E. coli* *recA fur* strain under iron-replete conditions. A *fur* strain was used to eliminate interaction between the Fur repressor of *E. coli* and the iron-regulated promoter of *Synechococcus* 7942. The iron-regulated reporter-gene construct was created by fusing the upstream region of *isiA* from *Synechococcus* 7942 to the coding region of the *cat* gene in the low-copy plasmid pACYC177. A low-copy plasmid was used so that even low-level expression of a repressor gene (present in a high-copy plasmid) would repress all copies of the reporter gene. To ensure complete repression by the target clone, the *cat* gene must be entirely under the control of the *isiA* promoter and must not be transcribed from promoters of drug resistance genes in the plasmid. Therefore, the residual promoter region of the *amp* gene was removed and the *isiA/cat* fusion construct was placed so that its transcription would be in the opposite direction to that of the resident *npt* gene.

An analysis of *isiA* promoter activity indicated that the fusion construct created chloramphenicol resistance in host *E. coli* cells when streaked on solid media, up to a chloramphenicol concentration of 22.5 µg ml⁻¹. This strain of *E. coli* was transformed with a *Sau3AI* partial chromosomal library from *Synechococcus* 7942, and 5000 colonies were screened for the loss of the chloramphenicol-resistance phenotype. False positives were eliminated by a second round of screening. The 80 positive clones remaining were tested for iron regulation by screening for chloramphenicol resistance under iron-deficient conditions. Six of the original 80 clones proved to be iron-regulated. Restriction endonuclease analysis showed that the plasmids from four of these clones contained identical 3 kb DNA fragments.

Sequence analysis of the *Sau3AI* clones containing the putative *fur* gene

DNA sequence analyses of two of these clones revealed an open reading frame that codes for a 147 amino acid protein with a sequence that is 36% identical to the amino acid sequence of Fur from *E. coli*. This putative cyanobacterial Fur protein contains the sequence HHXHXXCXXC which has been implicated in metal binding and is conserved in most other Fur sequences (Hennecke, 1990). Since the sequence of these *Sau3AI* clones contained only 45 bases in the upstream region of

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1  AAGTTTGGAGGCTCCGACTGCTGCGATCGCCGATCCTTTAGCTTCATTGGTGGCTGCCTA 60
61  GTTGCATCGCCCGTTCGCGCCACTCTTACTGTAGATCAGCAGGTTGGCGCGACTAGT 120
122  GGTGAGCTATGGGTGGGAAGCAATCGCTCATCTGGACAGCATAGGCTGCCGTTGCTTTT 180
181  TCGCCTTACCTGCTCAGACCCAAGCGGTGAGGAATACACGATTCTTGAAGCCTTGGGA 240
241  TCTCGTCGGCTATCATCTTCAATATGACGAGCTAGTTGACTCGCAATGACCTACACGCG 300
      M T Y T A
      1
301  GCCTCCCTCAAAGCGGAATGAACGAGCGAGGCTGGCGATTGACGCTCAGCGCGAAGAA 360
      A S L K A E L N E R G W R L T P Q R E E
      10      20
361  ATTCTGCGAGTCTTTCAAATCTCCAGCAGGTGAACACCTCAGTGTGAGGACCTTTAC 420
      I L R V F Q N L P A G E H L S A E D L Y
      30      40
421  AATCATTGCTGAGTCGCAATTCACCCATCAGCCTCTCGACTATCTATCGCACCTGAAG 480
      N H L L S R N S P I S L S T I Y R T L K
      50      60
481  CTAATGGCTCGCATGGTCTCTCGGAGAGCTAGATCTGGCCGAAGATCACAAGCATAC 540
      L M A R M G L L R E L D L A E D' H K H Y
      70      80
541  GAGCTCAATCAACCCCTGAAGCATCACCATCACTTGTATCTGCGTCAGTTGAGCAGACG 600
      E L N Q P L K H H H L I C V S C S K T
      90      100
601  ATTGAGTTCAAGAGCGATTCCGTGCTCAAATTTGGCGCAAGACTTCCGAGAAGGAGGGC 660
      I E F K S D S V L K I G A K T S E K E G
      110      120
661  TACCACCTCTTGGATTGTGAGCTCAGCTCACCATTATGCGCTGCGCAACCTGTGAGCGATCG 720
      Y H L L D C Q L T I H G V C P T C Q R S
      130      140
721  CTGGTTTGGTGCAGCTAGCGTAGCGTCCGCAAGCAACGACCAATGACAAATGGTGTGA 780
      L V STOP
      147
781  GGAAACGGCACTTGGCGAGCAGCTGCACTGGCAAGAGGTCTTGATAGAGCTGTTCGAG 840
841  GCGATCGAGGTTGCCCTCAAGGGTGTGA

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Fig. 1. Nucleotide sequence of *fur* and flanking regions. The deduced amino acid sequence is shown below the nucleotide sequence in one-letter code. The open reading frame of *fur* occupies nucleotide positions 285–726. A putative transcriptional stop site is underlined with inverted arrows. The nucleotides corresponding to the translational start site and stop site are in bold letters and underlined.

the *fur* gene, the *Synechococcus* 7942 *fur* gene was cloned on two, independent 1.6 kb *Pst*I fragments using the *Sau*3AI fragment as a probe. The combined sequence information from all *fur* clones is given in Fig. 1. The sequence downstream of the *fur* gene contains a potential transcriptional stop structure between nucleotides 491 and 575, 2 bases downstream from the last codon. The calculated stability of this palindrome is $\Delta G = -48.7 \text{ kcal mol}^{-1}$ (204 kJ mol^{-1}).

Insertional inactivation of the *fur* locus

To examine the functions of Fur in *Synechococcus* 7942, a mutant was created by insertional inactivation of the *fur* gene. A 1.2 kb DNA fragment containing the *cat* gene was inserted into the *Sac*I site, positioned at amino acid 86 of the *fur* gene, and this construct was used to transform wild-type cells of *Synechococcus* 7942. Because the construct was cloned into pBluescript, a plasmid that cannot replicate in *Synechococcus* 7942, chloramphenicol resistance must result from integration of plasmid DNA into the

Fig. 2. Southern blot hybridization analysis of *Synechococcus* 7942 clones transformed with pBSFURCAT and probed with the *Synechococcus* 7942 *fur* gene. Lane A 1, wild-type *Synechococcus* 7942 chromosomal DNA digested with *Acc*I; lanes A 2–9, chromosomal DNA from eight transformed clones digested with *Acc*I. Lanes P 1–9, same as A 1–9 but the restriction enzyme used was *Pst*I.

cyanobacterial genome. Chloramphenicol-resistant transformants were screened for sensitivity to ampicillin, a phenotype indicative of a double recombination event that replaces the wild-type *fur* genes with the mutant allele. Transformants were grown under high chloramphenicol concentrations ($35 \mu\text{g ml}^{-1}$) for 3 weeks to allow for the complete segregation of the mutated *fur* gene from the wild-type gene.

After this period of growth, two restriction endonucleases were used to digest chromosomal DNA from 40 putative *fur* mutants. *Pst*I and *Acc*I digestion of wild-type DNA results in *fur*-containing fragments of 1.6 kb and 3.0 kb, respectively. The insertionally inactivated *fur* gene contains an additional 1.2 kb of DNA in both the *Pst*I and the *Acc*I fragments. Southern blot analysis of the *fur* locus for all putative mutants showed two bands, the wild-type and a larger interrupted band (Fig. 2). In all cases the wild-type band was more intense than that of the inactivated gene. Since the insertionally inactivated form of the gene must be present in all cells for them to survive at high chloramphenicol concentrations ($35 \mu\text{g ml}^{-1}$), the Southern blot results indicate that both forms of the gene are present in these cyanobacterial cells, a condition we will refer to as heteroallelic. This condition is possible because of the polyploid nature of this and related strains of cyanobacteria (Binder & Chisholm, 1990). Although the wild-type form of the gene was more abundant in all the transformed cells that had been grown on high concentrations of chloramphenicol for 3 weeks, all of the transformed cells exhibited the mutant phenotype, in that their whole-cell absorption spectra exhibited a 5–6 nm blue shift in the main red chlorophyll absorption band when compared to wild-type cells grown in iron-replete medium (data not shown). This spectral shift is a characteristic symptom of iron deficiency (Ferreira & Straus, 1994), resulting from the appearance of the iron-stress-induced chlorophyll-binding protein IsiA (Burnap *et al.*, 1993). Preliminary comparisons of growth rates for the heteroallelic mutant cultures and wild-type cultures in

Fig. 3. Immunoblot analysis for the presence of ferredoxin (Fd) and flavodoxin (Flv) in the soluble protein fraction of *Synechococcus* 7942 strains. Lanes 1 and 2 contain protein from wild-type cells grown under iron-replete and iron-deficient conditions, respectively. Lanes 3 and 4 contain protein from heteroallelic *Synechococcus* 7942 *fur* grown under iron-replete and iron-deficient conditions, respectively. The antibodies were raised against ferredoxin and flavodoxin from *Synechococcus* sp. strain PCC 7002 and were kindly provided by Dr D. A. Bryant.

iron-replete and iron-deficient media indicated that there was surprisingly little difference in growth rates; however, such comparisons should be studied more carefully with a wild-type strain that carries a suitable *cat* construct for growth-rate comparisons under identical conditions.

Effects of the *fur* mutation on the expression of flavodoxin and ferredoxin

To examine the effect of the *fur* mutation on expression of the ferredoxin and flavodoxin genes, proteins were isolated from the soluble fraction of heteroallelic *fur* mutant and wild-type cells that were grown in iron-deficient and iron-replete conditions. These protein preparations were tested for the presence of flavodoxin and ferredoxin by immunoblot analysis (Fig. 3). Flavodoxin was present in the heteroallelic mutant cells grown in either iron-replete or iron-deficient conditions; in wild-type cells, flavodoxin could be detected only in iron-deficient cultures. Since the gene for flavodoxin is the second open reading frame of the *isiAB* operon this observation indicates that the *isiAB* operon has been derepressed in the heteroallelic *fur* mutant strain (Laudenbach & Straus, 1988). This deregulation is in agreement with the iron-stress spectral phenotype of the heteroallelic *fur* mutants grown under iron-replete conditions (see above). The expression of ferredoxin does not appear to be affected by the *fur* mutation.

Effect of the *fur* mutation on siderophore production

Siderophore biosynthesis pathways and the corresponding transport systems are negatively regulated by Fur in many species of purple bacteria (Braun & Hantke, 1991). Since *Synechococcus* 7942 produces a hydroxamate-type siderophore under iron-deficient conditions (Trick & Kerry, 1992), *fur* mutants were tested for the production of hydroxamate-type siderophores under iron-replete conditions. The Csáky test for hydroxamate siderophores

Fig. 4. Analysis of pulse-labelled soluble protein fraction from wild-type *Synechococcus* 7942 cultures grown under iron-replete (lane 1) and iron-deficient conditions (lane 2), and from heteroallelic *Synechococcus* 7942 *fur* grown under iron-replete (lane 3) and iron-deficient (lane 4) conditions.

was performed on wild-type and mutant cultures grown in BG11 and BG11-CH pF16. Although iron-deficient medium was required to induce siderophore production in the wild-type culture, the mutant culture showed another, constitutive, iron-stress symptom by producing high levels of siderophores in both BG11 and iron-deficient medium. In fact, the level of siderophores produced by the mutant culture in BG11 exceeded that of the wild-type culture in iron-deficient medium.

Soluble protein profile of *fur* mutant strain

Since the heteroallelic mutant displays three classic symptoms of iron stress in a constitutive manner, the soluble protein fraction of the mutant was analysed by SDS-PAGE to estimate the number of proteins that are constitutively produced as a consequence of the *fur* mutation (Fig. 4). For this analysis, the heteroallelic mutant and wild type cells were grown for 2.5 h in BG11 and BG11-CH pF16 containing $\text{Na}_2^{35}\text{SO}_4$. The resulting pulse-labelled protein profile revealed that at least seven proteins were derepressed in iron-replete conditions in the mutant strain. The corresponding proteins were observed to be under negative regulation by iron in the wild type. The sizes of these proteins were estimated to be: 142, 134, 132, 127, 123, 60 and 30 kDa. Interestingly, a 110 kDa protein which was induced under iron-deficient

Fig. 5. Measurements of β -galactosidase activity under the control of the *fhuF* promoter. Activity was measured for *E. coli* strains H1680 (Fur⁺; ■) and H1681 (Fur⁻; □), and for *E. coli* H1681 transformed with the *Sau3AI* clone containing the *Synechococcus* 7942 *fur* gene (▨). The error bars indicate SEM.

conditions in the wild type failed to appear in the heteroallelic mutant under iron-replete or iron-deficient conditions, while a 92 kDa protein maintained an iron-regulated expression pattern in both the wild-type and the mutant. These results indicate either that the 92 kDa protein is not under Fur regulation or that the residual copies of functional Fur repressor that are produced by the wild-type chromosomes are sufficient to maintain normal regulation of the gene for this protein.

Lack of interaction between the *Synechococcus* 7942 Fur homologue and the *E. coli* *fhuF* promoter

E. coli strain H1681 was transformed with the vector containing the *Synechococcus* 7942 *fur* homologue to test for interactions between *Synechococcus* 7942 Fur and a Fur-regulated-promoter of *E. coli*. This strain of *E. coli* has a Mu phage DNA insertion in the Fur-regulated gene *fhuF*. The insertion places the promoterless *lacZ* gene of the Mu phage under the transcription of the *fhuF* promoter. The transformed strains were assayed for β -galactosidase activity under iron-replete and iron-deficient conditions (Fig. 5). The results indicate that the *Synechococcus* 7942 Fur does not repress the *fhuF* promoter under iron-replete conditions, although the same *fur*-containing vector showed iron regulation of the *isiA/cat* reporter gene construct.

DISCUSSION

In attempting to isolate a cyanobacterial Fur homologue, all of the methods that have been used previously to clone *fur* from purple bacteria were tried: heterologous Southern hybridization, heterologous complementation of *E. coli* *fur* strains and the creation of *fur* mutants in cyanobacteria for complementation; however, none of these proved successful. Therefore, the approach of this work was to design a screen that utilized cyanobacterial genetic signals in the *E. coli* cloning system. The *in vivo* repression assay in *E. coli* resulted in the isolation of a Fur

<i>Synechococcus</i> PCC 7942	MTYTAAS.LKAEINERGWRLTPQREILRVFQNLPAEHLSEAED
<i>E. coli</i>	MTD.NNTALK..KAGLKVTL.P.RLKILEVLQE.PDNHVSAAED
<i>L. pneumophila</i>	M..EESQQLK..DAGLKITL.P.RIKVLQILBQ.SRNHLSAEE
<i>P. aeruginosa</i>	MVE.NSE.LR..KAGLKVTL.P.RVKILQMLDS.AEQRHMSAED
<i>N. meningitidis</i>	MEKFS.NIAQLK..DSGLKVTG.P.RLKILDLPET.HAEHLSEAED
<i>B. pertussis</i>	MSDQSELKNMGLKATFPRLKILD.IFR.K.SDLR.....HISAED
<i>C. jejuni</i>	MLIENVEYDVLLER.FKKILR..QGGLKYTK.Q.REVLLKTLYH.SDT.HYTPES
CONSENSUS	L R H E
<i>Synechococcus</i> PCC 7942	LYNHLSRNSP..ISLSTIYRILKLMARMGLL.RELDLAEDHGH
<i>E. coli</i>	LYKRLIDMGEE..IGLATVYRVLNQFDDAGIVTRH..NFEGGKS
<i>L. pneumophila</i>	VYKALLESSED..VGLATVYRVLTQFEAGLVSRH..NFEGGHS
<i>P. aeruginosa</i>	VYKALMEAGED..VGLATVYRVLTQFEAGLVVRH..NFDGSHA
<i>N. meningitidis</i>	VYRILLLEGVE..IGVATVYRVLTQFEAGILQRH..HFETGKA
<i>B. pertussis</i>	VYRALIAENVE..IGLATVYRVLTQFEAGILTRS..QFDTGKA
<i>C. jejuni</i>	LYMEIKQAEPLNNGIATVIVILNLLLEAEAMVTSI..SFGSAGK
CONSENSUS	Y
<i>Synechococcus</i> PCC 7942	Y.ELNQPLK.HHHHLICVSCSKTIEFKSD.SLVKIGAKTSEKE
<i>E. coli</i>	VFELTQQ..HHHDHLICLDCKGVIEF.SDDSIARQREIAAKH
<i>L. pneumophila</i>	VFELSQQ..EHHDHLVCVKCGRVVEEF.VDEIIE..QRQ.KAIA
<i>P. aeruginosa</i>	VFELADS..GHHDHMCVDTGEVIEF.MDAEIE..KRQ.KEIV
<i>N. meningitidis</i>	VVELDKG..DHDHIVCVKCGEVTEF.HNPEIE..ALQDK.IA
<i>B. pertussis</i>	VFELNDG..DHDHDLICTNCGTVFEF.SDPDIE..KRQYK.VA
<i>C. jejuni</i>	KYELANKP..HHDHMICKNCGKIEFE.NPIIE..RQQAIIA
CONSENSUS	EL HH H C EF
<i>Synechococcus</i> PCC 7942	GY...HLHL...L.DQQLTIH.GVC..PTC..QRSIV
<i>E. coli</i>	GIRL/TNHS.....LYL.YGHCAEGDCREDEHAHBGK
<i>L. pneumophila</i>	.ER.A.H.FKMT...DHALNT.YGTC..PQC..Q
<i>P. aeruginosa</i>	RER.G...FELV...DHNVL.Y.V.....R.KKK
<i>N. meningitidis</i>	E.....ENGYRI.VDHALYM.YGVC..SDC..QAKGKR
<i>B. pertussis</i>	.KD..NG.FVLESHAMV..LYVLYGIC..GNC..Q.KG.R
<i>C. jejuni</i>	KE...HGFKLTGHLMMQ.L...YGVC..GDCNNQ.KAKVKI
CONSENSUS	L

Fig. 6. Amino acid sequence comparisons of Fur sequences from purple bacteria and from *Synechococcus* 7942. Dots represent spaces introduced for optimal matching. The consensus line indicates matching in all sequences.

homologue from *Synechococcus* 7942. Amino acid sequence comparisons between DtxR and the Fur homologue showed no convincing homology. On the other hand, the amino acid sequence of the cyanobacterial Fur showed significant similarity to all known Fur sequences (Fig. 6) and contained the putative iron-binding domain of Fur repressors, HHHXHXCCXXC, and not of the DtxR repressors, HXXXCXXXH (Wang *et al.*, 1994).

Transformation of *Synechococcus* 7942 with an insertionally inactivated form of *fur* resulted in heteroallelic mutants that showed iron-stress symptoms in an iron-replete medium; these symptoms include constitutive flavodoxin and siderophore production. The persistence of the wild-type *fur* gene under conditions that strongly select for the mutant gene suggests that elimination of the functional *fur* gene may be lethal. The failure to inactivate *fur* by allelic exchange in *Pseudomonas aeruginosa* (Prince *et al.*, 1993), *Neisseria gonorrhoeae* (Berish *et al.*, 1993), *Neisseria meningitidis* (Karkoff-Schweizer *et al.*, 1994) and *Vibrio anguillarum* (Tolmasky *et al.*, 1994) indicates that the *fur*

mutation is also lethal in these purple bacteria. However, the polyploid nature of *Synechococcus* 7942 permits a situation where the mutant form of the *fur* gene can exist in the same cell as the wild-type form of the gene. Presumably the mutant phenotype appears because the replacement of wild-type forms of the gene with defective forms results in fewer functional repressor proteins per cell. Since, like many other repressors, Fur functions as a dimer (Coy & Neilands, 1991), the presence of incomplete Fur molecules may disrupt homodimer formation and convey phenotypic consequences that are more striking than one would expect from the relative abundance of the mutant form of the gene. In any case, the resulting competition for functional wild-type homodimers of Fur by the operators of Fur-regulated genes should lead to a derepression of those genes with the least stable Fur/operator complex. Such genes would be expected to be the first to be expressed during the onset of iron deficiency. Indeed, the symptoms of the heteroallelic mutants fall into the category of early symptoms of iron stress rather than iron starvation (Brown & Trick, 1992; Sandmann & Malkin, 1983).

Although the cyanobacterial Fur repressor and its putative binding sequence are similar to their purple bacteria counterparts, the Fur repressor of cyanobacteria is incapable of complementing a *fur* mutation in *E. coli* and showed no *in vivo* interaction with an *E. coli* Fur binding operator. This is probably why we were unable to isolate the cyanobacterial *fur* gene by genetic complementation of the *E. coli fur* mutant. The lack of heterologous repressor/operator interaction implies that the recognition sequences, as well as the Fur protein sequences involved in DNA interactions, have diverged substantially between the two species. In fact, the putative Fur-binding sequences of the *isiA* gene of *Synechococcus* 7942 have single base insertions, when compared to the Fur-binding consensus sequence of purple bacteria (Straus, 1994).

Since cyanobacteria and purple bacteria are believed to have diverged more than 2 billion years ago, before the development of an oxidizing atmosphere, the presence of related Fur repressor proteins in both of these groups indicates that this protein appeared early in microbial evolution. This would imply that iron-deficient conditions and the subsequent need to scavenge iron must have existed in the seas of Archaean and Proterozoic times, when one would have expected iron to be abundantly available in the form of the more soluble ferrous ion. However, the presence of banded iron formations (BIFs) in the sedimentary deposits of Archaean and early Proterozoic seas, 3.5–1.8 billion years ago, indicates the periodic recurrence of iron-limiting conditions even in these early times (Kump, 1993).

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