The ferric uptake regulator of *Pseudomonas aeruginosa* has no essential cysteine residues and does not contain a structural zinc ion

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The ferric uptake regulator (Fur) of *Pseudomonas aeruginosa* was expressed in *Escherichia coli* in its native form and as a fusion to the maltose-binding protein (MBP). Fur from the MBP fusion bound to MBP after proteolytic cleavage, and the two could only be separated by partial unfolding. The refolded protein was in the same conformation as native protein (as judged by circular dichroism and fluorescence spectroscopies) and was fully active in DNA-binding assays. As-prepared native Fur contained small amounts of Zn²⁺ that were easily removed by treatment with EDTA, and apo-protein could be reconstituted with approximately one Zn²⁺ ion per monomer. Thus, the *P. aeruginosa* Fur can probably accommodate a single Zn²⁺ ion bound to the metal-sensing site. The single cysteine residue of *P. aeruginosa* Fur aligns with a cysteine in other members of the Fur family that is essential for activity of the *E. coli* protein, and is believed to provide one of the ligands to a structural Zn²⁺ ion. This cysteine residue was shown to be dispensable for the *in vivo* activity of *P. aeruginosa* Fur, which is consistent with the suggestion that the *P. aeruginosa* protein does not contain a structural Zn²⁺ ion. Members of the Fur family contain a highly conserved His-His-Asp-His motif. Alanine substitutions of residues in this motif showed His-87 and His-89 of *P. aeruginosa* Fur to be essential for activity, whilst His-86 and Asp-88 are partially dispensable.

Keywords: Fur, iron regulation

INTRODUCTION

The ferric uptake regulator (Fur) is a widespread bacterial protein that regulates the expression of iron acquisition and storage systems in response to intracellular iron (Escobar *et al.*, 1999). Fur is a key regulator of iron metabolism, but in different organisms it clearly has roles in numerous other aspects of physiology (Escobar *et al.*, 1999). In the enteric bacteria, such as *Escherichia coli* and *Salmonella enterica*, members of the Fur regulon include siderophore synthesis and uptake genes, as well as genes involved in carbon metabolism and responses to oxidative stress (Escobar *et al.*

et al., 1999). There is also genetic evidence to suggest that Fur has a role in acid tolerance (Hall & Foster, 1996). Besides acting as a classical iron-dependent repressor of protein-coding genes, it has recently been found that the *E. coli* Fur also represses expression of a small regulatory RNA designated RhyA. RhyA itself down-regulates the expression of genes encoding proteins involved in iron storage and utilization (Massé & Gottesman, 2002). The discovery of RhyA provides a rationale for the apparent ability of Fur to work as an activator, and for the early observation that *fur* mutants of *E. coli* are unable to grow on succinate (Massé & Gottesman, 2002; Hantke, 1987).

Iron acquisition is a particular problem for pathogenic bacteria, since the bioavailability of iron in the host is often especially low. Hence, there is often a need for iron acquisition systems to be switched on when the pathogen encounters a host environment (Litwin & Calderwood,

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Abbreviations: CD, circular dichroism; Gdn.HCl, guanidinium hydrochloride; MBP, maltose-binding protein.

1993; Ratledge & Dover, 2000). Furthermore, there is good evidence to indicate that iron limitation is used, in some cases, as a signal to activate the expression of virulence genes, and that this regulation can ultimately depend on Fur. One example is the opportunistic pathogen *Pseudomonas aeruginosa*, in which Fur regulates the expression of the alternative sigma factor encoded by the *pvdS* gene, which is in turn required for the transcription of some virulence genes, including those encoding the endoprotease PrpL and exotoxin A (Leoni *et al.*, 1996; Wilderman *et al.*, 2001; Ochsner *et al.*, 1996; reviewed by Vasil & Ochsner, 1999).

Genetic characterization of *fur* mutants, and biochemical analysis of the Fur protein, suggested that the active species is a dimer with one Fe^{2+} ion bound to each monomer. Results from proteolysis and gene-fusion experiments indicated that the protein folds into an N-terminal DNA-binding domain and a C-terminal metal-binding and dimerization domain (Coy & Neilands, 1991; Stojiljkovic & Hantke, 1995). Early models for the mode of action of Fur had the protein functioning as a classical repressor, with the Fe^{2+} ion acting as co-repressor. This simple view has been challenged by two recent developments. The first of these is the identification of a structural Zn^{2+} ion in the *E. coli* protein that is apparently required for DNA binding, and is bound to a site distinct from the metal-sensing site (Jacquamet *et al.*, 1998; Gonzalez de Peredo *et al.*, 1999; Althaus *et al.*, 1999). Secondly, it has been suggested that the binding of Fur to DNA is directly inhibited by EDTA, which may require the conclusions of some earlier experiments to be re-evaluated (Althaus *et al.*, 1999). *E. coli* Fur containing the structural Zn^{2+} ion and with a vacant metal-sensing site has been shown to bind to DNA with high affinity, which may even call into question the role of Fe^{2+} as co-repressor (Althaus *et al.*, 1999). In this context, it is interesting that the *Bacillus subtilis* Fur apparently does not require Fe^{2+} for DNA binding activity *in vitro* (Bsai & Helmann, 1999). Ligands to the structural Zn^{2+} ion are believed to include Cys-92 and Cys-95, which have been shown to be essential for normal Fur activity in *E. coli* (Coy *et al.*, 1994; Gonzalez de Peredo *et al.*, 1999). Indeed, amongst a collection of Fur proteins substituted at all cysteine and histidine residues, those in which Cys-92 or Cys-95 were replaced by serine had much the most severe phenotypes (Coy *et al.*, 1994). These two cysteines are conserved in, for example, the Fur proteins of *Vibrio anguillarum* and *Bacillus subtilis*, which are also thought to contain a structural Zn^{2+} ion (Bsai & Helmann, 1999; Zheleznova *et al.*, 2000). On the other hand, only Cys-92 is conserved in some presumed Fur orthologues, including that from *P. aeruginosa*, and in other cases (such as *Pseudomonas putida*), neither cysteine is conserved. This raises the possibility of structural and/or mechanistic diversity in the Fur family, perhaps with some members not requiring a structural Zn^{2+} ion for activity (Zheleznova *et al.*, 2000). As a first step in exploring whether sequence diversity is reflected in the biochemical properties of Fur proteins, the Fur from *P. aeruginosa* has been further

characterized. It is shown that this protein does not contain a structural Zn^{2+} ion, and that its single cysteine residue is not essential for activity *in vivo*. Two histidine residues essential for *P. aeruginosa* Fur activity are also identified.

METHODS

Bacterial strains, plasmids and growth conditions. The strains of *E. coli* used were JRG2653 $\Delta ara \Delta (argF-lac) rpsL relA deoC ptsF \Delta fur \lambda bfd-lacZ$ and its isogenic *fur*⁺ parent JRG2652 (both gifts from Simon Andrews, University of Reading, UK), BL21 *ompT gal dcm hsdS_B* (λ DE3) for expression of pET21a clones, and JM83 *lacZ lacI* $\phi 80 \Delta lacZ$ M15 for routine DNA manipulations and expression of the maltose-binding protein (MBP)-Fur fusion protein. The strain of *P. aeruginosa* used was PAO1 (from Richard James, University of Nottingham, UK). The plasmids used were pUC18 for routine cloning procedures, pET21a (Novagen) for overexpression, and pMALc2 (New England Biolabs) for construction of a *malE-fur* fusion. Routine growth of bacteria was in Lennox broth (tryptone 10 g l⁻¹, yeast extract 5 g l⁻¹, NaCl 5 g l⁻¹). For assay of β -galactosidase, *E. coli* strains were grown in M9 minimal medium (Miller, 1992), supplemented as indicated with 50 μM Fe(III)citrate or with 20 μM bipyridyl.

DNA manipulations. The *P. aeruginosa fur* gene was amplified from chromosomal DNA purified from strain PAO1, using a primer (5'-CATATGGTTGAAAATAGCGAACTT-3') that incorporated an *NdeI* site at the start codon (underlined) of the coding region. The PCR product was cloned into pUC18, and sequenced to check that mutations had not been introduced. The *fur* gene was then excised from this clone using the vector *PvuII* sites and was blunt-end cloned into the *ScaI* site of pBR322 to generate pPAD24. The *fur* gene in pPAD24 is expressed from the *lac* promoter derived from pUC18, and the lower-copy-number vector was found to be required for stable maintenance of the clone. Mutations were introduced into a clone of the *fur* gene in pUC18 by a PCR-based method (Hutchings *et al.*, 2000); mutant genes were sequenced twice to confirm that the correct mutation had been introduced, and were then cloned on *PvuII* fragments into the *ScaI* site of pBR322. The *NdeI-EcoRI* fragment from the pUC18 *fur* clone was cloned into pET21a to generate a clone suitable for overexpression in BL21(λ DE3). For construction of an MBP-Fur fusion, *fur* was amplified by PCR using a 5' primer (5'-GGGAATTCATGGTTGAAAATAGCGAACTT-3') incorporating an *EcoRI* site immediately upstream of the start codon (underlined). PCR products were blunt-end cloned into *SmaI*-digested pUC18, and, from a clone in the correct orientation, *fur* was excised on an *EcoRI-BamHI* fragment (using the vector *BamHI* site) and cloned into pMAL-c2 (New England Biolabs). The intermediate pUC18 clone was sequenced to ensure that no mutations had been introduced. This procedure fused *fur* in-frame to *malE*; the gene fusion is predicted to encode a protein that adds the sequence Ile-Ser-Glu-Phe on to the N-terminus of Fur after cleavage with factor Xa.

Protein purification. Fur was purified from the pET21 clone in BL21(λ DE3) initially using the method of Ochsner *et al.* (1995). Subsequently a modified procedure was developed, which did not involve metal-affinity chromatography. Cultures (500 ml) were grown to an OD₆₅₀ of 0.5–0.6, induced with 1 mM IPTG, then incubated overnight. Cells were harvested, washed in 50 mM Tris/HCl (pH 7.9)–0.5 mM EDTA–50 mM NaCl, then resuspended in the same buffer containing 1 mM PMSF and 1 mM DTT. After sonication, the

cell-free extract was applied to a 300 ml DEAE cellulose ion-exchange column equilibrated with 50 mM Tris/HCl (pH 7.9)-50 mM NaCl, and then eluted with a linear 50–500 mM NaCl gradient in the same buffer. Fur-containing fractions (as judged by SDS-PAGE) were dialysed overnight in 20 mM Tris/HCl (pH 7.0) then applied to a 20 ml heparin-agarose column equilibrated with 20 mM Tris/HCl (pH 7.0). The column was washed with the same buffer, then Fur was eluted with 20 mM Tris/HCl (pH 7.0)-1 M NaCl.

For purification of the MBP fusion, 500 ml cultures were grown in Lennox broth containing 0.2% (w/v) glucose to an OD₆₅₀ of 0.5–0.6, induced with 1 mM IPTG, then incubated overnight. Cells were harvested, washed in 50 mM Tris/HCl (pH 7.9)-50 mM NaCl, then resuspended in the same buffer containing 0.5 mM EDTA, 1 mM PMSF and 1 mM DTT. Cells were disrupted by sonication, clarified, and the cell extract applied to a 15 ml amylose column equilibrated with 50 mM Tris/HCl (pH 7.9)-100 mM NaCl. The column was washed with the same buffer, then the fusion protein eluted with 50 mM Tris/HCl (pH 7.9)-100 mM NaCl-10 mM maltose. The fusion was treated with factor Xa, and then 3 M guanidinium hydrochloride (Gdn.HCl). The partially denatured Fur was purified by FPLC on an S75 gel-filtration column, then renatured by dialysis against 20 mM Tris/HCl (pH 7.0)-50 mM NaCl.

DNA-binding assays. For use in gel retardation assays, a 300 bp fragment of the *P. aeruginosa pvdS* promoter region was amplified by PCR and cloned into the *Sma*I site of pUC18. The fragment extended 315 bp upstream of the *pvdS* start codon, not including the 14 bp immediately 5' to the ATG. Thus, the fragment included the *pvdS* –35 and –10 sequences, and a predicted Fur-binding site overlapping the –35 sequence (Ochsner *et al.*, 1995). This fragment was excised with *Eco*RI and *Hind*III (using the vector sites, generating a fragment of total size 350 bp) and was labelled by end-filling with DNA polymerase I in the presence of [³²S]dATP α S. The procedure for gel retardation assays was modified from that of Althaus *et al.* (1999). Binding buffer (5 $\times$ concentrate) contained 100 mM Tris/HCl (pH 7.0), 25% (v/v) glycerol, 10 μ g herring sperm DNA μ l⁻¹, 5 mM MgCl₂, 200 mM KCl and 500 μ g bovine serum albumin ml⁻¹, and was supplemented with MnCl₂ as required. Binding reactions contained 2 μ l 5 $\times$ binding buffer, 1 μ l radiolabelled DNA, Fur to the desired final concentration, and distilled water to a final reaction volume of 10 μ l. The binding reaction was left at room temperature for 20–30 min before loading on to a 6% polyacrylamide gel. The gel was made up in 20 mM Tris, buffered to pH 7.0 with boric acid, and was run at 120 V for 80 min. The gel was transferred to 3MM Whatman paper, dried, and exposed to a Molecular Dynamics phosphorimager screen overnight. The screen was digitized and quantified using a Storm 840 laser scanner (Molecular Dynamics) and images were visualized and quantified using ImageQuant version 5.0 (Molecular Dynamics).

Spectroscopy. Fluorescence emission spectra were recorded on a Perkin Elmer LS50 fluorimeter using an excitation wavelength of 290 nm and excitation and emission slits of 5 nm. Scan speed was 1000 nm min⁻¹ and a mean of 10 scans was recorded. Both proteins were 13 μ M in 20 mM Tris/HCl (pH 8)-50 mM NaCl, and emission spectra of the buffer were subtracted. Circular dichroism (CD) spectra were collected on a Jasco J-710 spectropolarimeter with a scan speed of 100 nm min⁻¹ and a path length of 0.2 mm. Each sample was scanned three times, and a mean spectrum calculated. Fur samples were at 0.3–0.9 mg ml⁻¹ in 20 mM Tris/HCl (pH 8.0)-50 mM NaCl. The observed ellipticity in millidegrees (θ) was con-

verted to mean residue molar ellipticity (θ)_{MR} in deg cm² dmol⁻¹ residue⁻¹ according to equation 1:

$$(\theta)_{\text{MR}} = (\theta \times 10^{-4} M_r) / (c.l.n) \quad (1)$$

where M_r is the relative molecular mass of the protein, c is the concentration in g ml⁻¹, l is the path length in cm, and n is the number of residues.

Protein unfolding. Protein samples (0.14 mg ml⁻¹) were dialysed for 4 h at room temperature against varying Gdn.HCl concentrations, then incubated at 37 °C for a further 1 h before recording CD spectra as above. Baselines collected at the relevant Gdn.HCl concentrations were subtracted. The ellipticity at 222 nm (θ) was used to calculate the fraction of unfolded protein (f_u) according to equation 2:

$$f_u = (\theta - \theta_n) / (\theta_u - \theta_n) \quad (2)$$

where θ_u is the ellipticity at 222 nm of the fully denatured protein and θ_n is the ellipticity at 222 nm of the native protein. Data points were corrected for the slopes of the pre- and post-transitional regions of the curves using linear regression (Santoro & Bolen, 1988; Pace & Scholtz, 1997). The fraction of unfolded protein (f_u) was plotted against [Gdn] and the resultant curves fitted to equation (3) using Fig.P v2.98 (BIOSOFT):

$$f_u = \frac{\exp\{m([Gdn] - [Gdn]_{0.5})\} / RT}{1 + \exp\{m([Gdn] - [Gdn]_{0.5})\} / RT} \quad (3)$$

where [Gdn]_{0.5} is the concentration of Gdn.HCl at which the protein is 50% unfolded, and m is the rate of change of free energy with respect to [Gdn].

Analytical techniques. Electrospray mass spectrometry was done on a Micromass Platform I mass spectrometer calibrated with horse heart myoglobin. The solvent was 50% (v/v) acetonitrile and 0.1% (v/v) formic acid in water, and samples were run at a rate of 20 μ l min⁻¹. For electrothermal atomic absorption spectroscopy, all glassware was washed in 10% (v/v) nitric acid, and rinsed thoroughly in triple-distilled water followed by Milli-Q water. Fur was dialysed overnight against 50 mM Tris/HCl (pH 8.0)-50 mM EDTA-200 mM NaCl, then dialysed extensively against 50 mM Tris/HCl (pH 8.0)-200 mM NaCl. To assay zinc binding, 1 ml samples containing 10 μ M Fur in 50 mM Tris/HCl (pH 8.0)-200 mM NaCl were made to 150 μ M ZnCl₂, and left overnight at 4 °C, before the dialysis against 200 mM NaCl was repeated. All samples and ZnCl₂ standards were passed through a 0.2 μ m filter before analysis on a Philips PU9200 atomic absorption spectrophotometer with an electrographite cuvette. Absorption was at 213.9 nm with a 0.5 nm bandpass, and each sample was measured five times. Protein concentrations were determined with a bicinchoninic acid assay kit (Sigma), or using an absorption coefficient (280 nm) of 4218 M⁻¹ cm⁻¹ estimated for the protein cleaved from the MBP fusion according to the method of Gill & von Hippel (1989). β -Galactosidase was assayed according to the method of Miller (1992). DNA sequences were determined by MWG Biotech (Germany).

RESULTS AND DISCUSSION

Expression, purification and biophysical characterization of *P. aeruginosa* Fur

The *P. aeruginosa fur* gene was amplified and cloned into pET21a, with a view to achieving a high level of expression for subsequent purification. Expression of

Fig. 1. Unfolding of MBP and Fur purified from the MBP-Fur fusion. ■, Unfolding profile of MBP (which lacks 26 residues at the N-terminus and has a 21-residue C-terminal extension compared to wild-type MBP). □, Unfolding profile of Fur (which has an N-terminal tetrapeptide extension Ile-Ser-Glu-Phe). The fraction of protein unfolded (f_u , where 1 represents complete unfolding) was calculated according to equation 2 (see Methods), and the curves were fitted to the data points using equation 3.

Fur was indeed at a high level, and protein could be purified by following the method of Ochsner *et al.* (1995), involving ion-exchange and copper-affinity chromatographies. This purification protocol proved to be poorly reproducible, so an alternative method was devised (see Methods) involving anion-exchange, followed by affinity chromatography on heparin-agarose, yielding 16 mg pure protein from a 3 l culture. Electrospray mass spectrometry of the purified material gave an estimated mass of 15 232 Da, in good agreement with the mass predicted from sequence (15 235 Da). One long-term goal of Fur expression was to provide isotopically labelled material for NMR analysis of the protein structure. For this purpose, the yield was rather low, so protein fusion methods were investigated as an alternative approach to Fur expression and purification.

In the first instance, the maltose-binding protein (MBP) was chosen for fusion to Fur, since MBP has previously been used successfully for making fusions to transcriptional regulators (Chai & Stewart, 1998; Li *et al.*, 1994). The *fur* gene was fused to the *malE* gene encoding MBP in the vector pMALc2. This procedure fused Fur to the C-terminus of a derivative of MBP from which the signal peptide has been deleted. The fusion protein was purified by affinity chromatography on an amylose resin and was cleaved with factor Xa. Following cleavage with factor Xa (confirmed by SDS-PAGE), Fur and MBP co-eluted in a number of chromatographic separations (amylose affinity, heparin affinity and ion exchange), indicating the formation of a complex between the cleaved Fur and MBP. Some free MBP (but no Fur) was always seen in these preparations, suggesting a stoichiometric excess of Fur in the complex, which is consistent with an oligomeric form of Fur binding to MBP. A similar post-cleavage interaction has been observed with a fusion of the cystic fibrosis transmembrane regulator to MBP (Ko *et al.*, 1993).

Fig. 2. CD spectroscopy of recombinant Fur protein purified from an MBP fusion and from the native pET21a expression clone. The native Fur (dashed line) was at 0.5 mg ml⁻¹, and the protein cleaved from the MBP fusion (solid line) at 0.28 mg ml⁻¹, in 20 mM Tris/HCl (pH 8.0)-50 mM NaCl.

Separation of Fur from MBP was achieved by exploiting their different stabilities in Gdn.HCl. MBP was 50% unfolded with 1.1 M Gdn.HCl, while Fur required 3.2 M Gdn.HCl for 50% unfolding (Fig. 1). The mixture containing Fur and MBP was treated with 3 M Gdn.HCl, and the partially unfolded Fur was separated from the unfolded MBP by gel filtration. Subsequent removal of the Gdn.HCl by dialysis yielded Fur that was active, as judged by its ability to bind to DNA (see below). The yield of protein from this procedure was 30 mg from 1.5 l of culture. Electrospray mass spectrometry gave an estimated mass of 15 709 Da, close to the predicted mass of 15 711 Da for the protein that has the sequence Ile-Ser-Glu-Phe added to its N-terminus as a result of the cloning procedure. Some of the biochemical experiments reported below were done with material prepared in this way, and protein preparations from the two different sources were indistinguishable by all criteria tested.

The CD spectrum (Fig. 2) of Fur purified from the clone in pET21a is indicative of a high degree of α -helical content. Deconvolution of the spectrum with the program CDNN (http://bioinformatik.biochemtech.uni-halle.de/cd_spec/download.html) suggests a secondary structure content of 49% α -helix, 9.1% β -sheet, 15.5% β -turn and 17.8% random coil. The CD spectrum of the protein purified from the MBP fusion is similar (Fig. 2), and gives similar estimates of secondary structure content. Both proteins emitted a similar fluorescence maximum centred at ~350 nm on excitation at 290 nm (Fig. 3). Since Fur lacks tryptophan, this fluorescence must arise from a tyrosinate ion, as there is no other amino acid fluorophore reported to have this behaviour (Lakowicz, 1983). Although the pK_a of the hydroxyl group of tyrosine is ~10.3 in its electronic ground state, it can decrease to <4 upon excitation and give fluorescence of the type seen here for Fur. Studies of this phenomenon in other proteins have shown that tyrosinate fluorescence reflects the three-dimensional struc-

Fig. 3. Fluorescence emission spectra of Fur. In both cases protein concentration was 13 μ M in 20 mM Tris/HCl (pH 8)-50 mM NaCl, and excitation was at 290 nm. Fur was purified from the native pET21 expression clone (thin line) and from the MBP fusion (thicker line).

Fig. 4. Binding of Fur to the *pvdS* promoter. Fur purified from the MBP fusion was mixed with 35 S-labelled DNA in the presence of 100 μ M MnCl_2 . Protein concentrations were: lane 1, 0; lane 2, 50 nM; lane 3, 100 nM; lane 4, 150 nM; lane 5, 200 nM; lane 6, 250 nM. Binding reactions were resolved on a 6% native polyacrylamide gel and were visualized by phosphorimaging. The mobilities of the free and protein-bound DNA species are indicated.

ture of the protein, with the tyrosine hydroxyl involved in a hydrogen bond that also shifts the tyrosine UV absorption to the red (Dietze *et al.*, 1996; Groen *et al.*, 1996; Soengas *et al.*, 1997). The fluorescence emission of Fur was completely quenched by 6 M Gdn.HCl, which is consistent with its originating from a structural feature. In summary, these spectroscopic data confirm that (a) refolding of the Fur protein after Gdn.HCl treatment returns it to its native state, and (b) the addition of four residues to the N-terminus of Fur does not have a gross effect on the secondary structure of Fur.

DNA binding

Gel retardation analysis with Fur purified from the MBP fusion confirmed that this protein preparation is active for binding to DNA containing a Fur box (from the *P. aeruginosa pvdS* promoter; Ochsner *et al.*, 1995) in the presence of Mn^{2+} ions (Fig. 4). At similar protein concentrations, Fur failed to bind to a similarly sized

fragment not containing a Fur box (data not shown), confirming that binding was specific. As has been observed previously for Fur proteins from different sources, binding was inhibited in the presence of relatively low concentrations of EDTA. This has usually been explained by suggesting that the chelating agent removes from the protein the metal ion that is the co-repressor for Fur (in this case Mn^{2+}). However, it has recently been suggested that, for the *E. coli* Fur, EDTA may act by directly inhibiting the ability of the protein to bind to DNA (Althaus *et al.*, 1999). The two possibilities cannot easily be resolved in this case, but it is interesting to note that, at a similar Fur concentration, binding to the *pvdS* Fur box was not inhibited by 1 mM bipyridyl (data not shown).

P. aeruginosa Fur does not contain a structural zinc

The *E. coli* Fur protein is a zinc metalloprotein that contains a structural Zn^{2+} ion bound to a site that is distinct from the metal sensing site where Fe^{2+} binds (Jacquemet *et al.*, 1998; Gonzalez de Peredo *et al.*, 1999; Althaus *et al.*, 1999). The metal-sensing site can also accommodate Zn^{2+} , such that as-prepared Fur from *E. coli* can bind a total of two Zn^{2+} ions per monomer. Of these, one is easily removed by treatment with EDTA, whereas removal of the structural Zn^{2+} ion requires unfolding of the protein (Althaus *et al.*, 1999). The refolded apoprotein can be reconstituted with either one or two Zn^{2+} ions. Ligands to the zinc are believed to include the essential residues Cys-92 and Cys-95, and, probably, two histidine residues (Jacquemet *et al.*, 1998). It has been suggested (Zheleznova *et al.*, 2000) that some Fur family members, such as the protein from *P. aeruginosa*, might not contain the structural Zn^{2+} ion. Cys-95 is not conserved in the *P. aeruginosa* Fur, yet this protein is able to bind Zn^{2+} as shown by its ability to bind to an immobilized Zn^{2+} -affinity column. It was, therefore, of some interest to determine whether this protein also contains a structural Zn^{2+} ion.

Analysis of as-prepared *P. aeruginosa* Fur by electrothermal atomic absorption spectroscopy (EAAS) revealed the presence of a significant amount of zinc that varied in different preparations. Unlike the *E. coli* Fur, treatment with EDTA removed the bulk of this zinc, to yield protein containing 0.19 Zn^{2+} ions per monomer. Treatment of the as-prepared Fur with a large excess of ZnCl_2 followed by dialysis to remove unbound Zn^{2+} yielded protein containing 0.94 Zn^{2+} ions per monomer as judged by EAAS, again unlike the *E. coli* protein, which binds two Zn^{2+} ions under similar conditions. The simplest interpretation of these results is that the *P. aeruginosa* Fur binds a single Zn^{2+} ion in the metal-sensing site, and does not have the structural Zn^{2+} -binding site found in the *E. coli* protein. This distinction is consistent with the absence of Cys-95 in the *P. aeruginosa* protein, and is indicative of a degree of structural diversity in the Fur family. Structural diversity does not seem to be reflected in mechanistic diversity, at least to the extent that the *P. aeruginosa fur* gene can complement an *E. coli fur* mutant (Prince *et al.*, 1993; and

Table 1. β -Galactosidase activities directed by a chromosomal *bfd-lacZ* fusion in Δfur and *fur*⁺ strains transformed with plasmids expressing the *P. aeruginosa* Fur and its engineered derivatives

The data are means $\pm$ standard errors calculated from duplicate assays performed on three independently grown cultures.

Strain	Plasmid	β -Galactosidase activity (Miller units) in cultures grown in M9 medium supplemented with:		
		No suppl.	Iron	Bipyridyl
JRG2653 (Δfur)	pBR322	135 $\pm$ 9	126 $\pm$ 10	123 $\pm$ 5
JRG2652 (<i>fur</i> ⁺)	pBR322	34 $\pm$ 7	5 $\pm$ 1	56 $\pm$ 6
JRG2653	pPAD24 (wt Fur)	63 $\pm$ 7	21 $\pm$ 9	85 $\pm$ 9
JRG2653	pPAD24-C92S	29 $\pm$ 6	21 $\pm$ 6	94 $\pm$ 2
JRG2653	pPAD24-C92A	18 $\pm$ 5	10 $\pm$ 1	62 $\pm$ 23
JRG2653	pPAD24-H86A	79 $\pm$ 6	46 $\pm$ 4	80 $\pm$ 14
JRG2653	pPAD24-H87A	177 $\pm$ 35	155 $\pm$ 25	70 $\pm$ 12
JRG2653	pPAD24-D88A	134 $\pm$ 11	82 $\pm$ 8	69 $\pm$ 13
JRG2653	pPAD24-H89A	187 $\pm$ 77	211 $\pm$ 64	83 $\pm$ 13

see below). Addition of a ZnCl₂ solution to metal-free Fur did not produce significant changes to the CD or fluorescence spectra of the protein (data not shown), consistent with Zn²⁺ binding not causing large-scale conformational changes affecting the chromophores giving rise to the spectroscopic effects.

P. aeruginosa Fur forms disulfide-linked dimers

SDS-PAGE analysis of *P. aeruginosa* Fur showed that in the presence of DTT it behaves as a monomer, while in the absence of DTT a mixture of monomeric and dimeric species was seen (data not shown), with the relative amounts being batch-dependent. These data suggested that Cys-92 (the only cysteine in the protein) forms intermolecular disulfide bonds on air-oxidation, with freshly prepared protein having less of the dimer form than older preparations. This was confirmed by showing that Fur alkylated with iodoacetamide was unable to dimerize in the absence of DTT (data not shown). In a mass spectroscopic analysis of the *E. coli* Fur, no evidence for the occurrence of disulfide-bridged dimers was found (Michaud-Soret *et al.*, 1997).

Mutational analysis of *P. aeruginosa* Fur

The *fur* gene of *P. aeruginosa* is believed to be essential (Hassett *et al.*, 1996), so it is difficult to evaluate the phenotypes associated with mutant alleles in a null background. The *P. aeruginosa fur* gene was originally cloned by complementation of an *E. coli* mutant (Prince *et al.*, 1993), and *P. aeruginosa* Fur can bind to an *E. coli* Fur box *in vitro* (Ochsner *et al.*, 1995). Hence, it was reasoned that it should be possible to examine activity of the *P. aeruginosa* Fur in the heterologous *E. coli* background. Therefore, a reporter system was developed in which the *P. aeruginosa fur* gene was expressed in a strain of *E. coli* (JRG2653) mutant for the

endogenous *fur* gene and containing a chromosomal fusion of the Fur-regulated *bfd* promoter to *lacZ*. The *bfd* gene is linked to the *bfr* gene of *E. coli*, encodes a [2Fe-2S] protein, and is repressed by Fur in response to iron (Andrews *et al.*, 1989; Garg *et al.*, 1996; Stojiljkovic *et al.*, 1994). Measurement of β -galactosidase activity confirmed that the *P. aeruginosa fur* gene complements the *E. coli fur* mutant, and that the complemented strain shows iron-regulated expression of the *bfd* promoter (Table 1). Repression is less tight than that seen with the chromosomal *E. coli fur* gene (in JRG2652), which may reflect either poor expression of the *P. aeruginosa fur* gene or a reduced activity of the heterologous regulator. The *E. coli fur* mutant has a pleiotropic phenotype that includes the overproduction of siderophores and an inability to grow on succinate as the sole source of carbon and energy (Hantke, 1987). Both of these phenotypes were also complemented by the *P. aeruginosa fur* gene. The reporter system was then used to assay the consequences of substituting the single cysteine residue of *P. aeruginosa* Fur with either serine or alanine. Both mutants were able to act as repressors of the *bfd* promoter (Table 1), indicating that Cys-92 is dispensable for the activity of *P. aeruginosa* Fur. This conclusion was supported by the observation that both mutants restored growth on succinate and siderophore production to wild-type patterns (data not shown). Interestingly, the two cysteine mutants both seem to work better as repressors in the presence of the trace amounts of iron present in unsupplemented M9 medium than does the wild-type *P. aeruginosa* Fur (Table 1).

In sequence alignments of Fur proteins, there is a highly conserved HHDH motif in the central region of the protein (residues 86 to 89 in the *P. aeruginosa* protein). These four residues were substituted with alanine, and the mutants evaluated for their ability to repress the *bfd* promoter in response to iron (Table 1). Proteins with

substitutions H87A and H89A were inactive as repressors, suggesting that these histidines are essential for *P. aeruginosa* Fur activity. Accordingly, genes expressing these proteins also failed to restore growth on succinate, and repression of the synthesis of siderophores (data not shown). Proteins with substitutions H86A and D88A retained partial ability, as judged by their ability to regulate *bfd* (Table 1). Strains expressing these partially active proteins overexpressed siderophores and showed a reduced ability to grow on succinate.

Substitution of each of the three histidines (His-85, His-86 and His-89) with leucine in the *E. coli* protein had surprisingly little effect on Fur activity *in vivo* (Coy *et al.*, 1994). On the other hand, substitution of His-89 (*E. coli* numbering) with leucine led to loss of activity of the *Vibrio cholerae* Fur (Lam *et al.*, 1994), which is consistent with the phenotype of the H89A mutant of *P. aeruginosa* Fur (Table 1). Substitution of His-90 of the *S. typhimurium* Fur (equivalent to H89 of the *E. coli* protein) with arginine eliminated iron-mediated repression but had no effect on the role of Fur in the acid tolerance response (Hall & Foster, 1996). Although the *fur* gene of *P. aeruginosa* is thought to be essential (Hassett *et al.*, 1996), some *fur* alleles with altered phenotypes have been characterized. Manganese resistance has been used as a screen for the isolation of *fur* alleles in *P. aeruginosa*, and amongst several characterized were two mutants with substitutions of arginine and tyrosine at His-86 (Barton *et al.*, 1996). Strains carrying these mutations showed constitutive expression of siderophores but retained iron-regulation of exotoxin A expression, suggesting only a partial loss of Fur activity (Barton *et al.*, 1996). This phenotype is consistent with the retention of some activity for the H86A mutant reported here.

Clearly much remains to be learned about the structure and mechanism of the Fur proteins. Future work will be directed towards exploiting the improved expression systems described in this report, with the ultimate goal of establishing the three-dimensional structure of the *P. aeruginosa* Fur.

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