

Characterization of the DNA- and Metal-Binding Properties of *Vibrio anguillarum* Fur Reveals Conservation of a Structural Zn²⁺ Ion

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The ferric uptake regulator, Fur, represses iron uptake and siderophore biosynthetic genes under iron-replete conditions. Here we report in vitro solution studies on *Vibrio anguillarum* Fur binding to the consensus 19-bp *Escherichia coli* iron box in the presence of several divalent metals. We found that *V. anguillarum* Fur binds the iron box in the presence of Mn²⁺, Co²⁺, Cd²⁺, and to a lesser extent Ni²⁺ but, unlike *E. coli* Fur, not in the presence of Zn²⁺. We also found that *V. anguillarum* Fur contains a structural zinc ion that is necessary yet alone is insufficient for DNA binding.

Iron is essential for survival and virulence of bacterial pathogens; however, its concentration in host tissues is limited. To acquire iron, bacterial pathogens rely on iron uptake systems that consist of an iron siderophore (a small-molecule chelator) and membrane transport proteins, which import Fe³⁺-siderophore complexes into the cell (7, 18). These systems are regulated by the DNA-binding protein Fur (ferric uptake regulator) in response to iron availability (3, 11, 20). When the intracellular concentration of iron increases above a certain level, Fur represses the transcription of genes encoding components of the membrane transport system as well as enzymes involved in siderophore biosynthesis.

To date, the most extensive biochemical studies have been limited to the *Escherichia coli* Fur protein, which in the presence of various divalent metals that act as corepressors binds a conserved 19-bp operator sequence, the iron box, which is located in the promoter region of iron uptake genes (4, 5, 9). Moreover, these studies have indicated that *E. coli* Fur possesses two metal ion-binding sites. One, the corepressor binding site, uses histidines and carboxylate ligands to coordinate binding of Fe²⁺ (and two of its functional mimics, Mn²⁺ and Co²⁺) (1). This event promotes a conformational change that leads to DNA binding. The other cation-binding site is involved in protein structure and stability and binds Zn²⁺ with high affinity, using the thiols of Cys92 and Cys95 as two of the four coordinating ligands (2, 3, 14). These cysteines are essential for *E. coli* Fur activity both in vivo and in vitro (6).

Fur homologues have been identified in multiple bacteria, where they also regulate iron acquisition. In the fish pathogen *Vibrio anguillarum* strain 775, Fur represses production of a siderophore, anguibactin, and of Fe³⁺-anguibactin transport proteins in response to abundant iron (21). *V. anguillarum* Fur is closely related to its counterparts from other *Vibrio* species, including *Vibrio cholerae* (93% sequence identity), *Vibrio vulnificus* (91%), and *Vibrio parahaemolyticus* (91%) (Fig. 1). Yet *V. anguillarum* Fur is less homologous to *E. coli* Fur (76% sequence identity), and the two proteins have different numbers of cysteines and histidines (Fig. 1). Therefore, to test whether

V. anguillarum Fur is functionally distinct from *E. coli* Fur, we characterized the DNA- and divalent-cation-binding properties of *V. anguillarum* Fur. We also investigated the effect of chemical modification of cysteines on the ability of Fur to bind a consensus 19-bp iron box in a metal-dependent manner. Here, we report that Fur binds the iron box in the presence of Mn²⁺, Co²⁺, Cd²⁺, and to a lesser extent Ni²⁺ but, unlike *E. coli* Fur, not in the presence of Zn²⁺. In addition, *V. anguillarum* Fur contains a structural Zn²⁺ ion that appears to be required for DNA binding.

The *V. anguillarum fur* gene subcloned into the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible pT7-5 vector (21) was kindly provided by A. M. Wertheimer. *E. coli* BL21(DE3) cells transformed with this vector were induced with 1 mM IPTG. Fur, which contains nine histidines, binds tightly to nickel-nitrilotriacetic acid-agarose (Novagen) and is eluted in 1 M imidazole. Protein purity was assessed by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis. After dialysis against the storage buffer [0.1 M Tris HCl (pH 7.9), 0.1 M NaCl, and 0.1 to 0.2 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP HCl), in the presence or absence of 1 mM EDTA], the protein concentration was measured spectrophotometrically by using an extinction coefficient, ε₂₈₀, of 12,007 M⁻¹ cm⁻¹, which was determined from amino acid hydrolysis.

The affinity of Fur for DNA was measured by fluorescence anisotropy (16). As it is performed in solution, this method provides a true equilibrium measurement of binding. All measurements were done using a Beacon fluorescence polarization instrument (Pan Vera) with excitation and emission wavelengths of 494 and 520 nm, respectively. The DNA used in this work is a 25-bp double-stranded oligonucleotide that encompasses the 19-bp iron box from *E. coli* (5). Each DNA duplex was prepared by annealing two complementary 25-mers, one of which contained a 5' fluorescein label (Genosys and Oligos, Etc.). The sequence of the labeled nucleotide is 5'-GCAGAT AATGATAATCATTATCGGA-3'. The inverted repeat of the iron box is underlined. All fluorescence anisotropy measurements were carried out at 25°C in 1 ml of binding buffer (100 mM HEPES with potassium salt [pH 7.5], 250 mM potassium glutamate, 150 mM sodium chloride, 10 mM magnesium acetate, and 5% glycerol) (15), with 1 μg of poly(dI · dC) per ml and 2 nM labeled DNA duplex. Fur was titrated into the mixture, and measurements were made after a 30-s equilibra-

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TABLE 1. K_d s of *V. anguillarum* Fur for the consensus iron box operator

Fur	Metal ^a	K_d (nM)
Unmodified	Mn ²⁺	50 ± 2
Unmodified	Co ²⁺	30 ± 1
Unmodified	Cd ²⁺	50 ± 2
Unmodified	Ni ²⁺	230 ± 15
Unmodified	None	>2,000
Unmodified	Zn ²⁺	>2,000
DTNB modified	Mn ²⁺	500 ± 20

^a The metal ion concentration was 1 mM in each binding experiment.

that the corepressor-binding sites of these two proteins are similar.

Recent studies have shown that *E. coli* Fur cysteine residues 92 and 95 are ligands for a structural Zn²⁺ ion (2, 14). The binding of this divalent cation is stable and requires extreme treatment to effect its removal (2). Since these cysteines are conserved in *V. anguillarum* Fur, we wanted to determine whether *V. anguillarum* Fur, like its *E. coli* counterpart, contained a structural Zn²⁺ coordinated by cysteines. To do so, we measured the Zn²⁺ content of Fur protein using a Varian-Techtron flame atomic absorption spectrometer ($\lambda = 213.9$ nm). Fur that was dialyzed against storage buffer, with or without 1 mM EDTA, contained 1.4 ± 0.1 (mean \pm standard deviation from three measurements per sample) mol of Zn²⁺

per mol of Fur. Interestingly, Fur dialyzed extensively against 50 mM EDTA also contained 1.4 ± 0.1 mol of Zn²⁺ per mol of protein. These amounts are different from those for the *E. coli* Fur protein, for which 0.5 to 0.8 (17) or 0.9 (2) mol of Zn²⁺ was found per mol of EDTA-treated protein, but 2.1 mol of Zn²⁺ was detected per mol of EDTA-free protein (2). The presence of only one Zn²⁺ ion per monomer in *V. anguillarum* Fur versus two Zn²⁺ ions in *E. coli* Fur correlates with the inability of *V. anguillarum* Fur to use Zn²⁺ as a corepressor and suggests that the corepressor-binding site of *V. anguillarum* cannot bind Zn²⁺, whereas that of *E. coli* Fur can.

Next, we set out to determine the role of cysteines in Zn²⁺ coordination. To do so, we quantified the accessibility of the thiol side chains of the five cysteines of *V. anguillarum* as measured by their chemical modification by 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (10, 19). If Cys92, Cys95, and possibly other cysteine residues were taking part in Zn²⁺ ion coordination, their chemical modification would eliminate Zn²⁺ from the protein sample. Our results showed that in the presence of 1 mM EDTA under both nondenaturing and denaturing conditions, 5.2 mol of cysteine per mol of *V. anguillarum* Fur was modified, which indicates that all five cysteine residues were accessible to DTNB. After DTNB labeling, Fur was dialyzed against its storage buffer, and subsequent Zn²⁺ analysis indicated the presence of 0.15 ± 0.05 mol of Zn²⁺ per mol of DTNB-labeled Fur protein. Concomitant with this dramatic decrease in Zn²⁺ content is a 10-fold-lower affinity of

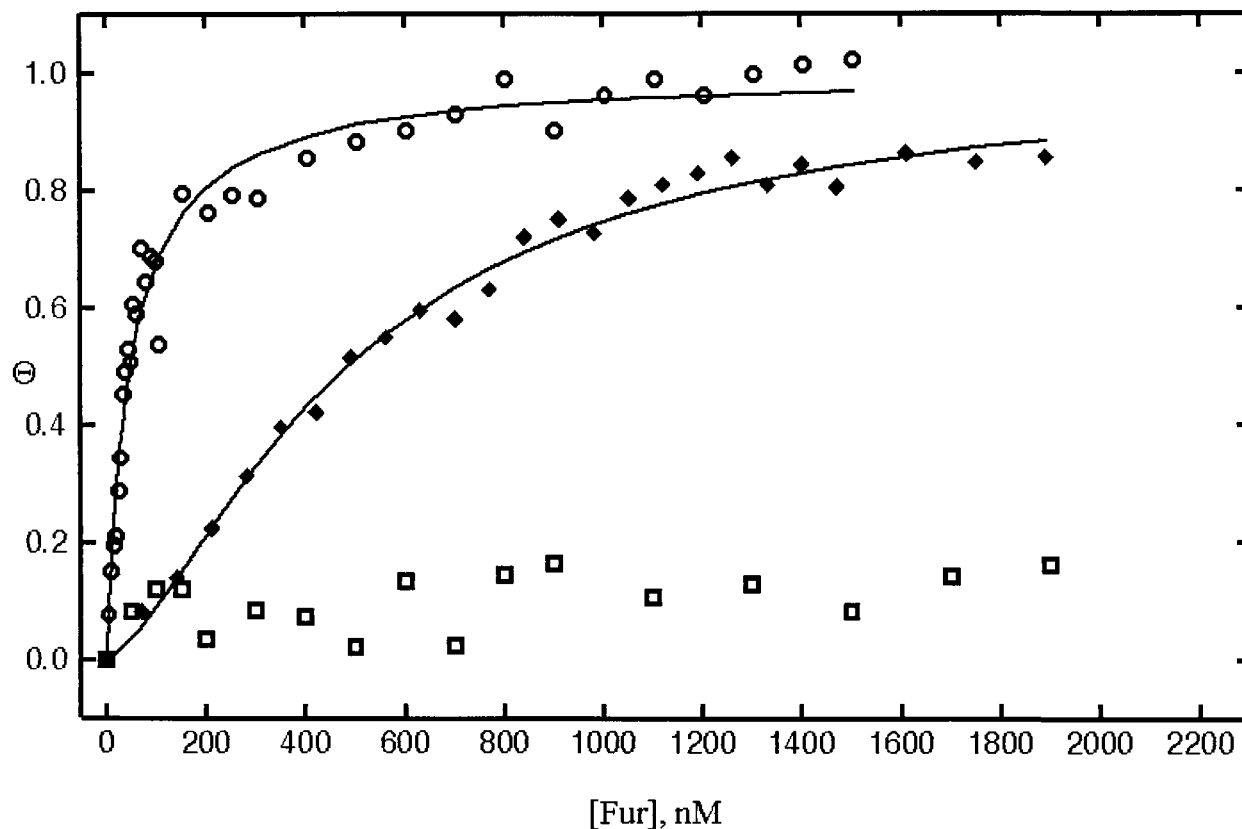


FIG. 2. Binding isotherms of unmodified Fur to the consensus iron box in the presence (○) and absence (□) of Mn²⁺ and binding of DTNB-modified Fur in the presence of Mn²⁺ (◆). Binding data were analyzed and fitted by nonlinear least-squares regression (Sigma Plot) to the following equation describing a cooperative binding model: $\theta = (A - A_0)/(A_{\max} - A_0) = [P]^h/(K_d + [P]^h)$, where θ is the fractional saturation of the operator with Fur, A is anisotropy, A_{\max} is maximum anisotropy, A_0 is initial anisotropy, $[P]$ is the total protein concentration, and h is the Hill or cooperativity coefficient, which was in the range of 2.0 to 2.5. The graph shows θ as a function of total Fur concentration.

DTNB-labeled Fur for the iron box ($K_d = 500$ nM) in the presence of 1 mM Mn^{2+} ion.

The results of our thiol modification experiments indicate that a Zn^{2+} ion likely serves as the structural cation in *V. anguillarum* Fur, because modification of all five cysteines eliminates Zn^{2+} and significantly diminishes DNA binding. These results are similar to those reported for the *E. coli* Fur, which binds Zn^{2+} at a structural site (14) using cysteines 92 and 95, and this Zn^{2+} stabilizes the protein (2). Specifically, mutagenesis of these two cysteines in *E. coli* Fur (6) as well as their chemical modification (8) results in diminished DNA binding. Further experiments are necessary to determine whether cysteines 92 and 95 are responsible for Zn^{2+} binding in *V. anguillarum* Fur. While these two cysteines are conserved between the *E. coli* and *V. anguillarum* Fur proteins, they are replaced in the Fur proteins of several other bacteria, such as *Pseudomonas putida* and *Bradyrhizobium japonicum* (Fig. 1) (13). Hence, it is unclear whether the structural Zn^{2+} found in *E. coli* and *V. anguillarum* Fur is universally used in Fur proteins from other bacteria and whether the presence of cysteines at positions 92 and 95 is indicative of the structural Zn^{2+} -binding site. Although necessary, this Zn^{2+} is not sufficient for high-affinity DNA binding by *V. anguillarum* Fur, which also requires the presence of a divalent corepressor ion, such as Mn^{2+} . On the contrary, *E. coli* Fur was recently reported to bind its operator with high affinity in the presence of only its structural Zn^{2+} (2). However, this finding will require additional investigation. Perhaps a side-by-side study of the two Fur proteins would clarify the basis of their different metal ion-binding properties.

In summary, our results reveal that the closely related *E. coli* and *V. anguillarum* Fur proteins possess comparable divalent cation- and DNA-binding characteristics, albeit with significant differences. These findings illustrate the overall conservation of function and metal specificity of Fur proteins from these different gram-negative bacteria and thus expand our understanding of these essential regulatory proteins.

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REFERENCES

- Adrait, A., L. Jacquamet, L. Le Pape, A. Gonzalez de Peredo, D. Aberdam, J. L. Hazemann, J. M. Latour, and I. Michaud-Soret. 1999. Spectroscopic and saturation magnetization properties of the manganese- and cobalt-substituted Fur (ferric uptake regulation) protein from *Escherichia coli*. *Biochemistry* **38**:6248–6260.
- Althaus, E. W., C. E. Outten, K. E. Olson, H. Cao, and T. V. O'Halloran. 1999. The ferric uptake regulation (Fur) repressor is a zinc metalloprotein. *Biochemistry* **38**:6559–6569.
- Bagg, A., and J. B. Neilands. 1987. Molecular mechanism of regulation of siderophore-mediated iron assimilation. *Microbiol. Rev.* **51**:509–518.
- Bagg, A., and J. B. Neilands. 1987. Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. *Biochemistry* **26**:5471–5477.
- Calderwood, S. B., and J. J. Mekalanos. 1988. Confirmation of the Fur operator site by insertion of a synthetic oligonucleotide into an operon fusion plasmid. *J. Bacteriol.* **170**:1015–1017.
- Coy, M., C. Doyle, J. Besser, and J. B. Neilands. 1994. Site-directed mutagenesis of the ferric uptake regulation gene of *Escherichia coli*. *Biometals* **7**:292–298.
- Crosa, J. H. 1997. Signal transduction and transcriptional and posttranscriptional control of iron-regulated genes in bacteria. *Microbiol. Mol. Biol. Rev.* **61**:319–336.
- Del Cardayre, S., and J. B. Neilands. 1991. Structure-activity correlations for the ferric uptake regulation (fur) repressor protein of *Escherichia coli* K12, p. 387–396. In R. B. Frankel and R. P. Blakemore (ed.), *Iron biominerals*. Plenum Press, New York, N.Y.
- de Lorenzo, V., S. Wee, M. Herrero, and J. B. Neilands. 1987. Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (*fur*) repressor. *J. Bacteriol.* **169**:2624–2630.
- Ellman, G. L. 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**:70–77.
- Escolar, L., J. Pérez-Martín, and V. de Lorenzo. 1999. Opening the iron box: transcriptional metalloregulation by the Fur protein. *J. Bacteriol.* **181**:6223–6229.
- Hamed, M. Y., J. B. Neilands, and V. Huynh. 1993. Binding of the ferric uptake regulation repressor protein (Fur) to Mn(II), Fe(II), Co(II), and Cu(II) ions as co-repressors: electronic absorption, equilibrium, and ^{57}Fe Mossbauer studies. *J. Inorg. Biochem.* **50**:193–210.
- Hamza, I., R. Hassett, and M. R. O'Brian. 1999. Identification of a functional *fur* gene in *Bradyrhizobium japonicum*. *J. Bacteriol.* **181**:5843–5846.
- Jacquamet, L., D. Aberdam, A. Adrait, J. L. Hazemann, J. M. Latour, and I. Michaud-Soret. 1998. X-ray absorption spectroscopy of a new zinc site in the fur protein from *Escherichia coli*. *Biochemistry* **37**:2564–2571.
- Leirno, S., C. Harrison, D. S. Cayley, R. R. Burgess, and M. T. Record, Jr. 1987. Replacement of potassium chloride by potassium glutamate dramatically enhances protein-DNA interactions *in vitro*. *Biochemistry* **26**:2095–2101.
- Lundblad, J. R., M. Laurance, and R. H. Goodman. 1996. Fluorescence polarization analysis of protein-DNA and protein-protein interactions. *Mol. Endocrinol.* **10**:607–612.
- Michaud-Soret, I., A. Adrait, M. Jaquinod, E. Forest, D. Touati, and J. M. Latour. 1997. Electrospray ionization mass spectrometry analysis of the apo- and metal-substituted forms of the Fur protein. *FEBS Lett.* **413**:473–476.
- Neilands, J. B. 1995. Siderophores: structure and function of microbial iron transport compounds. *J. Biol. Chem.* **270**:26723–26726.
- Riddles, P. W., R. L. Blakeley, and B. Zerner. 1983. Reassessment of Ellman's reagent. *Methods Enzymol.* **91**:49–60.
- Stojiljovic, I., A. J. Baumler, and K. Hantke. 1994. Fur regulon in gram-negative bacteria. Identification and characterization of new iron-regulated *Escherichia coli* genes by a fur titration assay. *J. Mol. Biol.* **236**:531–545.
- Tolmasky, M. E., A. M. Wertheimer, L. A. Actis, and J. H. Crosa. 1994. Characterization of the *Vibrio anguillarum fur* gene: role in regulation of expression of the FatA outer membrane protein and catechols. *J. Bacteriol.* **176**:213–220.