The Fur protein represses transcription of iron-responsive genes in bacteria. The discovery that Fur is a zinc metalloprotein and the use of surrogate metals for Fe\(^{2+}\) for in vitro studies question whether Fur is a direct iron sensor. In the present study, we show that the affinity of Fur from Bradyrhizobium japonicum (BjFur) for its target DNA increases 30-fold in the presence of metal, with a K\(_d\) value of about 2 nM. DNase I footprinting experiments showed that BjFur protected its binding site within the irr gene promoter in the presence of Fe\(^{2+}\) but not in the absence of metal, showing that DNA binding is Fe\(^{2+}\)-dependent. BjFur did not inhibit in vitro transcription from the irr promoter using purified components in the absence of metal, but BjFur repressed transcription in the presence of Fe\(^{2+}\). Thus, BjFur is an iron-responsive transcriptional repressor in vitro. A regulatory Fe\(^{2+}\)-binding site (site 1) and a structural Zn\(^{2+}\)-binding site (site 2) inferred from the recent crystal structure of Fur from Pseudomonas aeruginosa are composed of amino acids highly conserved in many Fur proteins, including BjFur. BjFur mutants containing substitutions in site 1 (BjFurS1) or site 2 (BjFurS2) bound DNA with high affinity and repressed transcription in vitro in an Fe\(^{2+}\)-dependent manner. Interestingly, only a single dimer of BjFurS2 occupied the irr promoter, whereas the wild type and BjFurS1 displayed one- or two-dimer occupancy. We suggest that the putative functions for metal-binding sites deduced from the structure of P. aeruginosa Fur cannot be extrapolated to bacterial Fur proteins as a whole.

Control of iron homeostasis is essential to most living organisms. Iron availability can be limited because it is predominately in the ferric form in aerobic environments. On the other hand, excessive intracellular iron concentration can be deleterious because it generates reactive oxygen species that damage cellular components (1, 2). In bacteria, studies on the control of iron homeostasis have focused largely on Fur (ferric uptake regulator), a regulatory protein that responds to cellular iron (reviewed in Ref. 3). Fur represses genes involved in high affinity iron transport under iron-replete conditions. It is also involved in numerous other facets of iron metabolism and in processes not obviously linked to iron (4–6).

The working model for Fur function posits that, when bound by ferrous (Fe\(^{2+}\)) iron, Fur binds the target operator within the promoter of the regulated gene to repress transcription. However, when iron is limited in the cell, the Fur protein is unbound by iron and no longer binds DNA with high affinity; hence, gene expression is derepressed. It was recently shown that binding of Fur to the promoter element is sufficient to block transcription in vitro, and thus Fur probably does not act to recruit another repressor or exclude an activator protein (7).

Several years ago it was discovered that Escherichia coli Fur is a zinc metalloprotein that binds two Zn\(^{2+}\) atoms per monomer (8). One of the zinc atoms is very tightly bound and is likely to have a structural function, whereas the second zinc can be easily removed with chelators. In addition, Fur containing one Zn\(^{2+}\) atom is sufficient for DNA binding, and the affinity does not increase when the second Zn\(^{2+}\) site is occupied (8). These observations question whether Fur is a direct iron sensor. Furthermore, most in vitro studies use a surrogate for Fe\(^{2+}\) such as Mn\(^{2+}\) because ferrous iron readily oxidizes to Fe\(^{3+}\) in air. Thus, the assumption that the surrogate metal is a substitute for Fe\(^{2+}\) is equivocal.

Several groups have shown that the structural Zn\(^{2+}\) atom of E. coli Fur is coordinated by Cys-92 and Cys-95 (9, 10). However, these cysteines are not conserved in many Fur proteins. Very recently, the Fur protein from Pseudomonas aeruginosa was crystallized in the presence of Zn\(^{2+}\), revealing two metal-binding sites (11). None of the coordinating amino acids composing these sites are cysteine. One of the Zn\(^{2+}\) atoms binds tightly and may be a structural ligand. The second metal-binding site can be occupied by Fe\(^{2+}\) and is a presumptive regulatory metal-binding site. Interestingly, the eight amino acids identified in the two metal-binding sites are highly conserved, leading Pohl et al. (11) to extrapolate their conclusions about the Fur protein from P. aeruginosa to other Fur proteins. Alteration of residues corresponding to some of these metal-binding residues in Fur proteins produces mutant bacterial strains with altered phenotypes (4, 12–14).

Fur from Bradyrhizobium japonicum (BjFur)\(^{3}\) typifies bacterial Fur proteins in that it complements an E. coli Fur mutant, mediates iron-dependent control of gene expression, and binds to a so-called Fur box, a consensus Fur-binding DNA element (7, 15, 16). However, BjFur also binds to DNA dissimilar to the Fur box within the promoter of the irr gene, whereas E. coli Fur does not bind that element in vitro (7). The irr gene from B. japonicum encodes Irr, a protein that mediates iron control of heme biosynthesis (17, 18). Irr expression is con-

\(^{3}\) The abbreviations used are: BjFur, B. japonicum Fur; EMSA, electrophoretic mobility shift assay; HMC, high mobility complex; LMC, low mobility complex; Ni-NTA, nickel-nitrilotriacetic acid; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
trolled both transcriptionally and post-transcriptionally, the former being regulated by BjFur. The amino acids identified in metal binding in P. aeruginosa Fur are conserved in the B. japonicum protein, whereas the cysteine involved in zinc binding in E. coli Fur are not present. In the present study, we demonstrate that BjFur is an iron-responsive DNA-binding protein in vitro and that iron confers on BjFur the ability to repress transcription. Furthermore, we show that the amino acids in BjFur corresponding to the preservative metal-binding sites of the P. aeruginosa protein are not required for iron-responsive DNA binding or transcriptional repression.

**MATERIALS AND METHODS**

**Chemicals and Reagents—** All chemicals were reagent grade and were purchased from Sigma, Fisher, VWR Scientific (West Chester, PA), or J. T. Baker Inc. Agar, purified noble agar, and yeast extract were purchased from Difco, and [α-32P]dCTP (3000 Ci/mmol) was purchased from PerkinElmer Life Sciences. Irro[32P]UTP (800 Ci/mmol) was purchased from ICN Biomedicals (Irvine, CA).

**Bacterial Strains, Plasmids, Media, and Growth—** E. coli strain DH5α was used for the propagation of plasmids and was grown at 37 °C on Luria-Bertani media with appropriate antibiotics. Plasmid pSkIron contains the 19-mer Fur box consensus sequence 5'GATAATGATAAT-3' cloned into the NdeI and BamHI sites of pET14b. pET14bjFur (Novagen, Madison, WI) contains the B. japonicum fur gene cloned into the NdeI and BamHI sites of pET14b. pET14jFur was derived from parental strain USDA 1110 (15). pET14ecFur contains the E. coli fur gene cloned into the NdeI and BamHI sites of pET14b.

**Overexpression and Purification of Fur—** Fur was overexpressed in E. coli strain BL21(DE3)(p lyS8) cells containing fur in pET14b initially grown on Luria-Bertani media containing chloramphenicol (25 μg/ml) and ampicillin (200 μg/ml). Cells were inoculated from an overnight culture grown in Luria-Bertani media containing chloramphenicol and ampicillin into 2 liters of fresh Luria-Bertani media with appropriate antibiotics, 50 μM MnCl2, and 1 mM radiolabeled DNA probe. Double-stranded DNA probes were produced by boiling and slowly cooling synthetic DNA oligonucleotides (Integrated DNA Technologies, Coralville, IA) in melting buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA) and then labeled with [α-32P]ATP (3000 Ci/mmol) with the Klenow fragment of DNA polymerase (Promega, Madison, WI). Following incubation, EMSA reactions were analyzed on 5% nondenaturing polyacrylamide gels in electrophoresis buffer (20 mM bis-Tris borate, pH 7.0) that were prerun for 30 min at 200-V constant voltage. After electrophoresis at 4 °C for 45 to 90 min at 200 V, gels were dried and autoradiographed. The Fur box probe was isolated as an 83-bp fragment from a NotI and XhoI digestion of pSkIron. A BanII and Hpal digest of pSKSBrir was used to isolate a 63-bp fragment containing the irr promoter. Autoradiograms were developed on BioMax film (Eastman Kodak Co.), scanned using a GS-700 densitometer (Bio-Rad), and signal intensities were quantified using a protocol modified from de Lorenzo et al. To determine the dissociation binding constant (Kd), binding reactions were titrated with various concentrations of Fur. Bound and unbound DNA were quantified by comparing relative signal intensities. Signal intensities were imported into Excel spreadsheets and analyzed using Graphpad Prism (Graphpad Software Inc., San Diego, CA).

**DNA 1 Footprint Analysis**—DNA footprint analysis examined the DNA region protected by Fur binding. 15 μl of Fur was incubated for 30 min at 4 °C in a 50-μl volume containing EMSA buffer, 125 ng of herring sperm DNA, 5 μg of bovine serum albumin, 100 μM MnCl2 or 100 μM freshly prepared FeSO4, and 1 μM irr promoter DNA probe end-labeled with [α-32P]dCTP with Klenow. 50 μl of room temperature binding buffer (50 mM Tris-HCl, 50 mM NaCl, and 0.5 mM MnCl2) was added, followed by the addition of 0.45 μg of RQ1 RNase-free DNase (Promega, Madison, WI) in 18 μl of 40 mM Tris-HCl (pH 7.0). The reaction was incubated for 2 min at room temperature. Reactions were stopped by the addition of 90 μl of stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS, and 100 μg/ml yeast RNA), and DNA was extracted with phenol:chloroform (1:1) followed by ethanol precipitation. G + A ladders of the labeled DNA were produced as described (21). Digested probe products were separated on a 15% denaturing polyacrylamide gel containing 7 M urea in 1X Tris borate-EDTA electrophoresis buffer. Autoradiograms were developed on BioMax film. The DNA probe used for DNA 1 footprint analysis was 3'-end-labeled; therefore the top of the gel represents the 5'-end.

**In Vitro Transcription Assays—** To directly examine the influence of B. japonicum Fur on transcription from the irr promoter, in vitro transcription assays were performed on irr promoter DNA. I rr promoter fragments were generated by PCR amplification of an irr sequence in pSKSBrir using the primers 5' -TTTGAATTCGTGACGAAATATGC-3' and 5'-TTTCTCGAGGCAGCTCGTGGTGAT-3', producing a 272-bp DNA fragment that terminated 80 base pairs downstream of the irr transcription start site determined previously (16). Fur was incubated for 30 min at 4 °C in EMSA buffer without glycerol, 1 μg of bovine serum albumin, 20 μM MgCl2, 10 mM 2-mercaptoethanol, 4 mM irr promoter template DNA, and either no added metal, 100 μM MnCl2, or 100 μM FeSO4. The final concentrations were appropriate for each metal at 1 μM. The samples were flushed with N2 to remove air. 0.5 unit of RNA polymerase (Epicerin, Madison, WI) was added, reactions were incubated at 37 °C for 10 min, after which 10 μl of a preheated NTP mixture (100 μM each of ATP, CTP, and GTP, 20 μM UTP, 8 μM [α-32P]UTP (800 Ci/mmol) in 10 μl of H2O) was added, and reactions were incubated for an additional 20 min. Products of transcription were run on a 15% denaturing polyacrylamide gel containing 7 m urea in 1X Tris borate-EDTA electrophoresis buffer along with a Low Range RNA ladder (Fermentas Inc., Hanover, MA) labeled with ATP [γ-32P]ATP with polynucleotide kinase (Promega, Madison, WI).

**Iron Absorption—** B. japonicum and E. coli Fur proteins were purified as described above and were analyzed directly or reconstituted using a procedure modified from Althaus et al. (8). 70–100 μM Fur protein was incubated with a 4-fold excess of ZnCl2 and a 10-fold excess of dithiothreitol in 20 mM Tris-HCl (pH 7.0) for 4 h at 4 °C. Unbound...
B. japonicum Fur and 32P-labeled EMSA analysis. EMSA was carried out using purified recombinant BjFur and 32P-labeled DNA. Samples were run on 5% nondenaturing polyacrylamide gels in the presence (A) or absence (B) of 100 μM MnCl2 and visualized by autoradiography. 0.1 pm DNA and 0–100 nM BjFur protein were used. C, autoradiograms were quantified and plotted as bound DNA versus BjFur concentration in the presence (closed squares) or absence (open squares) of MnCl2.

Metal was removed by passage through a Sephadex G-25 column (Roche Applied Science). Flameless atomic absorption analyses were performed on a PerkinElmer model 1100B spectrophotometer equipped with a model 700 graphite furnace and an AS-70 autosampler. Fur protein concentrations were determined in Bradford assays using bovine serum albumin standards, adjusted for a 120% overestimation as described previously (8).

RESULTS

Metal Increases the Affinity of B. japonicum Fur for DNA—Fur proteins mediate iron-dependent control of gene expression in vivo and bind metals in vitro. Thus, we wanted to determine the effects of metal on B. japonicum Fur (BjFur) binding to a cognate Fur-binding site. The irr gene from B. japonicum is transcriptionally regulated by BjFur and binds to an element within the irr promoter containing three imperfect direct repeat hexamers (7). The binding of recombinant BjFur to DNA corresponding to the BjFur binding site on the irr promoter was measured in electrophoretic mobility shift assays (EMSA) using Mn2+ as a surrogate metal. Titration of DNA with an increasing concentration of BjFur in the presence of Mn2+ revealed two complexes in EMSA, a high mobility complex (HMC) at lower protein concentrations and a low mobility complex (LMC) at higher concentrations (Fig. 1A). Previous work shows that the HMC and the LMC correspond to a Fur dimer and to two dimers or a tetramer, respectively (7). In the absence of Mn2+, Fur bound DNA much less well than in the presence of metal at each protein concentration tested (Fig. 1B). Furthermore, only the HMC was detected at 50 nM BjFur in the absence of Mn2+, whereas the LMC was predominant in the presence of Mn2+ at that protein concentration.

The effects of Mn2+ on the binding affinity of BjFur for the cognate DNA element were estimated from EMSA data by plotting bound DNA as a function of protein concentration (Fig. 1C). The Kd value of BjFur for DNA was 2.2 nM in the presence of Mn2+, which is in good agreement with what we reported previously (7). In the absence of metal, the binding affinity was nearly 30-fold lower, with a Kd of 60 nM. These observations show that Mn2+ substantially increases the affinity of BjFur for DNA.

BjFur Binds DNA in an Iron-responsive Manner in Vitro—We and others routinely use Mn2+ or another divalent metal as a presumed surrogate for Fe2+ for in vitro analysis of Fur (7, 23). However, the discovery that Fur is a zinc metalloprotein in at least some organisms calls into question the role of these other metals, and one report questions whether a regulatory Fe2+ is required for activity (8). EMSA analysis using Fe2+ is problematic because the DNA-protein complex must remain intact throughout the electrophoresis where ferrous iron can oxidize to the ferric form. Therefore, we assessed the dependence of Fe2+ on DNA binding activity by DNase I footprinting analysis, maintaining anaerobic conditions in the binding and digestion reactions (Fig. 2).

The irr promoter region was slightly protected by BjFur in the absence of metal compared with the control containing no protein (Fig. 2, first panel). However, in the presence of Fe2+, wild type BjFur bound the irr promoter and protected a region that includes the three imperfect direct repeat hexamers described previously (7). The protection was similar to that observed for Mn2+, indicating that Mn2+ was qualitatively similar to Fe2+ with respect to the effect on BjFur. Thus, BjFur is an iron-responsive DNA-binding protein.

Effects of Mutations in Putative Metal-binding Sites of Fur on DNA Binding in EMSA—The structure of Fur from P. aeruginosa crystallized in the presence of Zn2+ revealed two metal-binding sites (11). Each metal coordinates with amino acids that are conserved in many Fur homologs, including B. japonicum (Fig. 3), leading Pohl et al. (11) to extrapolate their findings for P. aeruginosa to Fur proteins from other organisms. Site 1 is proposed to be the regulatory Fe2+-binding site and is...
composed of amino acids corresponding to His-97, Asp-99, Glu-118, and His-135 in B. japonicum (Fig. 3). The mutant BjFurS1 lacking site 1 was constructed by changing these residues to alanine. Surprisingly, this mutant bound DNA in a metal-responsive manner similar to the wild type EMSA analysis (Fig. 4, also see below). Titration of DNA with BjFurS1 in the presence of Mn$^{2+}$ showed a concentration-dependent formation of a HMC and an LMC similar to the wild type, and both proteins had similar $K_d$ values of 2.2 and 4.5 nM for the wild type and the mutant, respectively (Fig. 4, A and B). These data indicate that conserved amino acids attributed to metal sites 1 and 2 are not required for metal-dependent DNA binding activity.

Site 2 is proposed to bind a structural zinc ligand and is composed of amino acids corresponding to His-43, Glu-91, His-97, Asp-99, Glu-100, and Glu-111 in B. japonicum Fur (Fig. 3). We constructed a BjFur mutant in which each of these residues was substituted with alanine. This mutant, BjFurS2, bound $irr$ promoter DNA with a $K_d$ value of 1.8 nM, similar to the wild type DNA (Fig. 4). Thus, the residues implicated in metal site 2 are not necessary for the DNA binding activity of BjFur. This observation led us to ask whether zinc is associated with purified recombinant BjFur1 in the presence of Mn$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$ (Fig. 4, also see below). Titration of DNA with BjFurS1 in the presence of Mn$^{2+}$ showed a concentration-dependent formation of a HMC and an LMC similar to the wild type, and both proteins had similar $K_d$ values of 2.2 and 4.5 nM for the wild type and the mutant, respectively (Fig. 4, A and B). These data indicate that conserved amino acids attributed to metal sites 1 and 2 are not required for metal-dependent DNA binding activity.

EMSA analysis with the wild type and mutant proteins using Fur box consensus DNA as the radiolabeled probe (Fig. 5). The footprinting analysis with mutant proteins protected the DNA promoter only in the presence of metal, but full protection was observed in the absence of metal, even up to 100 nM DNA (Fig. 4, A and B). Wild type BjFur binds DNA in the absence of metal but with lower affinity (Fig. 1). Thus, BjFurS1S2 does not behave as an unmetalated Fur protein but rather as an inactive one. Although no structural analysis of BjFurS1S2 was carried out, it is likely that the extensive mutagenesis compromised the structural integrity of the protein.

The $irr$ promoter element to which BjFur binds is dissimilar to the Fur box consensus sequence. E. coli Fur binds the Fur box only, whereas BjFur binds both elements with similar affinity composed of amino acids corresponding to His-97, Asp-99, Glu-118, and His-135 in B. japonicum (Fig. 3). The mutant BjFurS1 lacking site 1 was constructed by changing these residues to alanine. Surprisingly, this mutant bound DNA in a metal-responsive manner similar to the wild type EMSA analysis (Fig. 4, also see below). Titration of DNA with BjFurS1 in the presence of Mn$^{2+}$ showed a concentration-dependent formation of a HMC and an LMC similar to the wild type, and both proteins had similar $K_d$ values of 2.2 and 4.5 nM for the wild type and the mutant, respectively (Fig. 4, A and B). These data indicate that conserved amino acids attributed to metal sites 1 and 2 are not required for metal-dependent DNA binding activity. The footprinting analysis with mutant proteins protected the DNA promoter only in the presence of metal, but full protection was observed in the absence of metal, even up to 100 nM DNA (Fig. 4, A and B). Wild type BjFur binds DNA in the absence of metal but with lower affinity (Fig. 1). Thus, BjFurS1S2 does not behave as an unmetalated Fur protein but rather as an inactive one. Although no structural analysis of BjFurS1S2 was carried out, it is likely that the extensive mutagenesis compromised the structural integrity of the protein.

The $irr$ promoter element to which BjFur binds is dissimilar to the Fur box consensus sequence. E. coli Fur binds the Fur box only, whereas BjFur binds both elements with similar affinity.
Fe$^{2+}$ or Mn$^{2+}$ (Fig. 2), and therefore site 2 amino acids are not required for DNA binding activity or metal responsiveness. However, BjFurS2 protected a smaller region of the *irr* promoter than did BjFur or BjFurS1. This region corresponds to two direct repeat sequences described previously (7) required for formation of the high mobility complex, and thus the footprint is in excellent agreement with the EMSA analysis (Fig. 4). The mutant protein BjFurS1S2 did not protect the *irr* promoter in the absence or presence of metal, which is consistent with the conclusion that this mutant cannot bind DNA.

**BjFur Is an Iron-responsive Transcriptional Repressor in Vitro**—Recently we provided direct evidence that binding of a bacterial Fur protein to the *cis*-acting promoter element is sufficient to repress transcription using an *in vitro* system composed of purified components (7). A promoter mutation that compromised BjFur binding also mitigated transcriptional repression. Here we sought to address whether repression of the *irr* promoter by BjFur was iron-responsive using this *in vitro* system (Fig. 6). In the absence of BjFur, an 80-nucleotide RNA was synthesized from a double-stranded DNA template. When BjFur was included in the absence of metal, there was no repression of RNA synthesis. However, in the presence of Fe$^{2+}$, transcription was strongly repressed by BjFur, with a similar result using Mn$^{2+}$ as the regulatory metal. Mutant protein BjFurS1 also showed iron-dependent transcriptional repression and to a lesser extent with Mn$^{2+}$ as well. These data show that BjFur is an iron-responsive transcriptional repressor in *vitro* and that the site 1 amino acids are not necessary for this regulated activity. BjFurS1S2 was unable to repress transcription from the *irr* promoter, which is consistent with an inability to bind DNA. These observations also show that Fe$^{2+}$ or Mn$^{2+}$ do not in themselves inhibit transcription. EMSA analysis and DNase I footprinting indicate that BjFurS2 only occupies the *irr* promoter as a dimer (Figs. 2 and 4). Nevertheless, BjFurS2 repressed transcription in the presence of Fe$^{2+}$ (Fig. 6), indicating that a dimer is sufficient as a repressor. This conclusion agrees with previous work showing that transcription from a mutant *irr* promoter that allows only dimer occupancy is repressed by wild type BjFur (7).

**DISCUSSION**

In the present study, we demonstrate that Fe$^{2+}$ activates *B. japonicum* Fur (BjFur) with respect to DNA binding and transcriptional repression activities *in vitro*. Therefore, iron-dependent regulation of genes mediated by Fur in *vivo* is most likely because of a direct effect of Fe$^{2+}$ on Fur. *E. coli* Fur binds DNA with a *K*_D value of ~20 nM when bound by one structural Zn$^{2+}$ atom only or when the second Zn$^{2+}$ site is also occupied (8). Those observations suggest either that *E. coli* Fur does not have a regulatory metal-binding site that enhances activity or else the second Zn$^{2+}$ is not a surrogate for Fe$^{2+}$ *in vitro*. It is plausible that Fe$^{2+}$ would have increased the affinity of the protein for the *cis*-acting element in the protein preparations, as has been deduced in earlier work (24). In the present study, BjFur bound DNA in the absence of added metal, but the affinity increased substantially in the presence of added Fe$^{2+}$ or Mn$^{2+}$ (Figs. 1 and 2).

In a previous study, we provided direct evidence that BjFur binding to the cognate *cis*-acting element is sufficient to block transcription *in vitro* without the need for additional regulatory factors (7). Here, we showed that Fe$^{2+}$ controls transcriptional repression *in vitro* using purified components (Fig. 6), and this regulation can be attributed to the effects of Fe$^{2+}$ on DNA binding affinity. BjFur binding to the *irr* promoter probably inhibits occupation by RNA polymerase or else it prevents the formation of an open complex needed to initiate transcription. Neither mechanism would require the addition or exclusion of another regulatory protein. Collectively, the *in vitro* experiments fit a relatively simple model of regulation whereby Fur senses Fe$^{2+}$ directly to effect affinity for a promoter element of iron-controlled genes. Recently, iron was shown to increase the affinity of the *Helicobacter pylori* Fur protein for some operator DNA elements but decrease the affinity for other elements (25). In that case, a model must accommodate a Fur protein with activity when unbound by iron but with different DNA sequence specificity. Similarly, a *B. japonicum* Fur mutant displays a phenotype with respect to expression of the heme biosynthesis gene *hemA* under iron limitation (16), indicating that the protein has an activity under those conditions as well.

The recent crystal structure of the *P. aeruginosa* Fur protein and accompanying spectroscopic data indicate a high affinity Zn$^{2+}$-binding site with a putative structural function and a lower affinity site that can also be occupied by Fe$^{2+}$. The coordinating amino acids are highly conserved among Fur proteins from many organisms, including *B. japonicum*, suggesting that generalizations can be made regarding these metal sites. However, the present work shows clearly that such extrapolations cannot be made *prima facie*. The BjFurS1 mutant containing substitutions in all four amino acids corresponding to the putative regulatory iron site of *P. aeruginosa* is iron-responsive with respect to DNA binding and transcriptional repression activities (Figs. 2 and 6). Furthermore, BjFurS1 formed both a high mobility complex and a low mobility complex in EMSA analysis and had a similar binding affinity for the cognate DNA elements as did the wild type (Figs. 4 and 5). From this we suggest that the conclusions drawn for *P. aeruginosa* Fur do not represent bacterial Fur proteins as a whole. It will be important to analyze the effects of similar mutations on function in the *P. aeruginosa* protein.

The BjFurS2 mutant containing amino acid substitutions corresponding to putative structural Zn$^{2+}$-binding residues also bound DNA with high affinity, was iron-responsive, and was able to repress transcription. Therefore, this mutant is a functional protein. It is interesting to note that purified recom
binant *P. aeruginosa* Fur bound to zinc in one study (11) but not in another (22). Therefore the role of zinc and the site 2 residues remains to be elucidated in that protein. We cannot rule out that zinc binds BjFur with low affinity that is removed by dialysis or desalting, but a structural zinc atom should bind with high affinity as was found for *E. coli* (11).

Although BjFurS2 binds DNA with high affinity and is functional, the substitutions in the mutant protein are not completely innocuous. BjFurS2 occupied the *irr* promoter only as a dimer as indicated by the HMC in EMSA and did not form the LMC at higher protein concentrations (Fig. 4). However, this trait is very different from that expected of a protein that cannot coordinate a tightly bound metal necessary for the integrity of the monomer. From this we conclude that BjFurS2 is not involved in binding an essential structural Zn$^{2+}$ as envisioned for *P. aeruginosa* Fur. We do not know whether the inability of BjFurS2 to form an LMC is related to metal or some other feature of the protein. The ability of BjFurS2 to repress transcription agrees with our previous study showing that dimer occupancy is sufficient to inhibit transcription (7). In that work, dimer occupancy was created by mutation of the promoter or by using a low concentration of protein with the normal promoter, whereas in the present study it was the result of an altered protein.

We suggest that a unified model for Fur function cannot be assumed from studies of relatively few organisms and that Fur is a functionally and structurally more diverse protein within the eubacterial kingdom than has been realized previously.

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The Ferric Uptake Regulator (Fur) Protein from *Bradyrhizobium japonicum* Is an Iron-responsive Transcriptional Repressor *in Vitro*

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