A Novel DNA-binding Site for the Ferric Uptake Regulator (Fur) Protein from Bradyrhizobium japonicum*

Yali E. Friedman and Mark R. O'Brian‡

From the Department of Biochemistry and Witebsky Center for Microbial Pathogenesis and Immunology, State University of New York at Buffalo, Buffalo, New York 14214

The Fur protein is a global regulator of iron metabolism and other processes in many bacterial species. A key feature of the model of Fur function is the recognition of a DNA element within target promoters with similarity to a 19-bp AT-rich palindromic sequence called a Fur box. The irr gene from Bradyrhizobium japonicum is under the control of Fur. Here, we provide evidence that B. japonicum Fur (BjFur) binds to the irr gene promoter with high affinity despite the absence of DNA sequence similarity to the Fur box consensus. Both Escherichia coli Fur and BjFur bound a synthetic Fur box consensus DNA element in electrophoretic gel mobility shift assays, but only BjFur bound the irr promoter. BjFur maximally protected a 30-bp region in DNase I footprinting analysis that includes three imperfect direct repeat DNA sequences. BjFur bound a high mobility complex and a low mobility complex with DNA in electrophoretic gel mobility shift assays corresponding to occupancy by a single dimer and two dimers or a tetramer, respectively. A mutation in the downstream direct repeat DNA sequence allowed high mobility complex formation only. In vitro transcription from the wild type irr promoter or from a mutated promoter that allowed only dimer occupancy was repressed by Fur, indicating that the dimer can be a functional repressor unit. Our findings identify a novel DNA-binding element for Fur and suggest that the Fur box consensus may not completely represent the target sequences for bacterial Fur proteins as a whole. In addition, Fur binding to a target promoter is sufficient to repress transcription in vitro.

Control of iron homeostasis is essential to most living organisms. Iron is required for many cellular processes but can be unavailable because it is insoluble in its predominant ferric (Fe\(^{3+}\)) form. However, excess iron is toxic because it catalyzes the formation of reactive oxygen species that can damage DNA, protein, and lipids. Organisms have specific mechanisms to control iron acquisition and to store it in an inert form. 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. Fur represses genes that are involved in high affinity iron transport under iron replete conditions and that are derepressed when the metal is scarce. In addition, Fur is involved in numerous other facets of iron metabolism and also in processes not obviously linked to iron, such as acid shock response (1), synthetic pathways (2), and the production of toxins and other virulence factors (3). Fur is the founding member of a family of regulators that also includes Zur (4, 5), PerR (6), and Irr (7, 8). These proteins differ in function and have different DNA-binding sites but are all involved in metal-dependent control of gene expression.

Fur homologs are found in many bacterial genomes, but the protein has been studied in relatively few of them. Structural analysis of Fur and its DNA binding properties has been most extensively studied in Escherichia coli, Pseudomonas aeruginosa, and Bacillus subtilis, whereas analyses of fur mutants and the identification of genes under Fur control have also been studied in those bacteria and in several other organisms as well. P. aeruginosa Fur was crystallized as a dimer (9), which also appears to be its oligomerization state in solution (10, 11). It has been proposed that at least two Fur dimers occupy its target promoter based on the size of protected DNA in footprint analyses (12). In addition, Fur binds two zinc atoms/monomer; one zinc has a structural function, and the other may be the ferrous iron-binding site in vivo (9). However, the E. coli protein binds DNA when one or both metal sites is occupied by zinc (13). Furthermore, cysteines are zinc ligands in E. coli (13, 14) but not in P. aeruginosa (9). The working model for Fur function posits that, when bound by ferrous (Fe\(^{2+}\)) iron, Fur binds its target DNA within the promoter of the regulated gene to repress transcription. However, when iron is limiting in the cell, Fur protein is unbound by iron and no longer binds DNA with high affinity, hence gene expression is derepressed. It is generally assumed that Fur binding blocks access of RNA polymerase to the promoter to repress transcription, but this has not been demonstrated directly, nor have the possibilities that Fur excludes an activator or recruits a repressor been ruled out.

Central to the model is the so-called Fur box, a DNA-binding element for Fur that contains similarity to a 19-bp, AT-rich palindromic consensus sequence (Fig. 1). This consensus sequence was originally derived from examination of promoters of numerous Fur-regulated genes (12). Subsequent searches have yielded one promoter that matches the Fur box consensus exactly (15), with 14- or 15-bp matches out of 19 being more typical and 11 bp as a minimum match (15–17). Sequence similarity to a Fur box consensus within promoter regions of genes is taken as ab initio evidence for regulation by Fur. The assumption that Fur recognizes the two 9-bp inverted repeats of the palindrome was challenged by studies that interpret the consensus as three shorter hexameric repeats in a head-to-tail orientation (18) (Fig. 1). Yet a third interpretation of the consensus using B. subtilis Fur is a two 7-1-7 inverted repeat motif that accommodates two dimers (19). Thus, there is

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‡ To whom correspondence should be addressed: Dept. of Biochemistry, 140 Farber Hall, State University of New York at Buffalo, Buffalo, NY 14214. Tel.: 716-829-3200; Fax: 716-829-2725; E-mail: mrobrain@buffalo.edu.

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‡ To whom correspondence should be addressed: Dept. of Biochemis-
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Bradyrhizobium japonicum is a Gram-negative bacterium that lives as a free-living organism or in symbiosis with soybean. The B. japonicum fur gene was identified based on functional complementation of an E. coli mutant (20). It has also been characterized in Rhizobium leguminosarum (21), and homologs are found in the genomes of other taxonomically related organisms within the α-proteobacterial group as well. Fur is involved in controlling iron metabolism in B. japonicum (20, 22) but appears to play a lesser role in R. leguminosarum (21).

In addition to Fur, the Irs protein in B. japonicum is involved in iron metabolism, where it mediates iron-dependent regulation of heme biosynthesis (7). The irs gene is controlled by iron at both transcriptional (22) and post-translational levels (8, 23). Evidence for Fur-mediated transcriptional control is based on the observations that iron-dependent accumulation of irs mRNA is aberrant in a fur mutant, and extracts from E. coli cells harboring the B. japonicum fur gene bind to DNA upstream of the irs gene (22). However, the irs promoter region does not contain DNA sequence similar to the Fur box consensus sequence, indicating an important difference between the B. japonicum Fur protein and the other proteins on which the Fur box consensus sequence is based. In this study, we provide evidence that the functional pattern within that sequence.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—All chemicals were reagent grade and 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 Laboratories, Detroit, MI. [α-32P]dCTP (3000 Ci/mmol) was purchased from PerkinElmer Life Sciences. [α-32P]PUT4 (800 Ci/mmol) was purchased from ICN Biomedicals, Irvine, CA.

Bacterial Strains, Plasmids, Media, and Growth—E. coli strain DH5α was used for propagation of plasmids and was grown at 37 °C on LB media with appropriate antibiotics. Plasmid pSKIroN contains the 19-mer Fur box consensus sequence 5′-GATAAATGATAAATCTCATTC-3′ cloned into the ClaI site of pBluescript SK and was a gift from Dr. M. L. Vasili (24). pET14b/Fur (Novagen, Madison, WI) contains the B. japonicum fur gene cloned into the NdeI and BamHI sites of pET14b. The resultant protein expressed from pET14b contains a histidine tag that is used for purification.

Overexpression and Purification of Fur—Fur was overexpressed in E. coli strain BL21(DE3) (pLysS) cells containing fur in pET14b, initially grown on LB media containing chloramphenicol (25 μg/ml) and ampicillin (200 μg/ml). Cells were inoculated from an overnight culture grown in LB media containing chloramphenicol and ampicillin into 1 liter of fresh 2× YT containing antibiotics, 50 μg FeCl3, and 1 mm ZnCl2. Overexpression was induced in cells at mid-log phase by adding 20 ml/liter of 95% ethanol and a final concentration of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, and then incubating at 20 °C for 4 h with shaking. Cells were harvested by centrifugation at 4000 × g, washed in TNG (50 mM Tris-HCl, 50 mM NaCl, 5% glycerol, pH 7.4), and resuspended in 15 ml of phosphate binding buffer (5 mM imidazole, 300 mM NaCl, 10% glycerol, 50 mM NaPO4, pH 8.0, 10 mM phenylmethylsulfonyl fluoride, and 25.5 μg of aprotinin/5 g of cells. Cells were disrupted by passage twice through a French pressure cell at 1200 p.s.i. and clarified by centrifugation at 37,000 × g for 45 min. 1 ml of 50% Ni-NTA slurry (Qiagen Inc., Valencia, CA) was added to 4 ml of clear lysate and rocked for 60 min at 4 °C. The Ni-NTA slurry-protein mixture was poured into a column and washed three times with 25 ml of phosphate wash buffer (20 mM imidazole, 300 mM NaCl, 10% glycerol, 50 mM NaPO4, pH 8). Purified His-Fur was eluted with phosphate elution buffer (250 mM imidazole, 300 mM NaCl, 10% glycerol, 50 mM NaPO4, pH 8). The His tag was cleaved by adding 5 μg of thrombin (Novagen, Madison, WI) per 2 mg of His-Fur and incubating at room temperature for 2 h. To remove imidazole, Fur was further purified by retention through a 3-KDa nominal molecular weight limit Ultrafree column (Millipore, Billerica, MA). The His tag was removed by resuspending the retentate in 1.2 ml of 50% Ni-NTA/300 μl of purified Fur and loading on a column. The eluate was collected in fractions, and fractions containing purified Fur were combined. Protein was diluted to 1 μM working stock solution in electrophoretic gel mobility shift buffer (EMSA) binding buffer (10 mM Tris borate, 1 mM MgCl2, 40 mM KCl, 5% glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol, pH 7.5).

Electrophoretic Mobility Shift Assay—EMSA were used to determine DNA binding to Fur. The presence of manganese was necessary for DNA binding activity of Fur. Mn2+ presumably substitutes for Fe2+, but the latter is readily oxidized to Fe3+ in air. Using a protocol modified from de Lorenzo et al. (25), Fur was incubated for 30 min at 4 °C in a 20-μl volume of EMSA binding buffer supplemented with 50 ng of herring sperm DNA, 2 μg of bovine serum albumin, 100 μM MnCl2, and 100 μM DNA probe. 100 pM DNA probe was used for Kd determination and competition experiments. 1 μM DNA probe was used for all other experiments. Double-stranded DNA probes were produced by boiling and slowly cooling synthetic DNA oligonucleotides (Integrated DNA Technologies, Coralville, IA) in annealing buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and filled in with [α-32P]dCTP (3000 Ci/mmol). The 83-bp fragment was amplified from a 63-bp fragment used as nonspecific DNA. Autoradiograms were developed on 5% nondenaturing polyacrylamide gels in electro- phoresis buffer (20 mM Tris borate, pH 7.5) that were prerun for 30 min at 200 V of constant voltage. After electrophoresis at 4 °C for 45–90 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 BamHI and HpaII digest of pSKSB1rr was used to isolate a 69-bp fragment used as nonspecific DNA. Autoradiograms were developed on BioMax film (Eastman Kodak Co.) and scanned using a GS-700 densitometer (Bio-Rad), and signal intensities were detected and quantified.

Competition Assays—EMSA were used to determine the dissociation binding constant (Kd) binding reactions were titrated with various concentrations of Fur. Bound and unbound DNA was quantified by comparing relative signal intensities and analyzed using GraphPad Prism (Graph- pad Software Inc., San Diego, CA). DNA probes used to delimit sequence was sufficient for Fur binding in the irs promoter were initially synthesized as overlapping fragments and compared by EMSA. After identifying a 39-bp probe that was sufficient for both low and high mobility complex formation, base substitutions using nonspecific sequence were used to progressively alter the 5′ and 3′ ends of the probe, focusing on small DNA regions sufficient for formation of each individual complex and both complexes.

Comparison Assays—The relative affinity of B. japonicum Fur for Fur box and irs promoter DNA was determined by competition of one of the radiolabeled DNAs with the other unlabeled DNA in EMSA analysis. In the first case, 100 pM 32P-labeled irs promoter DNA was incubated with 50 ng of 5′ end-labeled pBSKIIrr DNA. Competing DNA was added, and DNA sequencing was observed as the result of the competition between Fur box and irs promoter DNA. Fur box DNA, or nonspecific DNA. In the second case, the experiment was carried out in the same way except that the Fur box was radiolabeled.

DNaSe I Footprint Analysis—DNase I footprint analyses examined the DNA region protected by Fur binding. Fur was incubated for 30 min
at 4 °C in a 50-μl volume EMSA binding buffer containing 125 ng of herring sperm DNA, 5 μg of bovine serum albumin, 100 μM MnCl₂, and 1 nM radiolabeled irradi promoter DNA. The DNA was labeled at one end with [³²P]dCTP and the klenow fragment of DNA polymerase. 50 μl of room temperature solution of 5 mM CaCl₂ and 10 mM MgCl₂ was added after 1 min followed by the addition of 0.45 units of RQ1 RNase-free DNase (Promega) in 18 μl of 40 μl Tris-HCl (pH 7.0). The reaction was incubated for 2 min at room temperature. Reactions were stopped by addition of 90 μl of stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS, 100 μg/ml yeast RNA). DNA was extracted with phenol:chloroform (1:1) followed by ethanol precipitation. G + A ladders of the labeled DNA were produced as described (26). Digested probe products were separated on a 15% denaturing polyacrylamide gel containing 7M urea in Tris borate EDTA electrophoresis buffer. Autoradiograms were developed on BioMax film. The DNA probe used for DNase I footprint analysis was radiolabeled at the 3’ end; therefore, the top of the gel represents the 5’ end.

Determination of the Mass of Fur-DNA Complexes—An EMSA-based method for determining the molecular weight of protein-DNA complexes was carried out as described previously (27). EMSA reactions as described above were run on non-denaturing gels with protein standards. Using autoradiography of Coomassie-stained gels, logarithms of relative mobility (Rₚ) for EMSA complexes and native proteins were plotted against acrylamide concentration, showing the relationship of the mobility of each species and gel concentration. The negative slope of Rₚ for each protein standard was plotted against molecular weight to generate a standard curve from which the estimated molecular weight of each EMSA complex was interpolated.

In Vitro Transcription Assays—To directly examine the influence of B. japonicum Fur on transcription from the irradi promoter, in vitro transcription assays were performed on native and altered irradi promoter DNA. Irradi promoter fragments were generated by PCR amplification of irradi sequence in pSKSBIrr using primers 5’-TTTGGATTCGTCGAC-

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**RESULTS**

*B. japonicum* Fur Binds to the *irr* Promoter—Previous work shows that the iron-dependent control of *irr* mRNA expression is aberrant in a *fur* mutant and that *E. coli* extracts that overexpress *B. japonicum* Fur (BjFur) bind to DNA corresponding to the *irr* gene upstream region (22). However, a Fur box-like element was not found in this region. To further study this interaction, we overexpressed and purified recombinant Fur proteins from *E. coli* (EcFur) and *B. japonicum* and examined their DNA binding activities by EMSA (Fig. 2). Recombinant BjFur bound to a 63-bp DNA fragment corresponding to the *irr* upstream region. EcFur, however, did not bind to this DNA; 50 nM BjFur was sufficient to bind all of the DNA in the EMSA assay (Fig. 2), but up to 150 nM EcFur did not bind the *irr* gene promoter (data not shown). The *B. japonicum* fur gene complements an *E. coli* mutant (20). Consistent with that observation, BjFur bound to the Fur box consensus sequence, as did EcFur (Fig. 2). Thus, BjFur has a unique DNA binding activity in addition to that found in the *E. coli* protein. This activity presumably allows BjFur to regulate the *irr* gene despite the absence of a Fur box consensus element in the *irr* promoter.

The affinity of BjFur for the *irr* promoter and Fur box consensus DNA was assessed by measuring bound DNA as a function of BjFur concentration in an EMSA assay (Fig. 3A). The dissociation binding constants (Kₐ) for the two DNA elements were nearly identical and estimated to be 5.1 and 4.4 nM for the *irr* promoter and the Fur box, respectively. The relative affinities of the two DNA elements for BjFur were also assessed by competition EMSA analysis (Fig. 3, B and C). Binding of BjFur to radiolabeled *irr* promoter DNA was competed out by unlabeled Fur box DNA as effectively as by unlabeled *irr* promoter DNA (Fig. 3B). Similar results were obtained when the experiment was repeated using radiolabeled Fur box DNA (Fig. 3C). Thus, the BjFur DNA-binding regions for each element are the same or overlapping. The data indicate that binding of BjFur to the *irr* promoter is similar to its binding to a known Fur target element, and therefore, it is likely to be physiologically relevant. These findings suggest that control of *irr* gene by Fur involves direct binding of Fur to the *irr* promoter.

The BjFur-binding Region of the *irr* Promoter Contains Three Imperfect Direct Repeat Sequences Dissimilar from the Fur Box Consensus—The BjFur-binding region in the *irr* promoter was further defined by DNase footprinting (Fig. 4). The promoter was maximally occupied at 10 nM BjFur, resulting in approximately a 30-bp protected region. EMSA analysis using various double-stranded DNA oligonucleotides representing different regions of the *irr* promoter also delimited the BjFur binding to this region (see below). The DNA binding region was found to extend from ~48 to ~19 with respect to the transcription start site (+1) and contains three imperfect direct repeat hexameric sequences (Fig. 5). Two tandem repeats of TGCTAC are preceded by a TGGGAG separated by 5 bp. The BjFur-binding site on the *irr* promoter has only 7 of 19 matches to the Fur box consensus (on the strand complementary to that shown in Fig. 5). The probability for a random match of 7 out of 19 is very high (0.1 based on calculations from Ref. 28). Furthermore, whereas the Fur box consensus can be interpreted as inverted repeat or direct repeat DNA, the BjFur-binding region of the *irr* promoter can only be interpreted as direct repeat DNA. From this, we conclude that binding of BjFur to DNA does not require the recognition of inverted repeat sequences.

**Binding of Fur to the *irr* Promoter Yields Two Complexes—Titration of *irr* promoter DNA with BjFur revealed two binding
species in EMSA analysis (Fig. 6). At low BjFur concentrations, a high mobility complex (HMC) was predominant, whereas a low mobility complex (LMC) was prevalent at 50 nM protein. The footprinting analysis indicated a concentration-dependent occupancy of the irr promoter by BjFur (Fig. 4), and the two EMSA complexes are consistent with that observation.

EMSA analysis was used to delimit the BjFur site on the irr promoter by first narrowing down the binding region to a 39-bp DNA fragment (Fig. 7). Subsequently, sequences on the left or right side of the 39-bp DNA were substituted with nonspecific sequence to keep the overall length constant. In addition, 5 and 50 nM BjFur were used for each DNA tested, which allows detection of the HMC and LMC, respectively. A fragment containing 29 bp of irr promoter DNA corresponding to the protected region in footprints gave both the HMC and LMC in the EMSA analysis (Fig. 7). Substitutions from the right side that eliminated some or all of the downstream direct repeat (DR3), but which kept DR1 and DR2 intact, resulted in loss of the LMC. However, the HMC was formed with that DNA and was observed at both 5 and 50 nM BjFur (Fig. 7B). Thus, DNA containing those two direct repeats was sufficient for formation of the smaller complex. Substitutions from the left side that eliminated some or all of DR1, leaving DR2 and DR3 intact, abrogated formation of the HMC. Thus, DR2 and DR3 are not equivalent to DR1 and DR2 in terms of binding BjFur, which may be due to differences in sequence or spacing between the repeats. Surprisingly, the LMC was observed with the DR2-DR3 fragment. However, a significant amount of DNA remained unbound with 50 nM protein, indicating that the affin-
The occupancy of BjFur on the mutated DNA was less than that for the wild type (Fig. 7B).

We wanted to determine the occupancy of the irr promoter by BjFur in each of the DNA-protein complexes. The acquisition of DNAs that formed only an HMC or LMC allowed the measurement of the apparent molecular weight of the protein-DNA complexes in native PAGE using gels containing different polyacrylamide concentrations (Fig. 8) (see “Experimental Procedures”). When compared with globular protein standards, the apparent molecular mass of the HMC was 56 kDa, which is close to the 60 kDa predicted for a BjFur dimer bound to a 39-bp DNA fragment. The LMC had an apparent molecular mass of 94 kDa, which is in good agreement with the 98 kDa expected of two BjFur dimers or a tetramer bound to a 39-bp DNA fragment. We suggest that BjFur can occupy the irr promoter as one or two dimers.

**Fig. 6.** BjFur forms high and low mobility complexes with irr promoter DNA. 

***Fig. 7.*** Delimiting regions necessary for HMC and LMC formation by EMSA analysis. As shown in A, subfragments of the original 63-bp irr promoter were tested for their ability to form the HMC and LMC. From a 39-bp fragment that yielded both complexes, *B. japonicum* DNA was replaced by nonspecific DNA from the left and right side (denoted by dashed lines) such that the length was maintained at 39 bp. Each fragment was tested by EMSA for complex formation and scored as shown. nt, nucleotides. B, EMSA using wild type 39-bp DNA and altered DNAs that yielded only the HMC or the LMC. 5 and 50 nM BjFur were used. The solid line denotes *B. japonicum* sequence, and the dashed line depicts nonspecific DNA. The arrows show the positions of the direct repeat sequences. free, no protein.

**Fig. 8.** Determination of the stoichiometry of BjFur bound to DNA in the HMC and LMC. Fur complexed with DNA that formed only the LMC or the HMC was resolved by nondenaturing PAGE along with marker proteins. As shown in A, the log of the relative mobility of the BjFur-DNA complexes and the standards versus polyacrylamide concentration was plotted. As shown in B, the slopes of the curves of the protein standards were plotted as a function of the molecular size (MS), and the molecular sizes of the HMC and LMC were estimated from the curve. The protein standards used were bovine carbonic anhydrase (open triangles, 29 kDa), chicken egg albumin (open circles, 45 kDa), bovine serum albumin monomer (open squares, 66 kDa), and bovine serum albumin dimer (open diamonds, 132 kDa). The HMC and LMC are denoted as closed squares and closed circles, respectively.
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To repress transcription of target genes (12). This model is based largely on work with Fur proteins from the taxonomically related organisms E. coli, P. aeruginosa, and Vibrio sp., but its general applicability is reinforced to some extent by work with the dissimilar organism B. subtilis. With respect to DNA recognition, the recent controversy has not been with the Fur element sequence but rather with the interpretation of the consensus as inverted repeat or direct repeat sequence (18, 19).

In the present study, we demonstrate that B. japonicum Fur recognizes a DNA element within the irr gene promoter that is dissimilar from the Fur box consensus sequence. The affinity of BjFur for the irr promoter was similar to that for the Fur box consensus, and irr mRNA accumulation is controlled by Fur (22). Thus, the DNA-protein interaction defined herein is physiologically relevant. The best alignment of the Fur box consensus to the 30-bp protected region of the irr promoter is only 7 of 19 residues and, accordingly, the element is not recognized by Fur from E. coli. This low match is predicted to occur with very high frequency (-9 x 10^5 sites/strand for a genome of 9 x 10^6 bp), and thus, the Fur box cannot be the basis of this binding site. Genes whose expression is affected in a fur mutant, but lack a Fur box element in their putative promoters, have been identified by microarray and proteomic analyses (29), but in those cases, Fur has not been shown to bind those promoters, and the effect of Fur may be indirect. To our knowledge, this is the first example of a DNA binding activity for a Fur protein that is disparate from recognition of the Fur box consensus. The findings suggest that a generalized model for Fur with respect to its target DNA needs to be expanded or modified. In addition, searches for Fur-regulated genes in genomes based on homology to an upstream Fur box consensus may exclude desired genes in organisms where Fur has not been characterized.

Whereas the Fur box consensus can be interpreted as inverted repeat or direct repeat sequence, the BjFur DNA-binding element in the irr promoter contains only direct repeat sequence. Therefore, a requirement for inverted repeat DNA for recognition by BjFur can be unequivocally ruled out. BjFur binds to both the Fur box consensus and the irr promoter element, and both elements contain three imperfect direct repeat hexamers. The simplest extrapolation from these findings is that BjFur recognizes direct repeat sequences in the Fur box as well as in the irr promoter element.

Although the novel DNA binding activity of BjFur must have a structural basis, there are no obvious differences between the B. japonicum protein and Fur from other organisms. Phylogenetic analysis (ClustalW; (30)) of numerous Fur homologs shows that the proteins cluster according to taxonomy, and the B. japonicum protein is not an outlier (data not shown). It is likely that the structural features of BjFur conferring the novel DNA binding are subtle and may be elucidated by mutational analysis and identification of other Fur proteins with that activity.

Fur proteins are likely to be dimeric in solution (10, 11), and the P. aeruginosa protein was crystallized as a dimer (9). A model for Fur based on the crystal structure of the unbound protein depicts two dimers binding to target DNA. This model accommodates the observation that Fur protects at least 30 bp of DNA in DNase I footprinting experiments, whereas a dimer should protect only ~20 bp (9). In that model, Fur recognizes three hexamers, with the central hexamer region in contact with both dimers. In the present study, BjFur maximally protected 30 bp of the irr promoter at a protein concentration that formed a low mobility complex comprising two dimers or a tetramer. These findings are in agreement with the proposed model based on the P. aeruginosa Fur structure. In addition, a high mobility complex comprising a BjFur dimer was predom-

**DISCUSSION**

Fur homologs are found throughout the eubacterial kingdom. A generalized model for Fur function posits that the protein binds ferrous iron when the metal is available, conferring the ability to recognize and bind to a Fur box DNA element to repress transcription of target genes (12).
in EMSA analysis at low protein concentrations and was the only complex observed at any concentration when DR3 was mutated (Figs. 7 and 9B). Thus, the irr promoter can be occupied by a dimer, and DR1 and DR2 appear to be sufficient for high affinity binding. However, DNA in which DR1 was altered, leaving DR2 an DR3 intact, did not yield a dimer, and therefore, the sequence or spacing of the repeats may be important since DR1 has a different sequence than the other repeats and is separated from DR2 by 5 bp. We suggest that the wild type promoter is occupied by a dimer at low BjFur concentrations and makes contact with DR1 and DR2. At higher BjFur concentrations, a second dimer binds to DR2 and DR3, and perhaps the second dimer is also stabilized by interaction with the first dimer. Thus, titration of DNA with Fur results in loss of the dimer in EMSA (Fig. 6) and additional protection of DNA in footprinting analysis (Fig. 4). Mutation of DR1 abrogates high affinity binding, but there may be sufficient DNA-protein and protein-protein interactions to form the low mobility complex at higher BjFur concentrations.

Fur is presumed to repress gene expression by binding to the target promoter and blocking transcription initiation. However, to our knowledge, the possibility that Fur repression involves the recruitment or exclusion of other regulatory factors has not been addressed. Here, we show that Fur was sufficient to repress transcription from the irr promoter in vitro. Furthermore, transcription from a mutant promoter that allowed only dimer occupancy was repressed by Fur as well, suggesting that a single dimer may be functional as a repressor and that the DNA containing two direct repeats can be a negative regulatory element.

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