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Fur-independent regulation of iron metabolism by Irr in *Bradyrhizobium japonicum*

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Bradyrhizobium japonicum **expresses both Fur and Irr, proteins that mediate iron-dependent regulation of gene expression. Control of *irr* mRNA accumulation by iron was aberrant in a *fur* mutant strain, and Fur repressed an *irr*::*lacZ* promoter fusion in the presence of iron. Furthermore, metal-dependent binding of Fur to an *irr* gene promoter was demonstrated in a region with no significant similarity to the Fur-binding consensus DNA element. These data suggest that the modest control of *irr* transcription by iron is mediated by Fur. However, Irr protein levels were regulated normally by iron in the *fur* strain, indicating that Fur is not required for post-transcriptional control of the *irr* gene. Accordingly, regulation of *hemB*, a haem biosynthesis gene regulated by Irr, was controlled normally by iron in a *fur* strain. In addition, the *hemA* gene was shown to be controlled by Fur, but not by Irr. It was concluded that Fur cannot be the only protein by which *B. japonicum* cells sense and respond to iron, and that Irr may be involved in Fur-independent signal transduction. Furthermore, iron-dependent regulation of haem biosynthesis involves both Irr and Fur.**

Keywords: *Bradyrhizobium japonicum*, iron metabolism, Fur, Irr, haem metabolism

INTRODUCTION

A considerable body of work on the regulation of iron homeostasis in bacteria has focused on Fur, a transcriptional regulator that controls genes in an iron-dependent manner. In the classic view, ferrous iron binds directly to Fur to confer binding to a defined *cis*-acting DNA element for transcriptional repression. Thus, Fur is both an iron sensor and a mediator of iron-dependent regulation. However, this model may not be universally applicable; Fur can bind DNA in the absence of metal (Althaus *et al*., 1999; Bsat & Helmann, 1999) and evidence for Fur as a positive effector or having activity in the absence of metal has been reported (Foster & Hall, 1992; Litwin & Calderwood, 1994).

Very recently, several Fur-like proteins have been identified that are not functional Fur homologues, but instead they are involved in maintenance of zinc homeostasis (Gaballa & Helmann, 1998; Patzer & Hantke, 1998), manganese-dependent response to oxidative stress (Bsat *et al*., 1998) or iron-dependent regulation of haem biosynthesis (Hamza *et al*., 1998). Additional *fur*-like genes have been identified from genome sequencing and from screens for genes involved in pathogenesis (Camilli & Mekalanos, 1995; Wang *et al*., 1996). There now appears to be a family of Fur proteins that are functionally diverse, but are all involved in metal-dependent regulation.

Irr from *Bradyrhizobium japonicum* may be the most divergent of the Fur-like proteins described thus far in that it is active only under metal limitation and contains a single cysteine residue rather than the multiple cysteines found in the other proteins. Moreover, *irr* gene expression is strongly regulated by iron whereas *fur* is essentially constitutive. Iron represses the *irr* gene moderately at the transcriptional level and strongly at protein turnover (Hamza *et al*., 1998; Qi *et al*., 1999). The latter mechanism involves iron-dependent binding of haem to a haem regulatory motif of the Irr protein which is necessary for its degradation (Qi *et al*., 1999). As a result, *irr* mRNA is diminished but detectable under high iron conditions, whereas protein levels are undetectable.

Haem is iron-protoporphyrin and Irr mediates iron control of the haem biosynthetic pathway (Hamza *et al*., 1998). δ-Aminolevulinic acid (ALA) synthase and ALA

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Abbreviation: ALA, δ-aminolevulinic acid.

dehydratase catalyse the first two steps of haem biosynthesis and are encoded by *hemA* and *hemB* respectively. Under iron limitation, Irr negatively regulates haem biosynthesis at *hemB*, and an *irr* strain shows constitutively high levels of *hemB* mRNA and protein. The *hemA* gene is also controlled by iron (Page *et al*., 1994), but a regulator for it has not been defined and is addressed herein. *B. japonicum* is the only organism described thus far that contains a Fur-like protein in addition to bona fide Fur that is involved in iron metabolism (Hamza *et al*., 1999). This led us to ask what the relationship between Fur and Irr may be, and whether Fur is involved in haem biosynthesis. Here we report that Fur is involved in control of *irr* transcription, but that post-transcriptional control by iron is Fur-independent and therefore *B. japonicum* must have at least two iron-dependent regulatory systems. We propose that these sensory mechanisms allow for differential control of the *hemA* and *hemB* genes.

METHODS

Chemicals and reagents. All chemicals were reagent grade and were purchased from Sigma or from J. T. Baker, NJ. Purified noble agar and yeast extract were obtained from Difco. $^{59}FeCl_3$, [α-^{32}P]dNTPs [3000 Ci mmol^{-1} (111 GBq mmol^{-1})] and [γ-^{32}P]ATP [3000 Ci mmol^{-1} (111 GBq mmol^{-1})] were obtained from Dupont-NEN Life Science Products. [α-^{32}P]UTP [800 Ci mmol^{-1} (29·6 GBq mmol^{-1})] used for RNase protection assays was obtained from ICN Biomedicals.

Bacterial strains, plasmids, media and growth. Bacterial strains used in this study are listed in Table 1. *B. japonicum* strain I110 was the parent strain used; strains LODTM5 and GEM4 are *irr* and *fur* mutants, respectively, and were described previously (Hamza *et al*., 1998, 1999) *B. japonicum* strains were routinely grown at 28 °C in GSY media as described previously (Frustaci *et al*., 1991). Cultures for growth of strain GEM4 were supplemented with 100 μg ml^{-1} each of spectinomycin and streptomycin, and LODTM5 was grown in the presence of 100 μg ml^{-1} each of kanamycin and streptomycin. The medium used for culturing cells under iron limitation was a modified GSY medium (0·5 × GSY) in which 0·5 g yeast extract l^{-1} was used instead of 1 g l^{-1} and no exogenous iron source was added. The actual iron concentration of the media was 0·23 μM as determined with a Perkin Elmer model 1100B atomic absorption spectrometer. High-iron media contained 6 μM added $FeCl_3$. Glassware was rinsed extensively with 6 M HCl and then washed with distilled water (Milli-Q PF plus), followed by rinsing with metal-free water (Milli-Q UV plus). *Escherichia coli* strains DH5α, XL-1 Blue or TB1 were used for propagation of plasmids. *E. coli* strains were grown at 37 °C on Luria–Bertani broth or 2 × yeast-tryptone medium with appropriate antibiotics. pMH15*fur* was provided by K. Hantke, University of Tübingen, Germany, and contains the *E. coli fur* gene cloned into pACYC184. pGDIrr-fuse contains a 608 bp *Sma*I/*Bam*HI fragment of the *irr* gene that includes 175 bp upstream of the transcription start site cloned into pGD499 (Ditta *et al*., 1985), resulting in an *irr*::*lacZ* transcriptional fusion. pSKDΔlac contains a deletion from the *Hin*dIII site in the multiple cloning site of pBluescript SK to an unidentified region upstream of the multiple cloning site created by digestion with T4 DNA polymerase. The plasmid does not encode β-galactosidase activity in *E. coli* strains harbouring the omega complementation fragment of β-galactosidase.

β-Galactosidase assay of *E. coli* liquid cultures. Analysis of iron-dependent repression of an *irr*::*lacZ* fusion by BjFur and EcFur in *E. coli* was carried out by measuring β-galactosidase activity in cells grown under high- or low-iron conditions. Cultures of *E. coli* strain DH5α harbouring pGDIrr-fuse and either pSKBJF800 (BjFur), pMH15*fur* (EcFur) or pSKDΔlac (control) were grown in LB media containing either 100 μM $FeCl_3$ or 200 μM α,α-dipyridyl for high- and low-iron conditions, respectively. β-Galactosidase activity was measured in cells as described by Miller (1972). Cells were grown aerobically at 37 °C to late-exponential phase. Cells were spun down and resuspended in 800 μl Z buffer (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 1 mM $MgSO_4.7H_2O$ and 50 mM β-mercaptoethanol, pH 7·0). One hundred microlitres of suspension, corresponding to 1×10^8 cells, was used per reaction. The data are represented in Miller units and each value is a mean of triplicate samples corrected for background. Absorbance of *o*-nitrophenol formed from ONPG by β-galactosidase was recorded at 420 nm and normalized for cell density at OD_{550}.

Gel mobility shift assay. *E. coli* strain H1780 with plasmids pMH15*fur*, pSKBJF800 and pSKSBIrr, was inoculated from an overnight culture into a fresh 250 ml 2 × YT liquid culture with antibiotics, 100 μM $FeCl_3$ and 1 mM IPTG. Cells were harvested at mid-exponential phase, washed twice and resuspended in TNG buffer [50 mM Tris, 50 mM NaCl and 5 % (v/v) glycerol, pH 7·4] and 1 mM PMSF. Supernatants were obtained by passage of the cells twice through a French pressure cell at 900 p.s.i. and clarification at 14000 ***g***. Protein concentration was estimated by the Bradford (1976) protein assay. On average, 11–16 mg total protein ml^{-1} was obtained

Table 1. Bacterial strains used in this study

Strain	Relevant genotype or trait	Source or reference
E. coli		
DH5α	Δ(*lacZYA–argF*)	Hanahan (1983)
H1780	JB1698, but *fur*	Hantke (1987)
B. japonicum		
I110	Small-colony derivative of USDA 3I1b110	Kuykendahl & Elkan (1976)
LO	Spontaneous Nal^r derivative of USDA 122 DES	O'Brian *et al*. (1987)
GEM4	I110, but *fur*::Ω cassette	Hamza *et al*. (1999)
LODTM5	LO, but *irr*::Tn*5*	Hamza *et al*. (1998)

per preparation. For gel mobility shift assays (modified from de Lorenzo *et al.*, 1988), 15 μg crude extract, 2·5 μg poly(dI-dC).poly(dI-dC) (Pharmacia Biotech) and 1×10^5 c.p.m. labelled DNA probe were mixed in 1 × binding buffer [10 mM bis-Tris borate, pH 7·5, 1 mM $MgCl_2$, 40 mM KCl, 5 % glycerol, 0·1 % (v/v) Nonidet P-40 and 1 mM DTT] and incubated on ice for 5 min in a 20 μl reaction volume. A 5 % nondenaturing polyacrylamide gel in electrophoresis buffer (20 mM bis-Tris borate, pH 7·5) was prerun for 15 min at 200 V constant voltage and loaded with 20 μl of the binding reaction mixture without dye. After electrophoresis at 4 °C for 2–3 h at 200 V, the gel was dried and autoradiographed. For assays in the presence of metal, 100 μM $MnCl_2$ was added to the assay mix, electrophoresis buffer and the polyacrylamide gel. Plasmid pSKES3, containing a 300 bp *Sma*I–*Hpa*I fragment (175 bp of upstream sequence from the *irr* transcriptional start site), was used to isolate various deletions of the *irr* upstream sequence for mobility shift experiments. The restriction-enzyme-digested DNA probes were either purified from 4 % GTG Nusieve agarose (FMC BioProducts) or 15 % acrylamide gels and radiolabelled at 30 °C for 1 h using [α-^{32}P]dNTP and the Klenow fragment of DNA polymerase I.

Analysis of ALA dehydratase and Irr protein. The presence of ALA dehydratase or Irr in whole cells or cell extracts was detected by immunoblot analysis of 10 or 15 % SDS-PAGE gels using antibodies raised against the respective protein. Anti-ALA dehydratase and anti-Irr antibodies were prepared previously (Chauhan & O'Brian, 1995; Hamza *et al.*, 1998) Cross-reactive material that bound to the membrane was analysed with peroxidase-conjugated goat anti-rabbit IgG and visualized by chemiluminescence by using the Renaissance kit (DuPont-NEN) according to the manufacturer's instructions. Autoradiograms were quantified using a Bio-Rad model GS-700 imaging densitometer in the transmittance mode and the Molecular Analyst software package, version 1.5. Several exposures of a single blot were analysed to be certain that the data were examined within the linear range of the densitometer.

Isolation of RNA and mRNA analysis. Total RNA was prepared and analysed as described previously (Chauhan & O'Brian, 1997) and quantified by measuring absorption at 260 and 280 nm. Cultured cells were grown to mid-exponential phase and steady-state levels of *irr*, *hemA*, *hemB* or *hemH* mRNA were analysed by the Ribonuclease Protection Assay kit (HybSpeed RPA; Ambion). Antisense RNA probes to the respective genes were synthesized and gel-purified using the T7 MAXIScript In Vitro Transcription kit (Ambion) as recommended by the manufacturer. The bands on autoradiograms of RNA gels were quantified using a Bio-Rad model GS-700 imaging densitometer in the transmittance mode as described above. The transcription start site of the *irr* gene was determined by primer extension analysis as described by Ausubel *et al.* (1994) using RNA isolated from *B. japonicum* cells grown in iron-limited media.

RESULTS

Fur regulates *irr* at the mRNA level

We showed previously that *irr* mRNA levels are iron-regulated (Hamza *et al.*, 1998) and that *B. japonicum* Fur has metal-dependent DNA-binding activity (Hamza *et al.*, 1999). Therefore, the effects of iron on *irr* mRNA expression were examined in the parent strain I110 and the *fur* strain GEM4. As demonstrated previously (Hamza *et al.*, 1998), *irr* mRNA was approximately

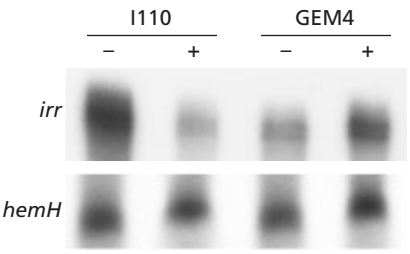

Fig. 1. Effects of a *fur* gene mutation on iron-dependent accumulation of *irr* mRNA. Cells from parent strain I110 or *fur* strain GEM4 were grown in media containing either no (−) or 6 μM (+) added $FeCl_3$. Cells were analysed for *irr* or *hemH* mRNA by RNase protection analysis. Two micrograms of total RNA was analysed per reaction. *hemH* is a control for a gene not regulated by iron.

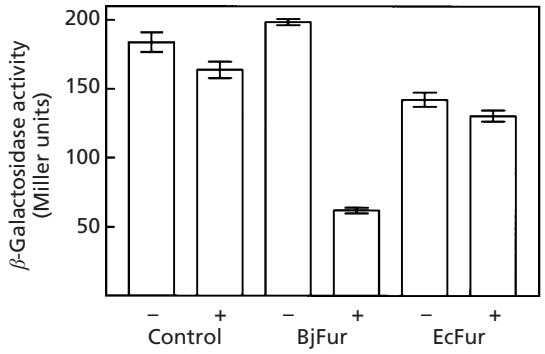

Fig. 2. Dependence of an *irr*::*lacZ* reporter on Fur and iron in *E. coli*. Strain DH5α cells bearing pGDIrr-fuse and either pSKBJF800 (BjFur), pMH15*fur* (EcFur) or pSKΔlac (Control) were grown in LB media containing either 200 μM of the iron chelator α,α-dipyridyl (−) or 100 μM $FeCl_3$ (+). β-Galactosidase activity was measured and expressed in Miller units as described in the text. The data are means of triplicate samples ± SD.

fivefold greater in iron-limited cells of the parent strain I110 compared with those grown in iron-rich media (Fig. 1). However, *irr* mRNA was not down-regulated in iron-replete cells of *fur* strain GEM4 compared with those grown under iron limitation, indicating that normal control of *irr* mRNA by iron was lost in the *fur* strain. By contrast, mRNA levels of *hemH*, a gene unresponsive to iron (Chauhan *et al.*, 1997; Hamza *et al.*, 1998), were not altered in the *fur* strain (Fig. 1). These data indicate that Fur is involved in iron-dependent control of *irr* mRNA accumulation. *irr* mRNA levels were lower in iron-limited cells of the mutant than those of the parent strain, which may indicate an activity for Fur when iron availability is low. If so, the activity would likely be indirect because data below suggest that Fur binds to the *irr* promoter in the presence of metal.

Evidence that Fur directly regulates the *irr* gene

We addressed the effects of Fur on *irr* promoter activity in *E. coli* cells using an *irr*::*lacZ* fusion (Fig. 2) along

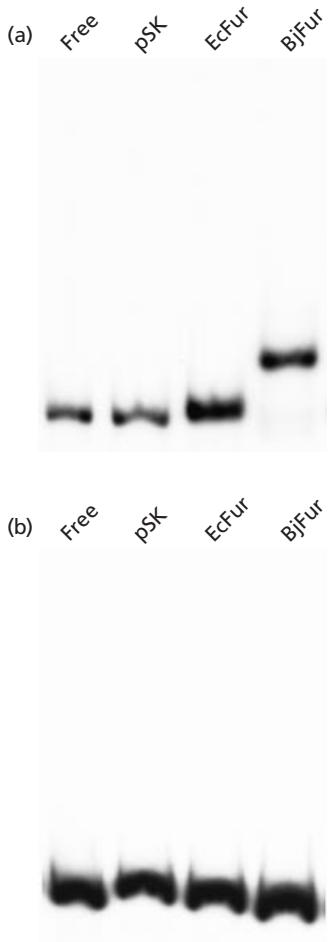

Fig. 3. Metal-dependent binding of BjFur to the *irr* gene upstream region. Gel mobility shift assays were carried out in the presence (a) or absence (b) of 100 μM $MnCl_2$ using a ^{32}P-labelled 191 bp *Sma*I–*Mlu*I fragment including nucleotides −175 to +16 of the *irr* upstream region (see Fig. 5). The protein samples used were from *E. coli fur* strain H1780 containing plasmids pSKBluescript(+) (pSK), pMH15fur (EcFur) or pSKBJF800 (BjFur). The DNA was also run in the absence of protein (Free).

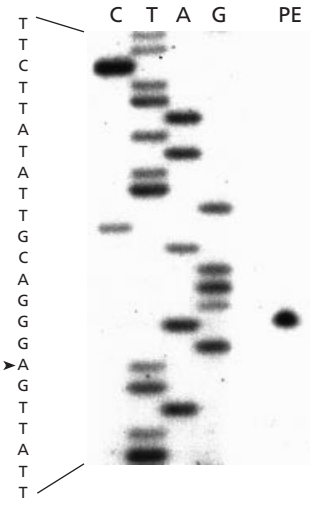

Fig. 4. Primer extension mapping of the 5′ end of *irr* mRNA. Total RNA was isolated from strain I110 cells grown under iron limitation. The oligonucleotide primer used is complementary to nucleotides between +29 and +46 of the *irr* gene. DNA sequencing reactions were carried out with the same oligonucleotide. The arrow indicates the 5′ end of the *irr* transcript.

with plasmid-borne *fur* genes of *B. japonicum* or *E. coli*. The *irr* promoter was active in *E. coli* as discerned by β-galactosidase activity, which was only slightly iron-responsive in control cells lacking a plasmid-borne *fur* gene. However, introduction of the *B. japonicum fur* gene resulted in over a threefold repression of activity in the presence of iron compared to cells grown under iron deficiency, indicating that a *B. japonicum* Fur (BjFur)-responsive element was present in the reporter gene fusion. Surprisingly, reporter activity was not significantly repressed by *E. coli* Fur (EcFur) compared to the control, indicating an activity for BjFur that is absent in EcFur. We observe that *lacZ* reporter fusions are not sensitive tools in *B. japonicum* when mRNA synthesis levels are easily discernible, even in the repressed or uninduced state (S. Chauhan & M. R. O'Brian, unpublished observations). Consistent with this, β-galactosidase activity was repressed only 30–50% by iron in *B. japonicum* harbouring pGDIrr-fuse (data not shown) even though iron affects *irr* mRNA synthesis and steady-state levels three to fivefold (Hamza *et al.*, 1998; Fig. 1). Thus, we did not use the reporter for further experimentation in *B. japonicum* cells.

The effects of Fur on *irr* gene expression were assessed further by gel mobility shift assays using *irr* gene upstream DNA and overexpressed Fur proteins. In addition, the assays were carried out in the presence or absence of Mn^{2+}, which has been shown to substitute for Fe^{2+} as a cofactor of Fur *in vitro* (de Lorenzo *et al.*, 1988). Mn^{2+} is used because Fe^{2+} is readily oxidized to Fe^{3+} in air, which is not functional as a Fur cofactor. A 191 bp *Sma*I–*Mlu*I fragment corresponding to positions −175 to +16 with respect to the *irr* transcription start site was bound by BjFur in the presence of Mn^{2+} but not in its absence (Fig. 3). Collectively, the data in Figs 1–3 indicate that BjFur controls the *irr* gene directly and is a negative regulator in the presence of metal. Recombinant Irr did not bind to its promoter region in either the absence or presence of metal, suggesting that the *irr* gene does not directly regulate its own expression (I. Hamza & M. R. O'Brian, unpublished data). Interestingly, EcFur did not form a complex with the *irr* upstream region in a gel mobility shift assay (Fig. 3), which is consistent with the *irr*::*lacZ* reporter gene data (Fig. 2). These findings indicate that BjFur has a DNA-binding activity not present in EcFur.

BjFur binds to a unique region in the *irr* upstream region

Both BjFur and EcFur bind the Fur box consensus sequence (Hamza *et al.*, 1999) and therefore the inability

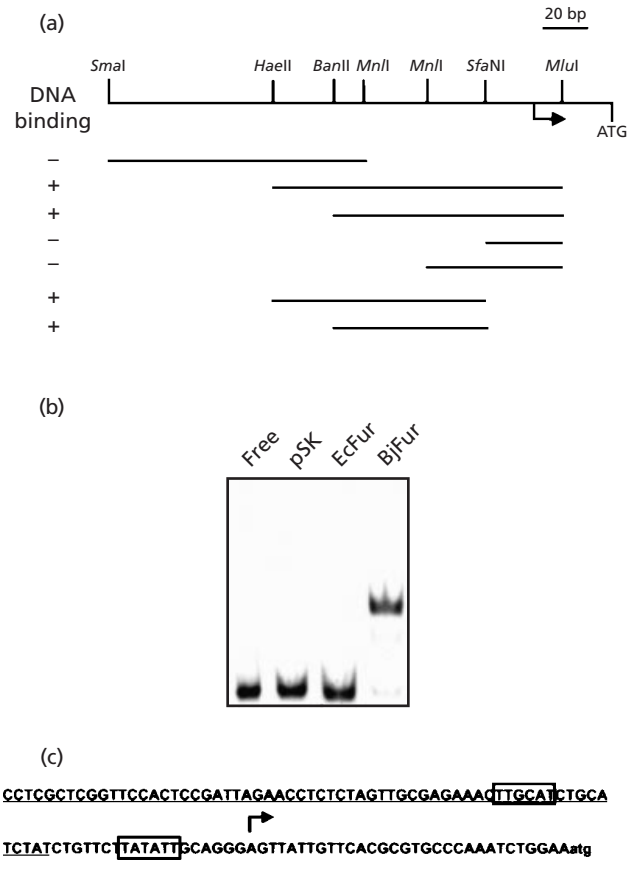

Fig. 5. Localization of the BjFur-binding region upstream of the *irr* gene. (a) Gel mobility shift assays were carried out using ^{32}P-labelled subclones of the *irr* upstream region. '+' denotes that BjFur bound to the DNA element in the presence of metal as indicated by a mobility shift. '–' denotes no mobility shift. The bent arrow denotes the transcription start site of *irr*. 'ATG' denotes the translation initiation codon. (b) Mobility shift assays using the 63 bp *Ban*II–*Sfa*NI DNA fragment containing nucleotides –82 to –20 and extracts containing BjFur or EcFur, and controls containing either extracts with no Fur protein (pSK) or no protein (Free). (c) Nucleotide sequence of the 63 bp *Ban*II–*Sfa*NI fragment that binds to BjFur (underlined) and adjacent DNA (GenBank accession number AF052295). The bent arrow denotes the transcription start site. The boxed DNA shows sequences with positional and sequence similarity to the –35 and –10 promoter region genes from *B. japonicum* and other bacteria. The lower case 'atg' denotes the translation initiation codon.

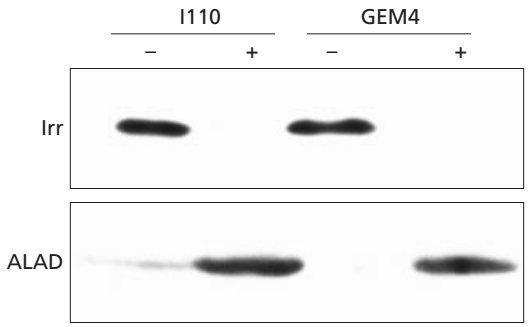

Fig. 6. Iron-dependent accumulation of Irr and ALA dehydratase (ALAD) in wild-type parent strain I110 and *fur* strain GEM4. Cells were grown in media containing either no (–) or 6 μM (+) added $FeCl_3$. Irr and ALA dehydratase proteins were detected in whole cell extracts by Western blot analysis using anti-Irr or anti-ALA dehydratase antibodies. Twenty-five micrograms of protein extract was loaded per lane.

of EcFur to bind to the *irr* upstream region suggested a BjFur-binding site that is dissimilar from the Fur box consensus. Indeed, examination of the *irr* gene upstream region revealed no element with significant similarity to the Fur box consensus sequence. To further localize the BjFur-binding region, the transcription start site of *irr* was determined by primer extension analysis (Fig. 4) and restriction fragment subclones of the 191 bp *Sma*I–*Mlu*I fragment upstream region were analysed by a gel mobility shift assay (Fig. 5). The smallest subclone tested that bound BjFur was a 63 bp *Ban*II–*Sfa*NI DNA fragment corresponding to nucleotides –82 to –20 of the *irr* promoter region (Fig. 5c). This fragment contains no substantial similarity to the Fur consensus sequence (best alignment is 7 of 19 nucleotides) and was not bound by EcFur (Fig. 5b). Both the BjFur and EcFur protein preparations used in the analysis bound to the Fur box consensus DNA (data not shown). We suggest that BjFur has a unique DNA-binding activity not found in EcFur in addition to its Fur-box-binding activity.

Control of Irr protein level by iron is independent of Fur. Irr protein turns over rapidly in the presence of iron and is undetectable in cells grown in iron-replete media. (Hamza *et al*., 1998; Qi *et al*., 1999). To further address the post-transcriptional control of the *irr* gene, we analysed the effects of iron on Irr protein accumulation by immunoblot analysis in parent strain I110 and *fur* mutant strain GEM4 (Fig. 6) Interestingly, the *fur* strain showed an iron-dependent Irr expression pattern similar to the wild-type (Fig. 6) despite the aberration in message accumulation (Fig. 1). These findings confirm the conclusion that post-transcriptional control is the predominant influence on expression of the *irr* gene. Furthermore, the normal iron-dependent accumulation of Irr in the *fur* strain indicates that post-transcriptional control of *irr* by iron is not Fur-dependent, and therefore *B. japonicum* must have another mechanism for sensing and responding to iron.

Fur does not directly control expression of the haem biosynthesis enzyme ALA dehydratase

The haem biosynthesis enzyme ALA dehydratase, encoded by *hemB*, is iron-regulated in *B. japonicum*, and Irr mediates negative control of that gene in response to iron limitation (Chauhan *et al*., 1997; Hamza *et al*., 1998). Consequently, *hemB* mRNA and protein levels are repressed over 100-fold in iron-limited cells of the wild-type, but remain high in an *irr* mutant strain. To determine whether Fur was involved in iron-dependent ALA dehydratase expression, immunoblot analysis was carried out using total protein from cells of parent strain

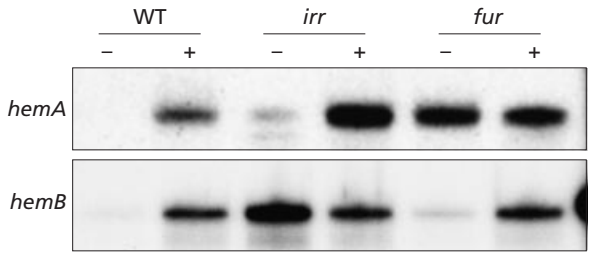

Fig. 7. Effects of a *fur* or *irr* gene mutation on iron-dependent accumulation of *hemA* and *hemB* mRNA. Cells from wild-type (WT) parent strain I110, *irr* strain LODTM5 or *fur* strain GEM4 were grown in media containing either no (−) or 6 μM (+) added $FeCl_3$. Cells were analysed for *hemA or hemB* mRNA by RNase protection analysis. Two micrograms of total RNA was analysed per reaction.

I110 and *fur* strain GEM4 grown in low- or high-iron media (Fig. 6). ALA dehydratase accumulated to high levels in cells of both the wild-type and mutant grown in iron-replete media and was very low in iron-limited cells; thus regulation was essentially normal in the *fur* strain. These findings indicate that Fur is not required for full expression of *hemB*. The normal regulated expression of ALA dehydratase in the *fur* strain was likely due to the fact that Irr protein, which controls ALA dehydratase, remained iron-dependent in the mutant (Fig. 6).

Fur and Irr mediate iron-dependent control of *hemA* and *hemB*, respectively

The *B. japonicum hemA* and *hemB* genes are both regulated by iron (Hamza *et al*., 1998; Page *et al*., 1994), but only a regulator of *hemB* has been described previously (Hamza *et al*., 1998). The current work indicates that Fur and Irr can mediate cellular responses to iron independently, and a region with weak similarity to the Fur box sequence was found in the *hemA* promoter by Page *et al*. (1994). Therefore, regulation of *hemA* and *hemB* transcripts by iron was examined in *irr* and *fur* strains by RNase protection analysis (Fig. 7). Transcripts of *hemA* and *hemB* were low in cells grown under iron deprivation and high under iron-replete conditions in parent strain I110. As shown previously (Hamza *et al*., 1998), control of *hemB* by iron was lost in the *irr* strain, resulting in elevated transcript even under iron deprivation. *hemB* mRNA levels were controlled normally in *fur* strain GEM4, which is in agreement with the observed protein levels (Fig. 6) and shows that *hemB* is not regulated by Fur. By contrast, *hemA* mRNA levels were unresponsive to iron in the *fur* strain, with the maintenance of high levels under both high- and low-iron conditions (Fig. 7), and thus Fur is involved in iron-dependent expression of *hemA*. However, *hemA* was regulated normally in strain LODTM5, indicating that Irr does not control that gene. These data strongly indicate different mechanisms for the regulation of *hemA* and *hemB* by iron, and show that Irr and Fur can act independently of each other.

DISCUSSION

In the present study, we demonstrate that Fur controls the *irr* gene at the mRNA level in a metal-dependent manner and that it binds to the *irr* promoter in a region dissimilar to the Fur box consensus. Screens for DNA elements from *Salmonella typhimurium* (Tsolis *et al*., 1995) or *Pseudomonas aeruginosa* (Ochsner & Vasil, 1996) that bind to Fur by an *in vitro* selection procedure or an *in vivo* titration assay, respectively, did not reveal elements with significant deviation from the iron box consensus sequence. However, *fur* genes have been identified in numerous bacteria in which the respective proteins have not been extensively characterized, and thus new properties for Fur may be found in those organisms. The *fur* gene of *Rhizobium leguminosarum* has been cloned (De Luca *et al*., 1998) and it would be interesting to assess the DNA-binding properties of that protein. The significance of the transcriptional control of *irr* by iron is not obvious since the accumulation of Irr was essentially normal in the *fur* strain under the conditions examined. It is possible that an increase in the *irr* mRNA level upon a decrease in the cellular iron concentration would facilitate Irr synthesis, but the steady-state level of protein is more greatly affected by post-transcriptional control.

The iron responsiveness of Irr accumulation in the *fur* strain shows that *B. japonicum* must have a mechanism for sensing and responding to the cellular iron level in addition to Fur. Haem mediates iron-dependent degradation of Irr (Qi *et al*., 1999) and thus haem may be the form of iron to which Irr responds. Also, iron may in some way activate the protein(s) that degrades Irr. In either event, the findings indicate that aspects of iron metabolism need not be under the control of the Fur regulon in *B. japonicum*. Furthermore, Irr is a regulatory protein and therefore ALA dehydratase may be one of numerous iron-dependent cellular processes that do not require Fur. Two-dimensional PAGE analysis of proteins from *S. typhimurium* and *Vibrio cholerae* wild-type and *fur* strains revealed proteins regulated by iron, but which are Fur-independent (Foster & Hall, 1992; Litwin & Calderwood, 1994). Thus, alternative mechanisms of regulating iron-dependent processes may be generally applicable in bacteria that express Fur. The need for multiple iron regulatory proteins will likely be clearer when the form of iron to which Irr responds is known, and when the respective regulons are more completely characterized.

The presence of two systems for mediating iron control of gene expression was underscored further by examination of the haem biosynthesis genes *hemA* and *hemB*. Both genes are regulated by iron, but analysis of mutants shows that *hemA* is only affected by Fur whereas *hemB* is controlled only by Irr under the conditions examined. Furthermore, the aberration in *hemA* expression in the

fur strain was found under low iron, indicating a direct or indirect role for Fur under those conditions. Although many studies, including this one, demonstrate activity in the presence of metal, other studies show that Fur can bind to DNA in the absence of metal (Althaus *et al*., 1999; Bsat & Helmann, 1999), and that it can have a physiological function under iron limitation (Foster & Hall, 1992). Finally, ALA synthase and ALA dehydratase, the respective *hemA* and *hemB* products, are part of a pathway committed to the same products; thus it is intriguing that they are regulated by iron via different systems. A role for ALA dehydratase in addition to haem synthesis has been reported in animals (Guo *et al*., 1994), but no similar function has been described in bacteria. The substrates for ALA synthase, glycine and succinyl coenzyme A, are involved in numerous cellular processes whereas ALA metabolized by ALA dehydratase is a committed intermediate. It is possible that ALA synthase and ALA dehydratase need to be coordinated with different enzymes even though they are part of the same pathway. Furthermore, *B. japonicum* can acquire ALA from its soybean host in symbiosis (Chauhan & O'Brian, 1993; Sangwan & O'Brian, 1991); thus the need for the two enzymes may be different in that context.

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