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# Metal-specific control of gene expression mediated by *Bradyrhizobium japonicum* Mur and *Escherichia coli* Fur is determined by the cellular context

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## Abstract

*Bradyrhizobium japonicum* Mur and *Escherichia coli* Fur are manganese- and iron-responsive transcriptional regulators, respectively, that belong to the same protein family. Here, we show that neither Mur nor Fur discriminate between $Fe^{2+}$ and $Mn^{2+}$ *in vitro*, nor is there a metal preference for conferral of DNA-binding activity on the purified proteins. When expressed in *E. coli*, *B. japonicum* Mur responded to iron, but not manganese, as determined by *in vivo* promoter occupancy and transcriptional repression activity. Moreover, *E. coli* Fur activity was manganese-dependent in *B. japonicum*. Total and chelatable iron levels were higher in *E. coli* than in *B. japonicum* under identical growth conditions, and Mur responded to iron in a *B. japonicum* iron export mutant that accumulated high levels of the metal. However, elevated manganese content in *E. coli* did not confer activity on Fur or Mur, suggesting a regulatory pool of manganese in *B. japonicum* that is absent in *E. coli*. We conclude that the metal selectivity of Mur and Fur depends on the cellular context in which they function, not on intrinsic properties of the proteins. Also, the novel iron sensing mechanism found in the rhizobia may be an evolutionary adaptation to the cellular manganese status.

## INTRODUCTION

Approximately 40% of all proteins require a bound metal to function (Andreini *et al.*, 2008). Because metals can be both scarce and toxic, cells must regulate gene expression to control metal-dependent processes and maintain homeostasis. Members of the Fur family of bacterial transcriptional regulators sense and respond to the cellular status of a specific divalent metal to regulate genes under their control (Bsat *et al.*, 1998, Gaballa and Helmann, 1998, Hamza *et al.*, 1998, Patzer and Hantke, 1998, Ahn *et al.*, 2006, Diaz-Mireles *et al.*, 2004, Chao *et al.*, 2004, Platero *et al.*, 2004).

Fur is the first and perhaps best studied member of this family. Fur binding to $Fe^{2+}$ confers activity on the protein, resulting in the recognition and binding to cis-acting elements within the promoters of genes within its regulon. The Fur family regulators Zur, Nur and Mur sense and respond to $Zn^{2+}$, $Ni^{2+}$ and $Mn^{2+}$, respectively, and appear to be mechanistically similar

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to Fur, except that they recognize different metals (Shin *et al.*, 2011, Ahn *et al.*, 2006, Diaz-Mireles *et al.*, 2004, Platero *et al.*, 2007, Hohle and O'Brian, 2010). The PerR protein found in *Bacillus subtilis* and some other gram-positive bacteria is an $H_2O_2$-responsive regulator in which bound $Fe^{2+}$ catalyzes histidine oxidation to inactivate the regulator and derepress target genes (Lee and Helmann, 2006).

Fur is the global iron-dependent transcriptional regulator in *E. coli*, *Bacillus subtilis* and many other bacteria, and its wide phylogenetic distribution suggests that it is evolutionarily ancient. Fur has been described predominantly as a repressor, but examples of Fur-dependent activation have been reported (Yu and Genco, 2012). High affinity transport genes are among those repressed by Fur, which are expressed under low iron conditions to scavenge available iron from the environment.

*Bradyrhizobium japonicum* lives as a free-living organism or as the symbiont of soybean, where it converts atmospheric nitrogen to ammonia within plant cells of root nodules. *B. japonicum* belongs to the α-Proteobacteria, a diverse taxonomic group that that includes intracellular pathogens, symbionts, photosynthetic organisms, bacteria that degrade environmental pollutants, and the abundant marine bacterium *Pelagibacter ubique* (Rappe *et al.*, 2002). *B. japonicum* and many related organisms do not use Fur for global iron-dependent transcriptional control. Instead, the Irr protein, also a Fur family member, positively and negatively regulates iron stimulon genes (O'Brian, 2015). Unlike other Fur family proteins, Irr does not directly bind its regulatory metal, but rather it senses and responds to an iron-dependent process, namely the synthesis of heme (Qi and O'Brian, 2002). Irr interacts directly with ferrochelatase, the enzyme that catalyzes the insertion of ferrous iron into protoporphyrin in the final step of heme biosynthesis. Irr is degraded (Qi and O'Brian, 2002) or inactivated (Singleton *et al.*, 2010) by heme when iron is sufficient, but is active under iron limitation.

The Mur protein from *B. japonicum* and other rhizobia share about 50% similarity to *E. coli* Fur, and has been co-opted to respond to manganese rather than iron (Chao *et al.*, 2004, Diaz-Mireles *et al.*, 2004, Platero *et al.*, 2004, Hohle and O'Brian, 2009, Menscher *et al.*, 2012). *Magnetospririllum gryphiswaldense* and *Caulobacter crescentus* are α-Proteobacteria, but are more distantly related to the rhizobia, and their Fur homologs are iron-dependent regulators (da Silva Neto *et al.*, 2009, Deng *et al.*, 2015). The metal-binding residues of *M. gryphiswaldense* Fur have been elucidated by X-ray crystallography (Deng *et al.*, 2015), and they are conserved in *B. japonicum* Mur as well as in Fur proteins from *E. coli*, *Pseudomonas aeruginosa* and other organisms (Fig. S1). Thus, the basis of metal specificities of Mur and Fur proteins in vivo are not readily apparent from amino acid sequences.

The function of Mur as a manganese-responsive regulator is consistent with observations indicating that *B. japonicum* is more reliant on manganese under non-stressed conditions than *E. coli* and perhaps other organisms as well. A *B. japonicum mntH* mutant is almost completely defective in high-affinity $Mn^{2+}$-uptake, and has a severe growth defect under normal growth conditions (Hohle and O'Brian, 2009). Further, *B. japonicum* employs an outer-membrane channel specific for $Mn^{2+}$ (Hohle *et al.*, 2011). Lastly, *B. japonicum* has a

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single pyruvate kinase, PykM, that uses $Mn^{2+}$ rather than $Mg^{2+}$ (Hohle and O'Brian, 2012). The reliance on manganese is likely true for the rhizobia in general as judged by phenotypes of manganese transport mutants within that group (Anderson *et al.*, 2009, Davies and Walker, 2007, Chao *et al.*, 2004).

In the present work, we show that the metal selectivity of *B. japonicum* Mur and *E. coli* Fur are dependent on the cellular context in which they function, and has implications into the evolution of the unusual iron sensing mechanism in the rhizobia that is mediated by Irr.

## RESULTS

### B. japonicum *Mur and* E. coli *Fur have similar metal-binding properties* in vitro

*B. japonicum* Mur and *E. coli* Fur share 49% similarity at the amino acid level, and respond to manganese and iron, respectively, *in vivo*. We wanted to determine if the metal responsiveness by *B. japonicum* Mur *in vivo* is due to differences in affinities between $Mn^{2+}$ and $Fe^{2+}$. Dissociation binding constants ($K_d$) of Mur for $Mn^{2+}$ and mole ratios (N) were determined using isothermal titration calorimetry (ITC) (Fig 1). Each monomer of the Mur dimer bound one molecule of $Mn^{2+}$ with a $K_d$ value of 2.04 μM (Fig 1A,E). Furthermore, each Mur monomer bound one molecule of $Fe^{2+}$, with a $K_d$ value of 1.53 μM, similar to that seen with $Mn^{2+}$ (Fig 1B,E). *Rhizobium leguminosarum* Mur was also reported to bind both metals similarly (Bellini and Hemmings, 2006). Because Mur bound both metals similarly, we wanted to determine if *E. coli* Fur showed differences in metal binding. Fur bound 1 $Mn^{2+}$ or $Fe^{2+}$ ion per monomer with similar $K_d$ values of 1.15 μM and 1.25 μM, respectively (Fig 1C, D, and E). We conclude that Mur and Fur each bind $Mn^{2+}$ and $Fe^{2+}$ *in vitro* with similar affinities, which cannot explain metal selectivity *in vivo*.

### Mur and Fur bind DNA tightly irrespective of the regulatory metal bound

We sought to determine whether the affinity of $Mn^{2+}$-bound Mur for DNA differed from that of $Fe^{2+}$-bound Mur *in vitro*. Electrophoretic mobility shift assay (EMSA) reactions were carried out by titrating Mur in reactions with a 39 bp fragment containing the Mur binding site found within the *mnoP* gene promoter (Hohle *et al.*, 2011). Mur bound DNA with a $K_d$ of 33.1 nM in the absence of metal (Fig 2A,D). In the presence of $Mn^{2+}$, Mur bound the *mnoP* DNA with about 100-fold greater affinity, with a $K_d$ value of 0.31 nM (Fig 2A,D). A similar $K_d$ value of 0.19 nM was observed in the presence of $Fe^{2+}$ (Fig 2A,D), showing that $Mn^{2+}$ does not confer greater DNA binding on Mur compared with $Fe^{2+}$. Similar experiments were carried out with *E. coli* Fur, using a 39 bp fragment of DNA containing the Fur box located within the *E. coli fiu* gene promoter. Binding of Fur was undetectable in the absence of metal (Fig 2B,D). Fur bound the *fiu* DNA with a $K_d$ value of 0.74 nM when bound by $Fe^{2+}$ (Fig 2B,D). When $Fe^{2+}$ was replaced with $Mn^{2+}$, the affinity was modestly greater with a $K_d$ value of 0.39 nM (Fig 2B,D). Thus, $Fe^{2+}$ does not confer greater DNA-binding activity on Fur compared with $Mn^{2+}$.

Mur is capable of binding the Fur box within the *E. coli fiu* gene promoter (Friedman and O'Brian, 2003), providing a control for the target DNA. Mur bound the *fiu* promoter with a subnanomolar $K_d$ value in the presence of either metal (0.20 and 0.71 nM for $Mn^{2+}$ and $Fe^{2+}$

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respectively). The data confirm that DNA-binding activities of Mur or Fur require a bound metal, but show that $Mn^{2+}$and $Fe^{2+}$ confer similar activities on each regulator. Apo-Mur bound *mnoP* promoter DNA with greater affinity than it bound the *E. coli fiu* DNA (Fig. 2A, C). However, the *mnoP* promoter is unbound by Mur in *B. japonicum* cells grown in low manganese medium (Hohle *et al.*, 2011), and therefore the weak binding activity of apo-Mur is unlikely to be physiologically relevant.

### *Mur is Mn-responsive in* B. japonicum, *but Fe-dependent in* E. coli

Mur occupies the promoter region of *mnoP* in a manganese-responsive manner *in vivo*, as determined by crosslinking/co-immunoprecipitation experiments using anti-Mur antibodies to precipitate bound DNA (Hohle *et al.*, 2011) (Fig. 3A). Correspondingly, quantitative real time PCR (qPCR) showed that the *mnoP* gene transcript was low in *B. japonicum* cells when grown in 20 μM $MnCl_2$ compared to when grown in low manganese medium (Fig. 3D). Mur occupancy and *mnoP* expression were independent of the iron status (Fig 3A,D).

To assess whether the selectivity of Mur was dependent on cellular environment, we expressed *B. japonicum mur* in *E. coli* by replacing the *fur* ORF with that of *mur*. The *E. coli* cells were grown in the same media as was *B. japonicum*. We confirmed that the *mur* gene was expressed in *E. coli* by Western blot analysis (Fig. S2). In *E. coli*, Mur occupied the promoter region of the *fiu* gene when grown in the presence of iron (Fig. 3B), but not in its absence. Moreover, occupancy of the *fiu* promoter by Mur was independent of the manganese status even though the presence of protein was confirmed under that growth condition (Fig. S2). Consistent with the promoter occupancy, Mur-responsive expression of the *fiu* gene was dependent on iron, not manganese (Fig. 3B and E). The *fiu* gene was derepressed under all metal conditions in a *fur* mutant, confirming that the regulation observed in the $mur^+$ *E. coli* strain was Mur dependent (Fig. S3). Thus, Mur is an iron-responsive transcription factor in *E. coli* cells

To determine if the iron-dependent activity of Mur observed in *E. coli* was influenced by the target gene, we introduced the *B. japonicum mnoP* gene in *E. coli* expressed from its own promoter containing the Mur binding site. The promoter region of *mnoP* in *E. coli* was occupied by Mur, and gene transcription was repressed, when iron was added to the growth medium, independent of the manganese concentration (Fig. 3C and F). Metal dependent gene expression was lost in an *E. coli fur* mutant (Fig. S3). Collectively, the findings suggest that the metal selectivity of Mur depends on the cellular context in which it functions, not on intrinsic properties of the protein.

### *Fur is an iron-responsive regulator in* E. coli, *but responds to manganese in* B. japonicum

Because Mur responded to iron instead of manganese when expressed in *E. coli*, we sought to determine the metal specificity of *E. coli* Fur in *B. japonicum*. In *E. coli*, Fur occupied the *fiu* promoter when iron was present in the growth medium (Fig. 4A). Furthermore, the gene transcript of *fiu* was significantly reduced when grown in 20 μM $FeCl_3$ compared to when it is grown in the absence of iron (Fig. 4D), corresponding to iron-dependent repression in *E. coli*. The *E. coli fur* gene was expressed in *B. japonicum* by replacing the ORF of the *mur*

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gene with that of *fur* so that transcription was initiated from the *mur* promoter. Expression of *fur* under all growth conditions examined was confirmed by Western blot analysis using anti-Fur antibodies (Fig. S2). Fur occupied the *mnoP* promoter in *B. japonicum* when manganese was added to the growth medium (Fig. 4B), resulting in low *mnoP* transcript under that growth condition (Fig. 4E). The addition of 20 μM $FeCl_3$ to the growth medium did not confer activity on Fur in *B. japonicum* as determined by low occupancy of the *mnoP* promoter and derepression of the *mnoP* gene (Fig. 4B, E). This iron concentration was sufficient for Fur activity in *E. coli*.

We wanted to determine whether the manganese-dependent activity of Fur in *B. japonicum* was due to the target DNA binding site. Because the *fiu* promoter was silent in *B. japonicum*, we constructed a Fur-responsive promoter in which the Mur binding site of the *B. japonicum mntH* promoter was replaced with the Fur box of the *E. coli fiu* gene. This chimeric promoter was placed upstream of the *fiu* open reading frame on a plasmid and introduced into *B. japonicum* cells. Fur occupied the *mntH*/*fiu* promoter in a manganese dependent manner (Fig. 4C). Moreover, *fiu* gene transcript expressed from the modified promoter was repressed in cells grown with 20 μM $MnCl_2$ compared to when no exogenous manganese was added (Fig. 4F). Fur did not occupy the *fiu* promoter region *in vivo* in cells grown in low manganese medium in the presence of 20 μM $FeCl_3$ (Fig. 4C). Metal-dependent repression of *mnoP* and *fiu* was lost in a *B. japonicum mur* mutant (Fig. S3) confirming that manganese-mediated expression of the $fur^+$ *B. japonicum* strain depended on Fur. The findings suggest that metal selectivity of Fur depends on the cellular context in which it functions, not on intrinsic properties of the protein.

### *Mur responds to very high levels of iron* in vivo *in* B. japonicum

A *B. japonicum* mutant defective in the iron export gene *mbfA* was shown to accumulate higher intracellular levels than the wild type (Sankari and O'Brian, 2014). We wanted to determine if high concentrations of iron are capable of causing Mur to respond to iron in *B. japonicum*. Mur occupancy of the *mnoP* promoter was examined in *B. japonicum* wild type and *mbfA* cells grown under low manganese conditions with 0, 20, or 100 μM $FeCl_3$ added to the growth medium. Mur occupancy of the *mnoP* promoter was low in both the wild type and *mbfA* strains grown under low iron (Fig 5A). Interestingly, Mur occupied the *mnoP* promoter in the *mbfA* mutant grown in 20 μM $FeCl_3$ (Fig 5A), but not in the wild type. However, Mur occupied the *mnoP* promoter in the wild type strain when grown in 100 μM $FeCl_3$ (Fig 5A). *mnoP* gene transcripts were low under conditions where Mur occupied the promoter region (Fig. 5B). This suggests that high intracellular concentrations of iron can promote Mur binding *in vivo*. Because Mur normally regulates the manganese transport genes *mntH* and *mnoP*, we assume that the high iron levels that confer Mur activity represent a dysregulated state.

Because Mur responded to high iron in *B. japonicum*, we wanted to determine if Fur responds to high concentrations of manganese in *E. coli*. Cells were grown under low iron conditions with 0, 20, or 100 μM $MnCl_2$ added to the growth medium. Fur did not occupy the *fiu* promoter *in vivo* or repress transcript levels at any manganese concentration tested

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(Fig. 6A), whereas high occupancy and *fiu* repression were observed with the 20 μM $FeCl_3$ control (Fig. 6A, B). Thus, manganese-dependent Fur activity in *E. coli* was not achieved.

## Intracellular levels of iron correlate with iron-responsiveness of Fur and Mur

Both Fur and Mur are iron-dependent in *E. coli*, but respond to iron in *B. japonicum* only when grown in very high iron media or when the iron exporter gene *mbfA* is deleted. Therefore, we wanted to address whether *E. coli* accumulates more iron than *B. japonicum* under the same growth conditions. We measured the cellular iron content in *B. japonicum* and *E. coli* grown in medium containing 0, 20 or 100 μM $FeCl_3$. When no manganese was added to the medium, *E. coli* accumulated more iron than was observed in *B. japonicum* at the same iron growth condition (Table 1). *E. coli* contained 214 nmol Fe/mg protein when grown in 20 μM Fe, which was over 4-fold more iron than found in *B. japonicum*. Under that condition, Fur and Mur are active in *E. coli*, but not *B. japonicum*. The *B. japonicum* iron content could be increased either by mutation of the *mbfA* gene (Table 2) (Sankari and O'Brian, 2014) or by growing in high iron media (100 μM Fe), both of which resulted in Mur activity in those cells (Fig 5A,B). Thus, activity correlated with the cellular iron content.

We also measured the cellular iron content as described above, except that 20 μM or 100 μM $MnCl_2$ were also included in the growth medium (Table 1). We found that manganese did not affect the cellular iron content in *B. japonicum* or *E. coli*. Therefore, we can rule out that the observed manganese-responsiveness of Mur or Fur in *B. japonicum* is an indirect consequence of altering the iron content. In addition, we measured the iron content in *mur*$^+$ *E. coli* cells and *fur*$^+$ *B. japonicum* cells, and found them to be similar to their respective wild type cells under the same iron and manganese regimens (Table S1 and S2). Therefore, the observed metal-responsive activities of Mur or Fur in their heterologous hosts cannot be explained by changes in the iron content caused by their expression.

## The chelatable iron pool correlates with Mur and Fur activity in B. japonicum *and* E. coli

Most iron within cells is tightly associated with proteins, and therefore the regulatory iron that can be sensed by Fur (or Mur) is likely only a portion of the total iron content. We analyzed iron that could be chelated by the iron binding compound desferrioxamine as described previously (Woodmansee and Imlay, 2002). *B. japonicum* or *E. coli* cells were treated with the chelator, and bound iron was analyzed by EPR. No measureable signal was obtained for either bacterium when grown in medium with no added iron (Fig 7). When grown in 20 μM $FeCl_3$, *E. coli* contained 16.6 nmol/mg protein of chelated iron, whereas *B. japonicum* contained only 6.5 nmol/mg protein (Fig 7). Under these conditions, Fur and Mur were active only in *E. coli* cells. Interestingly, the chelatable iron pool of *B. japonicum* grown in 100 μM $FeCl_3$- was 18.2 nmol/mg protein (Fig. 7A,C), similar to the value obtained for *E. coli* when grown in 20 μM $FeCl_3$- (Fig. 7B,C). This corresponds to iron-responsive Mur activity seen in *B. japonicum* under these growth conditions (Fig. 5A,B). The findings show that chelatable iron is higher in *E. coli* than in *B. japonicum* when grown under identical conditions, and which correlates with iron-responsive gene expression.

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## Manganese-dependent activities of Mur and Fur do not correlate with total cellular manganese content

Mur and Fur are manganese-dependent regulators in *B. japonicum*, but not in *E. coli*. We measured the cellular manganese content in each cell type grown in 0, 20 or 100 μM $MnCl_2$, with no iron added to the medium (Table 1). The manganese content was higher in *B. japonicum* than in *E. coli* when cells were grown in manganese-limited media, but neither Fur nor Mur showed manganese-dependent activity in either cell type (Figs. 3 and 4). Previous work shows that the high affinity manganese transporter gene *mntH* is expressed in *B. japonicum* (Hohle and O'Brian, 2009), but not in *E. coli* (Anjem *et al.*, 2009) when grown in manganese-limited media, which is consistent with greater manganese content in *B. japonicum*. The manganese content in *B. japonicum* was 8.6 nmol Mn/mg protein in cells grown in 20 μM $MnCl_2$ with no iron added (Table 1), which was about twice as high as that found in *E. coli*. However, *E. coli* cells accumulated 68.9 nmol Mn/mg protein when grown in 100 μM $MnCl_2$, much higher than the 13.3 nmol Mn/mg protein found in *B. japonicum*. However, neither Fur nor Mur were active in *E. coli* under that growth condition (Figs 3B,C,E,F and 4A,D) despite having over 8-fold greater manganese than *B. japonicum* grown in 20 μM $MnCl_2$, where those regulators respond to manganese (Figs. 3A,D and 4B,C,E,F). These observations show that manganese-dependent activities of Mur and Fur do not correlate with the cellular manganese content. The findings implicate a regulatory pool of manganese in *B. japonicum* that is perceived by Mur or Fur that is absent in *E. coli*.

We measured the manganese content in *mur*$^+$ *E. coli* cells and *fur*$^+$ *B. japonicum* cells, and found them to be similar to the respective wild type under the same iron and manganese regimens (Table S1 and S2). Therefore, the observed metal-responsive activities of Mur or Fur in their heterologous hosts cannot be explained by changes in the manganese content caused by their expression.

## *Iron increases the manganese content in* E. coli, *but not in* B. japonicum

We measured the cellular manganese content as described above, except that 20 μM or 100 μM $FeCl_3$ were also included in the growth medium (Table 1). Iron in the medium did not affect the cellular manganese content in *B. japonicum*. However, we found that *E. coli* accumulated high levels of manganese in response to iron (Table 1). When *E. coli* cells were grown in 20 μM $MnCl_2$, the manganese content increased about 40-fold when 100 μM $FeCl_3$ was included in the medium compared to cells grown with no added iron. The manganese content reached 310 nmol/mg protein in *E. coli* cells grown with 100 μM each of $MnCl_2$ and $FeCl_3$.

Anjem et al (Anjem *et al.*, 2009) found that *E. coli* cells accumulate manganese in response to $H_2O_2$ stress due to activation of the manganese transporter gene *mntH* by the transcriptional regulator OxyR. Because iron can promote oxidative stress due to the Fenton reaction, we addressed whether the observed iron-dependent accumulation of manganese was an oxidative stress response by examining an *oxyR* mutant. The cellular manganese content of an *oxyR* mutant increased with increasing manganese in the medium (Table 3), as was observed in the wild type. However, the manganese levels were independent of iron in the mutant. Moreover, iron levels in the *oxyR* strain were similar to the wild type under all

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metal regimens tested. Thus, accumulation of manganese in response to iron in *E.coli* appears to be an oxidative stress response.

Mutation of the *oxyR* gene in *B. japonicum* had no effect on metal accumulation under all growth conditions tested (Table 3). Importantly, wild type *E. coli* cells grown in 20 μM Fe accumulated about 210 nmol Fe/mg protein and showed elevated manganese levels, whereas *B. japonicum* cells grown in 100 μM Fe contained about 235 nmol Fe/mg protein, but did not show elevated manganese levels. Thus, under comparable cellular iron levels, *E. coli* exhibited a stress response that was not observed in *B. japonicum*. These observations suggest that *B. japonicum* and *E. coli* manage iron differently.

Iron exposure resulted in *oxyR*-dependent manganese accumulation in *E. coli*. Thus we addressed whether iron affected the expression of the $Mn^{2+}$ transport gene. The *mntH* gene transcript level in *E. coli* wild type cells increased about 2-fold 30 minutes after 100 μM $FeCl_3$ was added to the growth medium (Fig. 8A). *mntH* transcript levels remained constant when iron was not added to the growth medium. Iron-responsive induction was lost in an *E. coli oxyR* mutant (Fig 8A), showing that *mntH* induction depends on OxyR. No iron-responsive induction of *mntH* was seen in *B. japonicum* (Fig 8B).

## DISCUSSION

In the present study, we found that the metal specificities of *B. japonicum* Mur and *E. coli* Fur are strongly dependent on the cellular environment in which they function rather than on the physical properties of the protein. In addition, *B. japonicum* and *E. coli* manage manganese and iron differently to affect homeostasis and gene expression. Finally, the inability of Mur or Fur to function as iron metalloregulators in *B. japonicum* may explain why it, and related organisms, sense iron differently than has been described in many other bacterial phyla.

Mur behaves as $Fe^{2+}$-responsive regulator in *E. coli* cells, and Fur responds to $Mn^{2+}$ rather than $Fe^{2+}$ in *B. japonicum* cells. Thus, *B. japonicum* confers manganese selectivity on these proteins. When grown identically in media containing up to 20 μM manganese, *B. japonicum* accumulates more manganese as long as iron is not also added (Table 1). Indeed, *E. coli* appears to need very little manganese unless challenged with hydrogen peroxide (Anjem *et al.*, 2009), whereas *B. japonicum* requires the metal under unstressed conditions (Hohle and O'Brian, 2012, Hohle and O'Brian, 2014). However, the total cellular manganese content is not sufficient to explain the behavior of Mur and Fur. Neither regulator was active in iron-limited *E. coli* cells containing 8-fold more cellular manganese than was found in *B. japonicum* cells where Mur was active (Table 1, Fig. 3 and 4). This suggests that the population of manganese perceived by Mur in *B. japonicum* differs from *E. coli* and does not correlate with total content of the metal.

Selectivity of metal sensing has been described in terms of affinity, access and allostery (Waldron *et al.*, 2009). We have ruled out affinity as the basis for selectivity by showing that Mur and Fur each bind $Fe^{2+}$ and $Mn^{2+}$ with similar affinities (Fig. 1). In addition, $Mn^{2+}$ and $Fe^{2+}$ confer similar DNA binding affinities on Fur and Mur (Fig. 2), thereby ruling out

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allostery as a basis of selectivity. The observed dependence on cellular milieu for metal selectivity by Fur and Mur strongly argues that access to metal is a major factor. The NmtR repressor from *Mycobacterium tuberculosis* responds to $Ni^{2+}$ in its natural host, but only to $Co^{2+}$ when expressed in *Synechococcus* PCC7942, which corresponds to a higher cellular nickel content in the natural host (Cavet *et al.*, 2002). The iron-specific transcription factor DtxR from *Corynebacterium diphtheria* mediates both iron-and manganese-responsive gene expression when expressed in *Bacillus subtilis*, and can be made highly manganese-specific upon mutation of its metal binding site (Guedon and Helmann, 2003). Thus, DtxR metal selectivity is based on both affinity and access.

Whereas affinity and allostery can be readily addressed *in vitro*, access to metal in cells is more difficult to characterize mechanistically. Metals are predominantly tightly associated with proteins, and only a portion of the total cellular content is perceived by a sensor such as Fur or Mur to act as so-called regulatory metal or a metal pool. Strategies associated with protein folding during biogenesis can ensure correct metallation of some enzymes (Tottey *et al.*, 2008, Leach and Zamble, 2007, Lee *et al.*, 1993), but Mur and Fur must bind metals reversibly to function as sensors. There may be a chaperone or some other trafficking mechanism that ensures that Mur is bound by the correct metal as has been described, for example, for copper trafficking in *Enterococcus hirae* (Cobine *et al.*, 1999). Further studies needs to be done to fully elucidate the mechanism in which Mur and Fur obtain their metal.

*E. coli* and *B. japonicum* manage iron differently as observed by the higher level of total and chelatable iron in *E. coli* when cells were grown under identical conditions (Table 1, Fig. 7). Although chelatable iron cannot be assumed to be a quantitative measure of the regulatory population perceived by Fur or Mur, it gives an assessment of iron that is not tightly bound in macromolecules. Our data suggests that the lower level of iron in *B. japonicum* cells allows Mur to function as a manganese-dependent regulator. Raising the cellular iron level by loss of the iron exporter MbfA or by growing cells in very high iron medium resulted in active Mur in *B. japonicum* even under low manganese conditions (Fig. 5A and B). Thus, high iron would be expected to interfere with manganese responsiveness. However, observations in *E. coli* indicate that low iron may be necessary but insufficient to render Fur or Mur manganese-responsive. The regulators are not active in *E. coli* grown in low iron media in the presence of manganese (Figure 3B, C, E, and F, Fig 4A and D). This reinforces the conclusion that there is a mechanism of manganese perception by Mur or Fur in *B. japonicum* apart from low iron levels that is missing in *E. coli*. We note that growth of an *E.coli* mutant that accumulates high levels of manganese was able to activate Fur (Martin *et al.*, 2015) when grown with 500 μM manganese, showing that Fur can be manganese-responsive under extreme conditions.

Increasing the iron level in cells by iron supplementation in the growth medium resulted in a concomitant increase in manganese levels as well in *E. coli*, but not *B. japonicum* (Table 1). A previous study reported that exposure of *E. coli* to $H_2O_2$ elevates manganese levels by *oxyR*-dependent activation of the manganese transporter gene *mntH* (Anjem *et al.*, 2009). Indeed, we found here that manganese accumulation by *E. coli* in response to iron also depended on *oxyR* expression, showing that iron elicits an oxidative stress response. When the cellular iron content was elevated in *B. japonicum* to levels found in *E. coli*, it did not

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elicit a manganese accumulation response (Table 1). It is somewhat counterintuitive that *E. coli*, which maintains iron at a higher level than *B. japonicum*, appears to be more stressed by the metal.

*B. japonicum* and related bacteria sense iron through an unusual mechanism via Irr not found in *E. coli* or other model systems (O'Brian, 2015), and the current work offers a plausible explanation for this. *B. japonicum* is very reliant on manganese (Hohle *et al.*, 2011, Hohle and O'Brian, 2012, Hohle and O'Brian, 2014), and the cellular accommodations to this are incompatible with Fur functioning as an iron-dependent regulator. The Irr protein does not respond to iron directly, but rather responds to an iron-dependent process, namely the synthesis of heme (Qi and O'Brian, 2002). Irr interacts directly with the heme biosynthetic enzyme ferrochelatase, which uses iron as a substrate, and heme triggers Irr inactivation under iron replete conditions. $Fe^{2+}$ and $Mn^{2+}$ share similar sizes and coordination geometries, potentially allowing mismetallation of proteins. However, because Irr binds heme rather than $Fe^{2+}$, it is able to discriminate iron in the form of heme from manganese, which is likely to be crucial in a cell containing a high level of bioavailable manganese. As a result, Irr can function as an iron-responsive regulator in a cellular environment where Fur cannot.

It is likely that this conclusion is generally applicable to the α-Proteobacteria that contain both Irr and Mur. Irr is a Fur family protein, and so it is likely that it arose from a gene duplication event. As a rhizobial ancestor adapted to a greater manganese metabolism, the *fur* gene product became manganese responsive, and the duplicated gene became *irr*. The latter event would have presumably required additional mutations that allow Irr to bind heme and recognize ferrochelatase. A bioinformatic and phylogenetic analysis of the a-Proteobacteria indicate that most species that contain Irr also have Mur (Rodionov *et al.*, 2006), which agrees with studies in which Irr and Mur have been described experimentally.

Some α-Proteobacteria within the Family Rhizobiaceae contain RirA as well as Irr to mediate iron-responsive gene expression (Ngok-Ngam *et al.*, 2009, Todd *et al.*, 2002, Viguier *et al.*, 2005, Ojeda *et al.*, 2012). Although the mode of iron sensing by RirA has not been elucidated, it is proposed to be active as an iron-sulfur protein (Rodionov *et al.*, 2006). This idea is consistent with the conclusion that these bacteria have evolved iron-sensing mechanisms that do not rely on binding to the free metal.

## MATERIALS AND METHODS

### Strains and media

Bacterial strains used in this study are listed in Table 4. *B. japonicum* strains were routinely grown at 29°C in glycerol-salts-yeast (GSY) medium as described elsewhere (Frustaci *et al.*, 1991). *E. coli* strains were routinely grown at 37°C in Luria-Bertani (LB) broth. For all experiments, *B. japonicum* and *E. coli* were grown in modified GSY medium, containing 0.5 g $l^{-1}$ yeast extract instead of 1 g $l^{-1}$ supplemented with $MnCl_2$ or $FeCl_3$ at the concentrations indicated for each experiment. The concentrations of manganese and iron present in the unsupplemented media were 0.2 μM and 0.3 μM, respectively, as determined using a Perkin Elmer model 1100B atomic absorption spectrometer.

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### *Construction of* B. japonicum *mutant strains*

To construct *Bjfur*$^{+}$, a *B. japonicum* mutant expressing *E. coli fur*, the open reading frame of *B. japonicum mur* and 500-bp flanking DNA on each side of it was amplified by PCR using USDA110 genomic DNA as the template, and ligated into pBluescriptSK+. The open reading frame was deleted using inverse PCR as previously described (Panek and O'Brian, 2004). The open reading frame of *E. coli fur* was amplified by PCR using BW25113 genomic DNA as template, and ligated into the inverse PCR product to create an in-frame *fur* construct run off of the native *B. japonicum mur* promoter. The construct was introduced into pLO1 (Lenz *et al.*, 1994), mobilized into the *mur* strain GEM4, and selected for single recombinant mutants on GSY medium containing 75 μg ml$^{-1}$ kanamycin, 100 μg ml$^{-1}$ spectinomycin, and 100 μg ml$^{-1}$ streptomycin. Double recombinants were then selected on GSY medium containing 5% sucrose. Double recombinants were confirmed using antibiotic sensitivity, PCR, and Western blot.

The open reading frame of *E. coli fiu* was amplified by PCR using BW25113 genomic DNA as template, and ligated into pBluescriptSK+. A *B. japonicum mntH*-promoter-*fiu* fusion was created so that the Mur binding site of *mntH* was replaced by the *fiu* Fur Box. The construct was introduced into a very low copy vector, pVK102, to create pVK102FurBox-mntHprom-fiu. The pVK102 construct was mobilized into USDA110, GEM4, and *Bjfur*$^{+}$, and selected on GSY medium containing 75 μg ml$^{-1}$ kanamycin and 75 μg ml$^{-1}$ tetracycline. Mutants were confirmed using PCR.

### *Construction of* E. coli *mutant strains*

To construct *Ecmur*$^{+}$, an *E. coli* mutant expressing *B. japonicum mur*, the open reading frame of *E. coli fur* and 500-bp flanking DNA on each side of it was amplified by PCR using BW25113 genomic DNA as the template, and ligated into pBluescriptSK+. The open reading frame of *fur* was deleted using inverse PCR. A cassette expressing the *camR* and *sacB* genes, conferring chloramphenicol resistance and sucrose sensitivity, respectively, was ligated into the inverse PCR product. The *camR*/*sacB* cassette containing the *fur* flanking DNA was isolated using PCR, purified using a QIAGEN PCR purification kit, and transformed into JW0669 using lambda Red recombineering (Datsenko and Wanner, 2000, Yu *et al.*, 2000). Constructs were verified using antibiotic resistance and sucrose sensitivity. The open reading frame of *B. japonicum mur* was amplified by PCR using USDA110 genomic DNA as template, and ligated into the inverse PCR product containing the *fur* genomic flanks. The *mur* gene containing the *fur* flanking DNA was isolated using PCR, purified using a QIAGEN PCR purification kit, and transformed into JW0669:*fur*::*camR*/*sacB* using lambda Red recombineering. Constructs were selected on LB containing 5% sucrose. The construct was confirmed using antibiotics sensitivity, colony PCR, and Western blot.

DNA including the open reading frame and promoter region of the *B. japonicum mnoP* gene was amplified by PCR using USDA110 genomic DNA as template, and ligated into pBluescriptSK+. The construct was introduced into pVK102, to create pVK102*mnoP* and transformed into BW25113, JW0669, and *Ecmur*$^{+}$. Constructs were confirmed by PCR and analysis of plasmid preparations.

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### *Overexpression and purification of* B. japonicum Mur *and* E. coli Fur

*B. japonicum* Mur and *E. coli* Fur proteins were overexpressed and purified as described elsewhere (Friedman and O'Brian, 2003). Purified protein was dialyzed one time against ITC dialysis buffer (20 mM Tris, pH 7.5 and 150 mM NaCl) containing 0.5 mM EDTA to chelate any metals bound to the proteins. The proteins were dialyzed 3× against ITC dialysis buffer without EDTA. The final dialysate was saved for protein dilutions and metal preparations for ITC analysis.

### Determination of binding affinities for manganese and iron for Mur and Fur using isothermal titration calorimetry (ITC)

All proteins, buffers, and metal solutions were degassed using a ThermoVac (ThermoScientific) at 24°C prior to running on ITC. Protein dilutions and metal salt preparations were carried out using the dialysate from the final dialysis step. Proteins were diluted to a monomer concentration of 30 μM before being loaded into the sample cell of a MicroCal VP-ITC. A 5 mM $MnSO_4$ solution was prepared by diluting a 1 M $MnSO_4$ stock solution. Titrations of manganese were carried out at a cell temperature of 25°C by injecting 1 μl of 5 mM $MnSO_4$ at a rate of 0.5 μl $s^{-1}$ every 90 seconds for 45 minutes, with reference power set to 20 μcal $s^{-1}$ and a stirring speed set to 242. Working stocks of $FeSO_4$ used for titrations were made fresh in degassed buffer to avoid oxidation of $Fe^{2+}$ to $Fe^{3+}$ and were 5.8 mM for Mur, and 7.0 mM for Fur. Titrations of iron were carried out in an anaerobic chamber at a cell temperature of 28°C by injecting 1 μl of $FeSO_4$ at a rate of 0.5 μl $s^{-1}$ every 90 seconds for 45 minutes, with reference power set to 20 μcal $s^{-1}$ and a stirring speed set to 242. Data were analyzed by a version of Origin modified for ITC data analysis as supplied by MicroCal. All experiments were carried out in triplicate.

### Determination of metal dependent binding affinities for Mur and Fur to DNA

Binding affinities of Mur and Fur were determined using electrophoretic gel mobility shift assays modified from that previously described (Hohle and O'Brian, 2009). Briefly, binding reactions containing 39-bp DNA fragments containing either the *B. japonicum mnoP* Mur binding site or the *E. coli fiu* Fur box were titrated with varying amounts of Mur or Fur in the presence of 0.1 mM $MnCl_2$, 0.1 mM $FeSO_4$, or no metal. 1 mM ascorbate was added to reactions containing $FeSO_4$ as a reducing agent. No metal was added to the non-denaturing polyacrylamide gel.

### In vivo binding of Fur or Mur to promoter DNA by cross-linking and immunoprecipitation

This technique was used to analyze the occupancy of the *mnoP* or *fiu* promoters by Mur or Fur *in vivo*. 50-ml cultures of USDA110, GEM4, and *Bjmur*$^+$ expressing *fiu* were grown under low or high manganese and low or high iron conditions to mid-log phase ($OD_{540}$ 0.35 - 0.4). 10-ml cultures of BW25113, JW0669, and *Ecmur*$^+$ expressing *mnoP* were grown under low or high manganese and low or high iron conditions to mid-log phase ($OD_{600}$ 0.35 - 0.4). *In vivo* cross-linking of DNA to protein and subsequent immunoprecipitation with antibodies specific to Mur or Fur were carried out as described elsewhere (Small *et al.*, 2009). Immunoprecipitated DNA (1 μl) was analyzed by qPCR using primers that amplify

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the promoter regions of interest. The data are expressed as the SQ of immunoprecipitated DNA normalized to the input.

### Analysis of RNA by quantitative real time PCR

Expression levels of selected genes were determined by qPCR with iQ SYBR supermix (BIO-RAD) using CFX-96 Touch Real Time PCR thermal cycler (BIO-RAD). RNA was isolated from *B. japonicum* and *E. coli* using a hot phenol-extraction method as previously described (Yang *et al.*, 2006). cDNA was synthesized from 1 μg total RNA using iScript cDNA synthesis kit (BIO-RAD). qPCR reactions were carried out as previously described (Hohle and O'Brian, 2009). Data are expressed as average of three triplicates and the standard deviation is represented by the error bars.

### Determination of intracellular iron and manganese using atomic absorption spectroscopy

Fifty milliliters of *B. japonicum* cultures were grown in 0, 20, or 100 μM $MnCl_2$ and 0, 20, or 100 μM $FeCl_3$ and harvested by centrifugation at 13,000 × g at 4°C for 5 min. The cell pellet was washed once with PBS containing 0.5 mM EDTA, twice with PBS, and twice with metal-free water. Cell pellets were digested in 69% $HNO_3$ (JT Baker, AAS grade) at 98°C for 2 hours, followed by an additional digestion in 5 mM $H_2O_2$ at 70°C for 2 hours. The intracellular iron content was determined on a Perkin Elmer Atomic Absorption Spectrometer model 1100B equipped with a model HGA 700 graphite furnace as previously described (Yang *et al.*, 2006). The same samples were used to measured intracellular manganese content as previously described (Hohle and O'Brian, 2009). Determination of intracellular iron and manganese in *E. coli* was done similarly, starting with 5 ml cultures. Protein concentrations were determined using the Bio-Rad protein assay using BSA as a standard.

### Determination of intracellular chelatable iron by electron paramagnetic resonance spectroscopy

The quantification of intracellular chelatable iron was determined using EPR spectroscopy modified from previously described (Woodmansee and Imlay, 2002). *B. japonicum* or *E. coli* cells (1 μl) were grown in low-metal medium supplemented with 0, 20, or 100 μM $FeCl_3$. Cell pellets were harvested and resuspended in low metal medium containing 20 mM desferrioxamine. Cultures were incubated, with shaking, at their respective growth temperatures (29°C for *B. japonicum*; 37°C for *E. coli*). After the incubation, cells were harvested, washed 3× in 20 mM Tris-HCl, pH 7.4, and resuspended in 0.5 ml Tris-HCl, pH 7.4 containing 10% glycerol. Final volume of cell suspension was approximately 1.2 ml. 0.4 ml of the cell suspension was loaded into 5-mm quartz EPR tube (Wilmad), immediately frozen in liquid nitrogen and stored at −80°C. Remaining cell suspension was used for protein quantification using the BIO-RAD protein assay. An iron standard curve was generated using 0.1 – 2.0 mM $FeSO_4$ solutions containing 1 mM desferrioxamine. Samples were run on a Bruker Elexsys E 500 EPR spectrometer. Each run was 100 scans consisting of 200 points and was acquired and processed using Bruker Xenon software. Final data was analyzed in Microsoft Excel. EPR settings were as follows: field center, 1570 G; field sweep, 400 G, sweep time, 4 sec. Samples were run in a dewar to maintain liquid nitrogen

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temperatures during the course of the run. Each sample was run in triplicate, with the average and standard deviations being reported.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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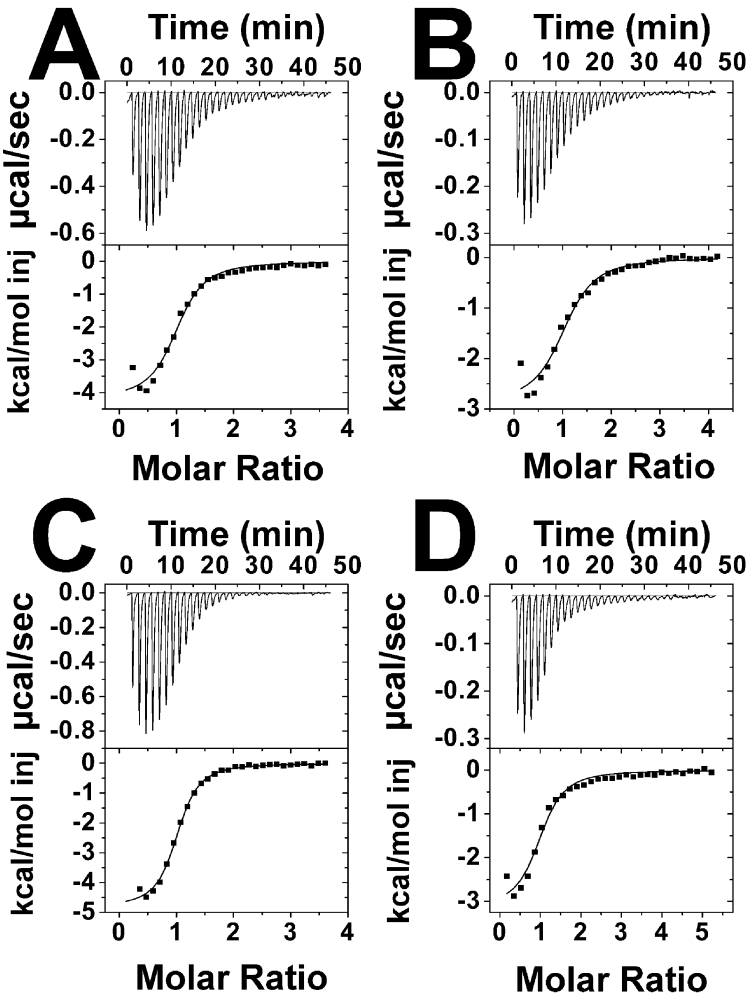


**E**

| | $K_d$ | N |
|---|---|---|
| **Mur +Mn** | 2.04 ± 0.17 | 1.00 ± 0.02 |
| **Mur +Fe** | 1.53 ± 0.08 | 1.00 ± 0.01 |
| **Fur +Mn** | 1.15 ± 0.03 | 0.98 ± 0.01 |
| **Fur +Fe** | 1.25 ± 0.11 | 1.00 ± 0.03 |

**Fig 1.**

ITC analysis of *B. japonicum* Mur and *E. coli* Fur titrated with $Mn^{2+}$ or $Fe^{2+}$.

ITC raw data collection trace for titration of (A) $Mn^{2+}$ to a sample of *B. japonicum* Mur, (B) $Fe^{2+}$ to a sample of *B. japonicum* Mur, (C) $Mn^{2+}$ to a sample of *E. coli* Fur, and (D) $Fe^{2+}$ to a sample of *E. coli* Fur. (E) Dissociation binding constant ($K_d$) values and calculated number of sites per monomer (N) of *B. japonicum* Mur and *E. coli* Fur for $Mn^{2+}$ and $Fe^{2+}$ were determined using a one-binding-site model fitted to the titration data taken in triplicate.

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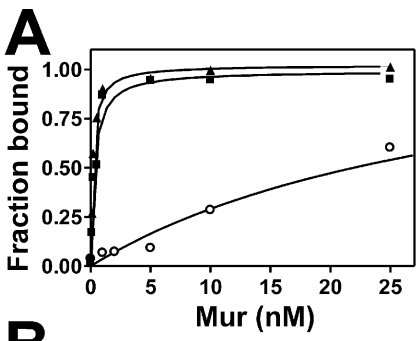


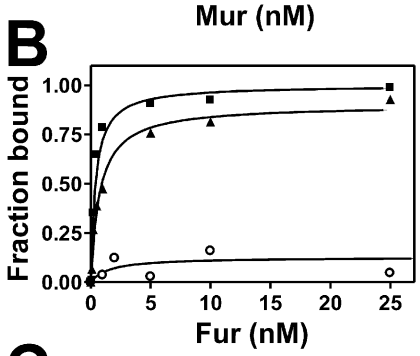


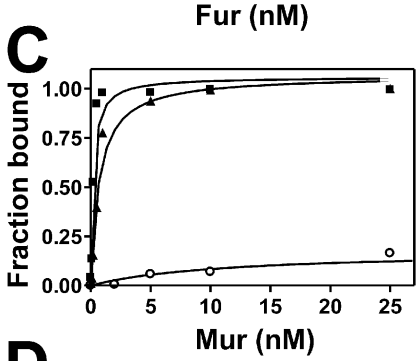


D

| Protein | metal | Probe | $K_d$ |
|---|---|---|---|
| Mur | Mn | *mnoP* | 0.31 |
| Mur | Fe | *mnoP* | 0.19 |
| Mur | none | *mnoP* | 33.1 |
| Fur | Mn | *fiu* | 0.39 |
| Fur | Fe | *fiu* | 0.74 |
| Fur | none | *fiu* | ND |
| Mur | Mn | *fiu* | 0.20 |
| Mur | Fe | *fiu* | 0.71 |
| Mur | none | *fiu* | ND |

**Fig 2.**

Effects of $Mn^{2+}$ and $Fe^{2+}$ on binding of *B. japonicum* Mur or *E. coli* Fur to the *mnoP* or *fiu* promoter *in vitro*.

Electrophoretic mobility shift assay (EMSA) analysis was carried out using (A) *B. japonicum* Mur and 100 pmol *mnoP* containing the *B. japonicum* Mur binding site, (B) *E. coli* Fur and 100 pmol *fiu* containing the Fur binding site and (C) Mur and the *fiu* binding site. The binding reactions were carried out either with no metal (open circles), 100 μM $Mn^{2+}$ (closed squares) or $Fe^{2+}$ (closed triangles). Bound and unbound DNA were resolved

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on a 5% non-denaturing polyacrylamide gel and visualized by autoradiography. Autoradiographs were scanned, and bands were quantified. (D). Dissociation binding constants ($K_d$) were calculated from the binding data.

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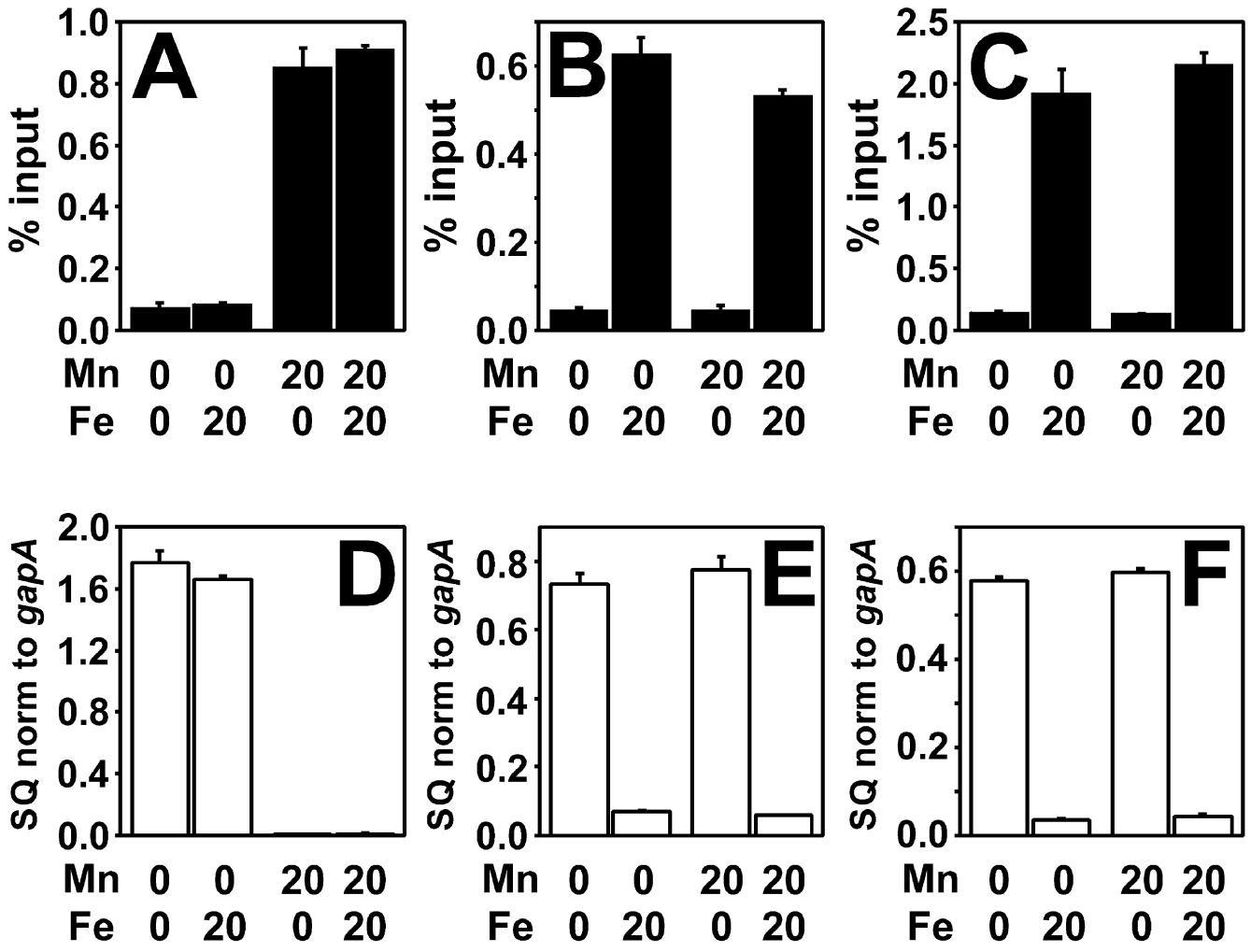


**Fig 3.**

Effect of metals on *in vivo* promoter occupancy and transcriptional repression by Mur in *B. japonicum* and *E. coli* cells.

Cells were grown in media with the presence or absence of 20 μM $MnCl_2$ or 20 μM $FeCl_3$. (A) *In vivo* occupancy of the *mnoP* promoter by Mur in *B. japonicum* cells (B) *In vivo* occupancy of the *fiu* promoter by Mur in *E. coli* cells. (C) *In vivo* occupancy of the *mnoP* promoter by Mur in *E. coli* cells. *In vivo* occupancy data are expressed as the relative starting quantity (SQ) of DNA normalized to the input, and are presented as the average of triplicate samples with the error bars representing the standard deviation. (D) Analysis of *mnoP* mRNA by qPCR in *B. japonicum* cells. (E) Analysis of *fiu* mRNA in *E. coli* cells. (F) Analysis of *mnoP* mRNA in *E. coli* cells. The data are expressed as the relative starting quantity (SQ) of *mntH* mRNA normalized to the housekeeping gene *gapA*, and are presented as the average of triplicate samples with the error bars representing the standard deviation.

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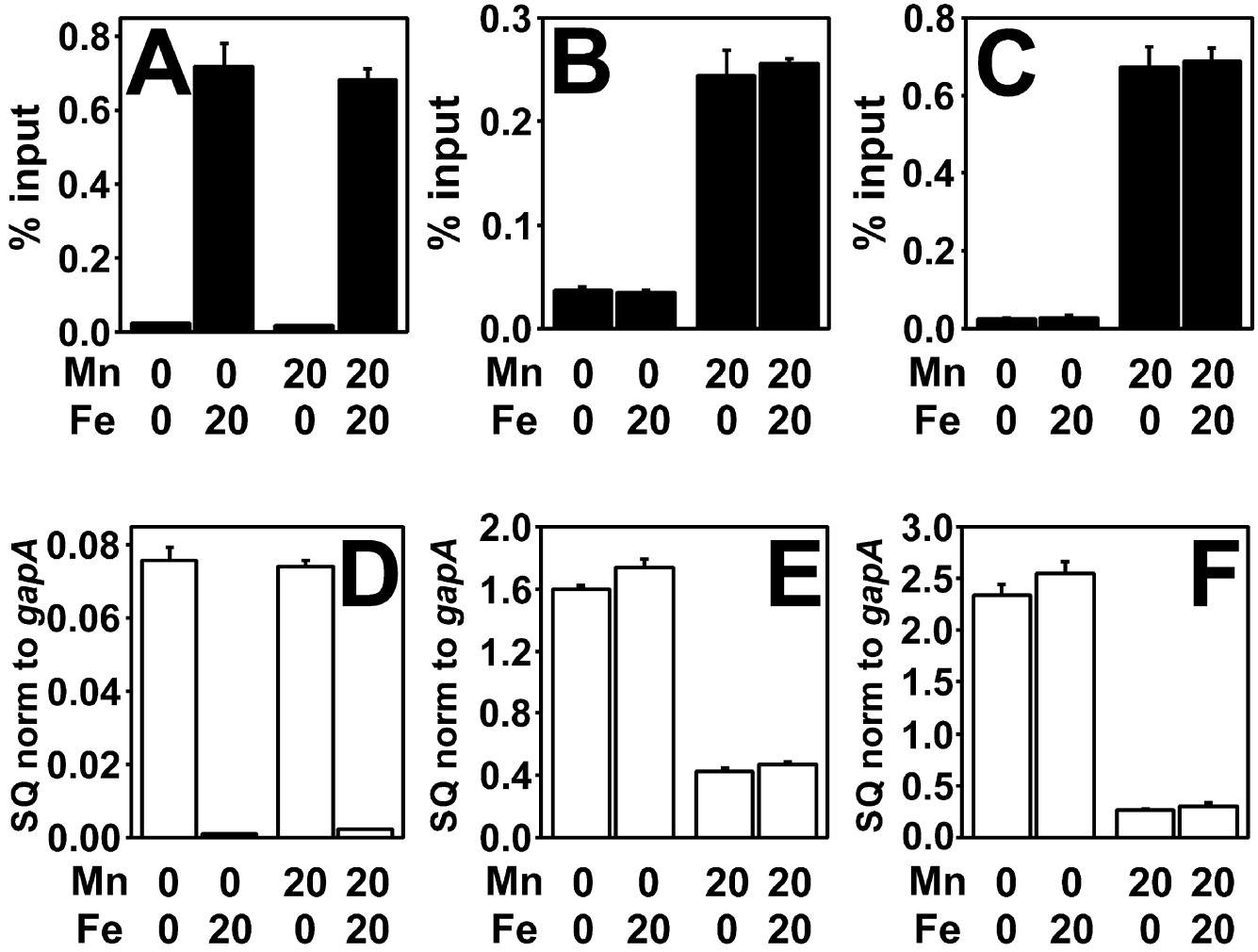


**Fig 4.**

Effect of metals on *in vivo* promoter occupancy and transcriptional repression by Fur in *B. japonicum* and *E. coli* cells.

Cells were grown in media with the presence or absence of 20 μM $MnCl_2$ or 20 μM $FeCl_3$. (A) *In vivo* occupancy of the *fiu* promoter by Fur in *E. coli* cells. (B) *In vivo* occupancy of the *mnoP* promoter by Fur in *B. japonicum* cells. (C) *In vivo* occupancy of the *fiu* Fur box in *B. japonicum* cells. The Mur-binding site within the *mntH* promoter was replaced by the *fiu* Fur box, and the chimeric promoter placed upstream of the *fiu* open reading frame. *In vivo* occupancy data are expressed as the relative starting quantity (SQ) of DNA normalized to the input, and are presented as the average of triplicate samples with the error bars representing the standard deviation. (D) Analysis of *fiu* mRNA by qPCR in *E. coli* cells grown as described above. (E) Analysis of *mnoP* mRNA in *B. japonicum* cells. (F) Analysis of *fiu* mRNA in *B. japonicum* cells. The data are expressed as the relative starting quantity (SQ) of *mntH* mRNA normalized to the housekeeping gene *gapA*, and are presented as the average of triplicate samples with the error bars representing the standard deviation.

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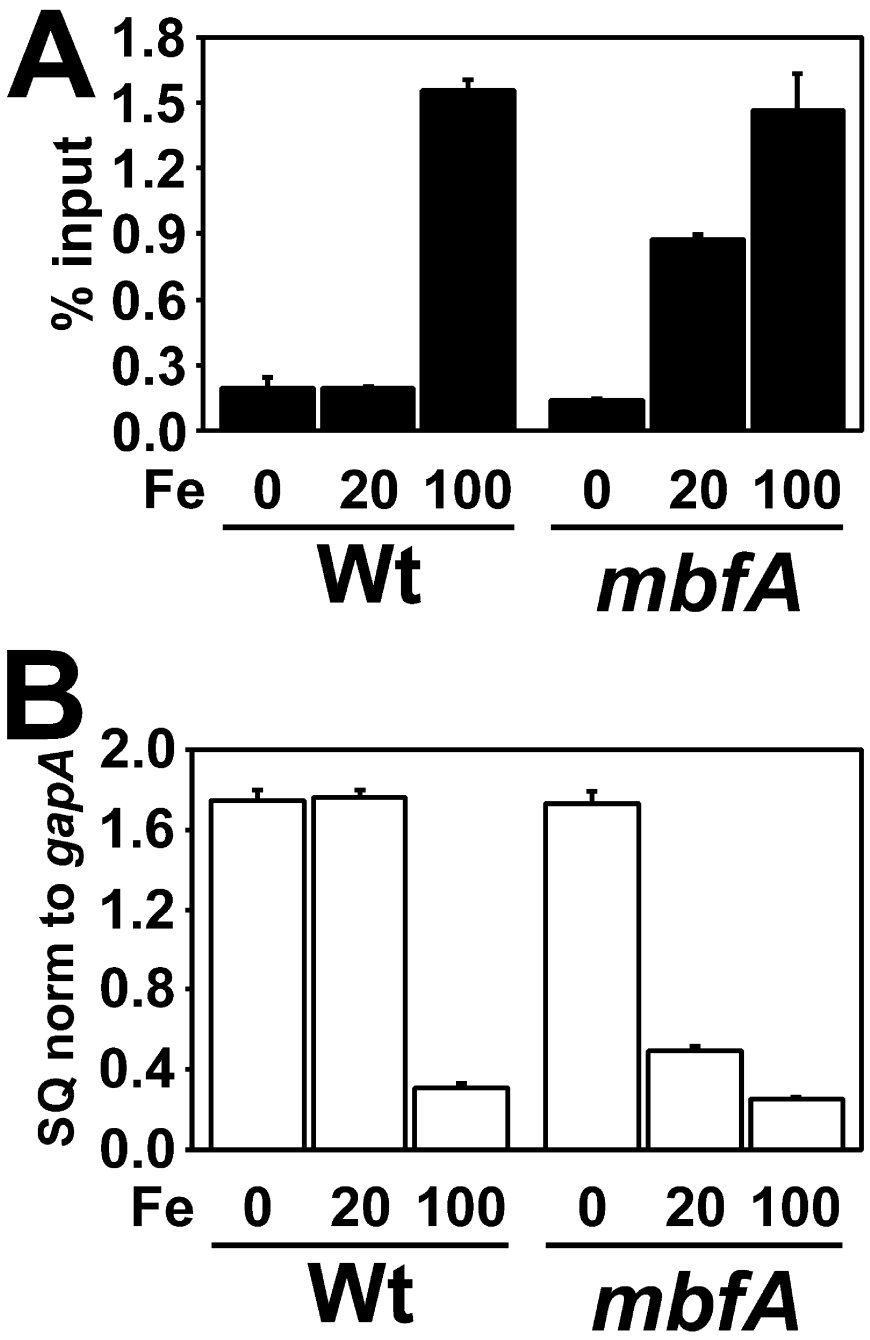


**Fig. 5.**

Effects of high intracellular iron on *in vivo* promoter occupancy and transcriptional repression by Mur in *B. japonicum* cells

The intracellular iron concentration was elevated by growth in media containing 100 μM $FeCl_3$ in the medium for *B. japonicum*, or by mutation of the iron exporter gene *mbfA*. Cells of the wild type and mutant were grown in media containing no added manganese and either 0, 20 μM or 100 μM $FeCl_3$. (A) *In vivo* occupancy of the *mnoP* promoter by Mur in *B. japonicum* wild type (Wt) or *mbfA* strain. Data are expressed as the relative starting quantity

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(SQ) of DNA normalized to the input, and are presented as the average of triplicate samples with the error bars representing the standard deviation. (B) Analysis of *mnoP* mRNA by qPCR in *B. japonicum* wild type or *mbfA* strain. The data are expressed as the relative starting quantity (SQ) of *mntH* mRNA normalized to the housekeeping gene *gapA*, and are presented as the average of triplicate samples with the error bars representing the standard deviation.

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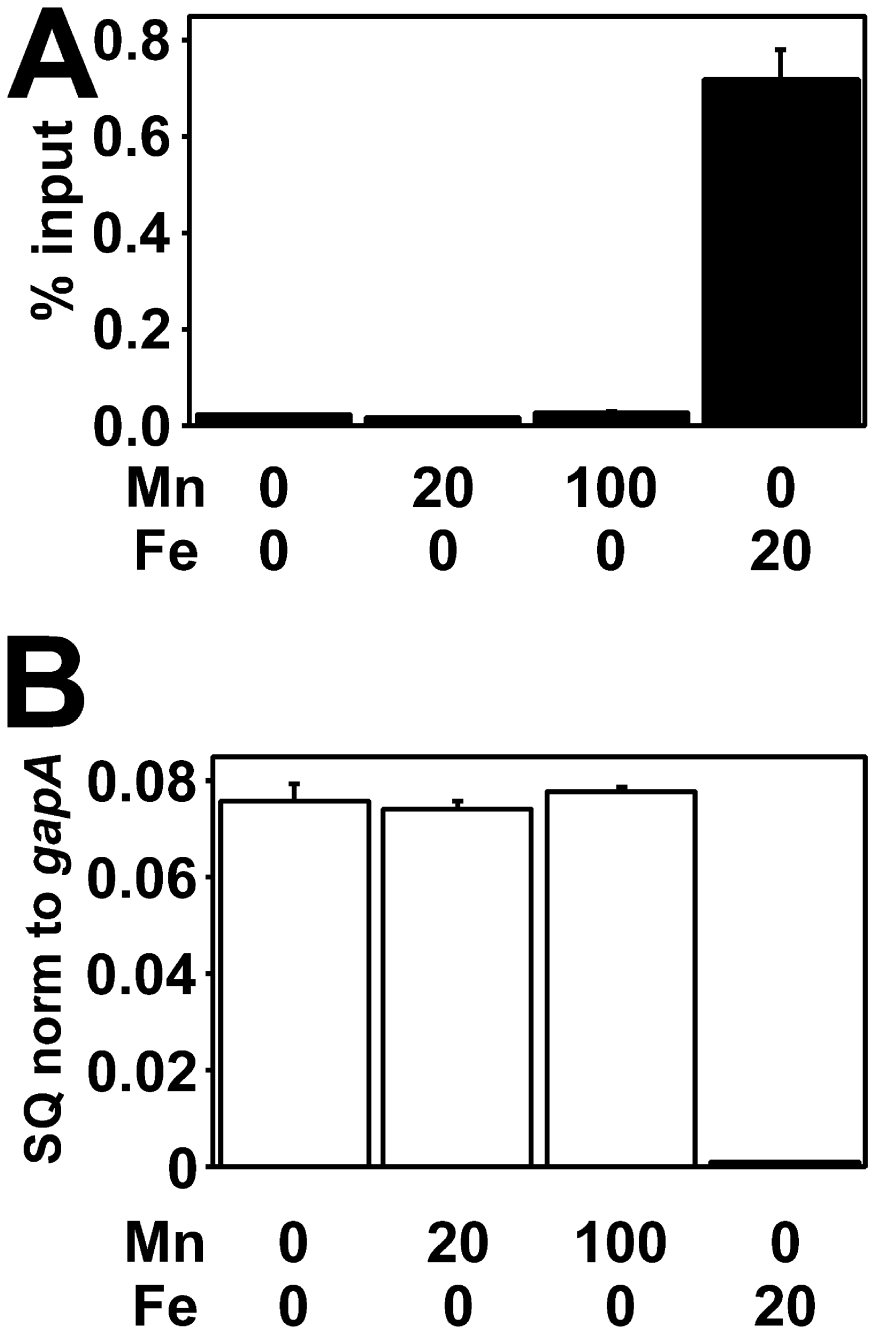


**Fig. 6.**

Effects of high intracellular manganese on *in vivo* promoter occupancy and transcriptional repression by Fur in *E. coli* cells

*E. coli* cells were grown in media containing no added iron and either 0, 20 μM or 100 μM $MnCl_2$, or 20 μM $FeCl_3$ in the absence of manganese. (A) *In vivo* occupancy of the *fiu* promoter by Fur in *E. coli* cells. Data are expressed as the relative starting quantity (SQ) of DNA normalized to the input, and are presented as the average of triplicate samples with the error bars representing the standard deviation. (B) Analysis of *fiu* mRNA in *E. coli* cells.

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The data are expressed as the relative starting quantity (SQ) of *mntH* mRNA normalized to the housekeeping gene *gapA*, and are presented as the average of triplicate samples with the error bars representing the standard deviation.

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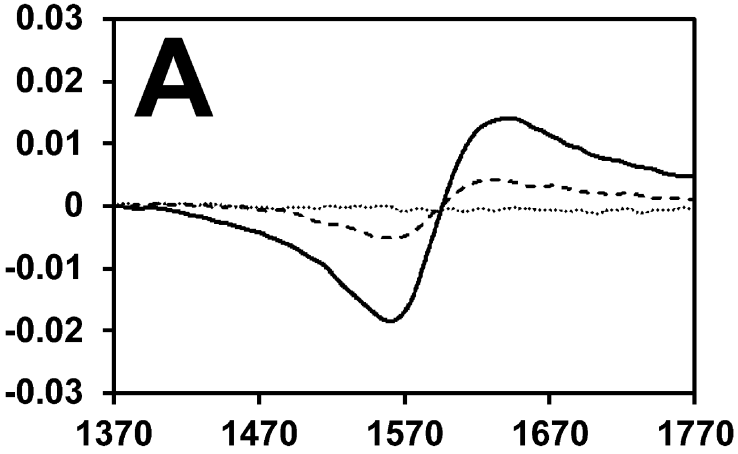


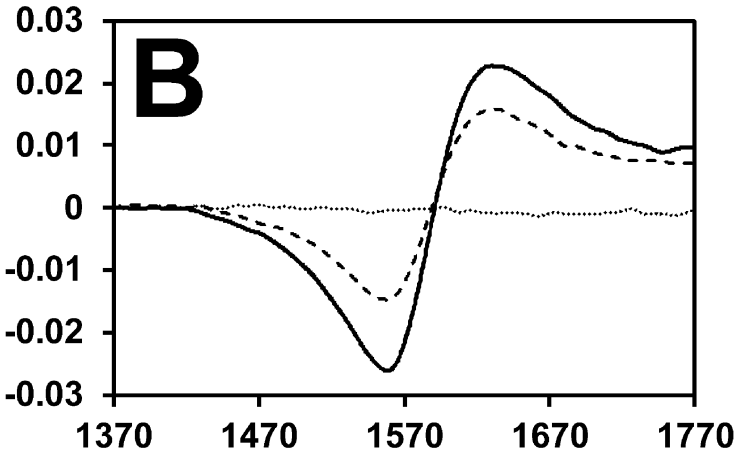


C

| [Fe] | *B.japonicum* | *E.coli* |
|---|---|---|
| 0 µM | ND | ND |
| 20 µM | 6.5 ± 0.6 | 16.6 ± 0.3 |
| 100 µM | 18.2 ± 0.2 | 31.9 ± 0.5 |

**Fig 7.**

Determination of intracellular chelatable iron in *B. japonicum* and *E. coli* cells by EPR. EPR raw data traces for (A) *B. japonicum* or (B) *E. coli* cells treated with the iron chelator desferrioxamine after growth in media containing no added iron (dotted line), 20 µM $FeCl_3$ (dashed line) or 100 µM $FeCl_3$. (solid line) $FeCl_3$. (C) Quantitation of intracellular chelatable iron as determined using EPR normalized to protein content. Data are expressed as average and standard deviation of triplicate trials. The data are expressed as nmol Fe per mg protein.

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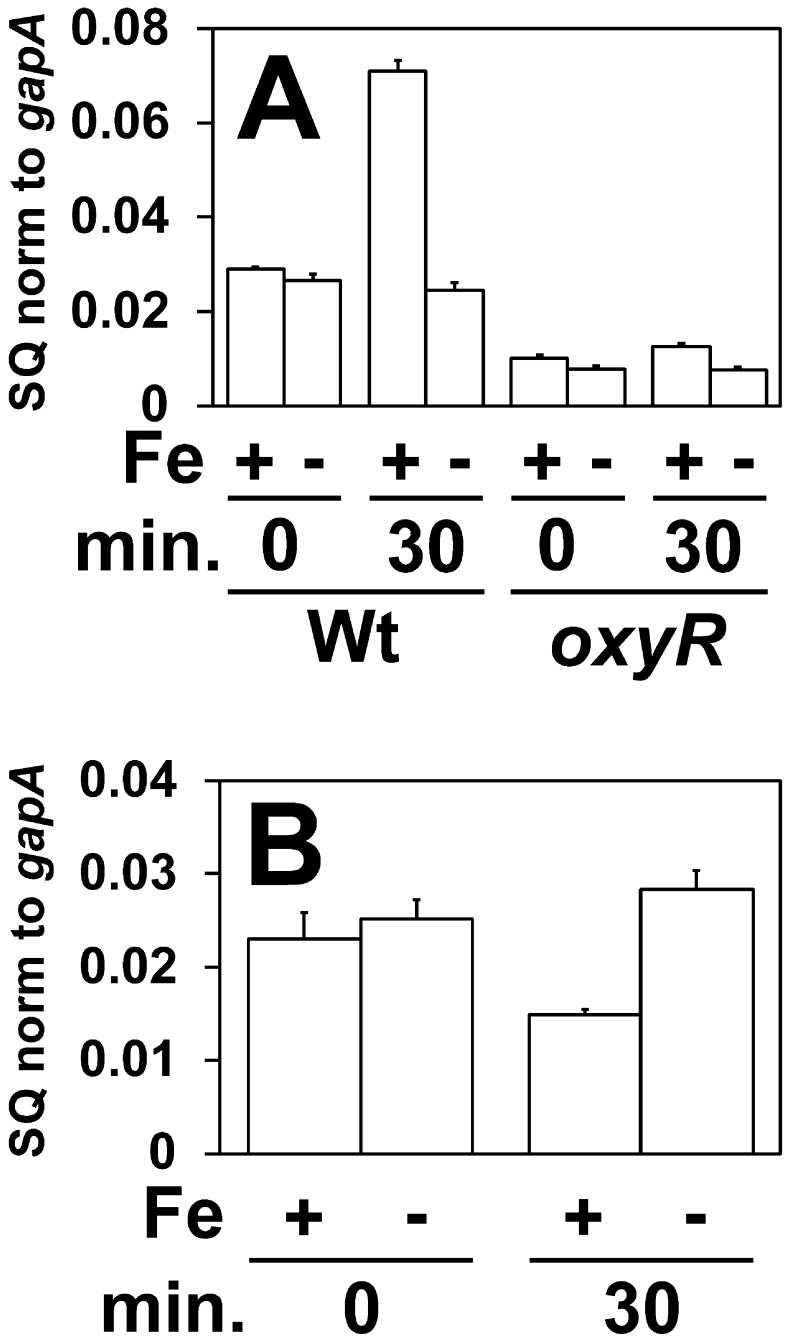


**Fig 8.**

Effects of iron on expression of the *mntH* gene in *E. coli* and *B. japonicum*

Cells were grown to mid log phase, then 0 or 100 μM FeCl3 was added to the medium, and cells were harvested after 30 minutes. (A) Analysis of *mntH* mRNA by qPCR in *E. coli* wild type (Wt) or *oxyR* strain. (B) Analysis of *mntH* mRNA in *B. japonicum* cells. The data are expressed as the relative starting quantity (SQ) of *mntH* mRNA normalized to the

housekeeping gene *gapA*, and are presented as the average of triplicate samples with the error bars representing the standard deviation.

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## Table 1

Determination of intracellular iron and manganese content of *B. japonicum* and *E. coli*. Data are expressed as average nmol metal per mg protein ±standard deviation of triplicate samples.

**Cellular iron content**

| Medium (μM) | | *B. japonicum* | *E. coli* |
|---|---|---|---|
| Mn | Fe | nmol Fe/mg protein | nmol Fe/mg protein |
| 0 | 0 | 0.96 ±0.01 | 2.0 ±0.02 |
| 0 | 20 | 46 ±3 | 214 ±3 |
| 0 | 100 | 307 ±5 | 1054 ±0 |
| 20 | 0 | 0.96 ±0.02 | 2.18 ±0.09 |
| 20 | 20 | 48 ±3 | 228 ±3 |
| 20 | 100 | 307 ±8 | 1268 ±52 |
| 100 | 0 | 0.95 ±0.00 | 2.09 ±0.01 |
| 100 | 20 | 47 ±1 | 274 ±2 |
| 100 | 100 | 307 ±7 | 1259 ±45 |

**Cellular manganese content**

| Medium (μM) | | *B. japonicum* | *E. coli* |
|---|---|---|---|
| Mn | Fe | nmol Mn/mg protein | nmol Mn/mg protein |
| 0 | 0 | 0.92 ±0.01 | 0.14 ±0.01 |
| 0 | 20 | 1.15 ±0.01 | 0.39 ±0.00 |
| 0 | 100 | 1.10 ±0.04 | 0.96 ±0.04 |
| 20 | 0 | 8.6 ±0.3 | 4.5 ±0.2 |
| 20 | 20 | 9.7 ±0.3 | 31 ±2 |
| 20 | 100 | 9.8 ±0.2 | 82 ±2 |
| 100 | 0 | 13.2 ±0.3 | 68 ±3 |
| 100 | 20 | 13.6 ±0.1 | 237 ±5 |
| 100 | 100 | 13.3 ±0.3 | 276 ±6 |

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**Table 2**

Determination of intracellular iron and manganese content of *B. japonicum* wild type and *mbfA* cells. Data are expressed as nmol metal per mg protein ±standard deviation of triplicate samples.

| Medium (μM) | | Cellular Iron Content nmol Fe/mg protein | | Cellular Manganese Content nmol Mn/mg protein | |
|---|---|---|---|---|---|
| Mn | Fe | Wt | *mbfA* | Wt | *mbfA* |
| 0 | 0 | 1.15 ±0.04 | 1.33 ±0.07 | 1.13 ±0.00 | 1.11 ±0.05 |
| 0 | 20 | 43 ±3 | 93 ±4 | 1.16 ±0.04 | 1.11 ±0.03 |
| 0 | 100 | 313 ±11 | 567 ±9 | 1.16 ±0.02 | 1.17 ±0.00 |
| 20 | 0 | 0.91 ±0.06 | 1.12 ±0.03 | 10.5 ±0.7 | 10.2 ±0.3 |
| 20 | 20 | 43 ±1 | 96 ±3 | 11.1 ±0.1 | 11.0 ±0.1 |
| 20 | 100 | 308 ±8 | 574 ±27 | 12.7 ±0.4 | 11.5 ±0.5 |

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## Table 3

Determination of intracellular manganese and iron content of *E. coli* and *B. japonicum* wild type and *oxyR* strains. Data are expressed as average nmol metal per mg protein ±standard deviation.

| | | *E. coli* | | *B. japonicum* | |
|---|---|---|---|---|---|
| **Medium (μM)** | | **nmol Mn/mg protein** | | **nmol Mn/mg protein** | |
| **Mn** | **Fe** | **Wt** | ***oxyR*** | **Wt** | ***oxyR*** |
| 0 | 0 | 0.33 ±0.01 | 0.38 ±0.02 | 1.57 ±0.03 | 1.38 ±0.04 |
| 0 | 20 | 0.62 ±0.01 | 0.53 ±0.01 | 1.47 ±0.17 | 1.53 ±0.08 |
| 0 | 100 | 0.91 ±0.04 | 0.70 ±0.01 | 1.53 ±0.05 | 1.51 ±0.04 |
| 20 | 0 | 2.6 ±0.1 | 3.1 ±0.2 | 13.2 ±0.2 | 10.7 ±0.2 |
| 20 | 20 | 27 ±2 | 1.7 ±0.1 | 12.8 ±0.2 | 11.1 ±0.2 |
| 20 | 100 | 102 ±1 | 2.1 ±0.1 | 11.6 ±0.2 | 10.2 ±0.2 |
| 100 | 0 | 67 ±1 | 64 ±5 | 16.0 ±0.3 | 16.7 ±0.8 |
| 100 | 20 | 220 ±4 | 73 ±1 | 17.6 ±0.6 | 16.8 ±0.3 |
| 100 | 100 | 311 ±2 | 56.3 ±0.1 | 16.9 ±0.4 | 16.2 ±0.3 |

| **Medium** | | **nmol Fe/mg protein** | | **nmol Fe/mg protein** | |
|---|---|---|---|---|---|
| **Mn** | **Fe** | **Wt** | ***oxyR*** | **Wt** | ***oxyR*** |
| 0 | 0 | 2.11 ±0.03 | 1.94 ±0.06 | 1.38 ±0.01 | 1.42 ±0.04 |
| 0 | 20 | 222 ±2 | 204 ±1 | 47 ±0.2 | 46 ±2 |
| 0 | 100 | 1132 ±8 | 1063 ±8 | 233 ±3 | 206 ±3 |
| 20 | 0 | 2.12 ±0.01 | 2.02 ±0.01 | 1.10 ±0.00 | 1.17 ±0.05 |
| 20 | 20 | 212 ±2 | 208 ±2 | 53 ±1 | 47 ±1 |
| 20 | 100 | 1255 ±14 | 1192 ±38 | 239 ±16 | 229 ±16 |
| 100 | 0 | 2.24 ±0.06 | 1.85 ±0.12 | 1.26 ±0.02 | 1.15 ±0.04 |
| 100 | 20 | 210 ±2 | 212 ±2 | 55 ±3 | 48 ±1 |
| 100 | 100 | 1236 ±20 | 1196 ±12 | 248 ±2 | 228 ±3 |

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**Table 4**

Strains used in this study

| *B. japonicum* | | |
|---|---|---|
| Strains | Relevant Characteristics | Reference |
| USDA110 | Parent Strain | |
| GEM4 | *mur*::Ω-cassette | (Hamza *et al.*, 1999) |
| Bjfur$^+$ | *B. japonicum mur* ORF replaced by *E. coli fur* ORF | This study |
| 110*mbfA*ΔΩ | *mbfA*:: Ω-cassette | (Sankari and O'Brian, 2014) |
| *oxyR* Δ Ω | oxyR:: Ω-cassette | (Panek and O'Brian, 2004) |
| Plasmids | | |
| pVK102FurBox-mntHprom-fiu | pVK102 with *fiu* fused to *mntH* promoter containing fiu Fur Box | This study |
| *E.coli* | | |
| Strains | Relevant Characteristics | Reference |
| BW25113 | *E. coli* parent strain | (Datsenko and Wanner, 2000) |
| JW0669 | *fur*::*kan* | (Datsenko and Wanner, 2000) |
| JW0669:kan::*camR*/*sacB* | *fur*::*camR*/*sacB* | This study |
| Ecmur$^+$ | *E. coli fur* ORF replaced by *B. japonicum mur* ORF | This study |
| JW3933-3 | *oxyR*::*kan* | (Datsenko and Wanner, 2000) |
| Plasmids | | |
| pVK102 mnoP | pVK102 with *mnoP* and promoter | This study |