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The *mntH* gene encodes the major Mn^{2+} transporter in *Bradyrhizobium japonicum* and is regulated by manganese via the Fur protein

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Abstract

The bacterial Nramp family protein MntH is a divalent metal transporter, but *mntH* mutants have little or no phenotype in organisms where it has been studied. Here, we identify the *mntH* homolog of *Bradyrhizobium japonicum*, and demonstrate that it is essential for Mn^{2+} transport and for maintenance of cellular manganese homeostasis. Transport activity was induced under manganese deficiency, and Fe^{2+} did not compete with $^{54}Mn^{2+}$ for uptake by cells. The steady state level of *mntH* mRNA was negatively regulated by manganese, but was unaffected by iron. Control of *mntH* expression and Mn^{2+} transport by manganese was lost in a *fur* strain, resulting in constitutively high activity. Fur protected a 35 bp region of the *mntH* promoter in DNase I footprinting analysis that includes three imperfect direct repeat hexamers that are needed for full occupancy. Mn^{2+} increased the affinity of Fur for the *mntH* promoter by over 50-fold, with a K_d value of 2.2 nM in the presence of metal. The findings identify MntH as the major Mn^{2+} transporter in *B. japonicum*, and show that Fur is a manganese-responsive regulator in that organism. Furthermore, Fe^{2+} is neither a substrate for MntH nor a regulator of *mntH* expression in vivo.

Keywords

bradyrhizobium; manganese; metal homeostasis

INTRODUCTION

Manganese is required for many biological processes as an enzyme cofactor, and serves as a protectant from oxidative stress. However, manganese can be toxic at high concentrations (Kehres and Maguire, 2003; Moore and Helmann, 2005; Papp-Wallace and Maguire, 2006; Que and Helmann, 2000), and thus manganese homeostasis is maintained in part by regulating acquisition of the metal from the environment. Manganese uptake by bacterial cells proceeds by the divalent metal transporters SitABCD (MntABCD) and MntH. SitABCD is an ABC-type transport complex that was initially described as an Fe^{2+} transporter (Bearden *et al.*, 1998; Zhou *et al.*, 1999), but is now known to transport Mn^{2+} as well. The high affinity of *Salmonella enterica* serovar Typhimurum SitABCD for Mn^{2+} compared to Fe^{2+} supports the argument that it functions physiologically as only a Mn^{2+} transporter (Kehres *et al.*, 2002a). However, abrogation of Fe^{2+} transport in that organism requires mutation of *sitABCD* as well as the ferrous iron transporter gene *feoB* (Boyer *et al.*, 2002; Perry *et al.*, 2007; Sabri *et al.*, 2008). In addition, iron-related phenotypes of *sitABCD* mutants in other bacteria have been

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described (Bearden *et al.*, 1998; Runyen-Janecky *et al.*, 2003; Sabri *et al.*, 2008; Zhou *et al.*, 1999).

MntH is a bacterial member of the Nramp family of divalent cation transporters originally described in eukaryotes (Horsburgh *et al.*, 2002; Kehres *et al.*, 2000; Makui *et al.*, 2000; Que and Helmann, 2000). Like SitABCD, MntH has preferential affinity for Mn^{2+}, but can also take up Fe^{2+} (Kehres *et al.*, 2000; Makui *et al.*, 2000). Bacterial *mntH* mutants generally do not have a growth defect or other phenotypes associated with manganese deficiency (Kehres *et al.*, 2000; Makui *et al.*, 2000), and characterization is carried out in strains where *mntH* is overexpressed from a plasmid. The lack of strong phenotypes is due in some cases to the presence of *sitABCD*, which carries out a similar function (Boyer *et al.*, 2002; Horsburgh *et al.*, 2002).

In numerous bacterial species, the *sitABCD* and *mntH* genes are regulated by Mn^{2+} via MntR, a transcriptional repressor that binds to target promoters when complexed with the metal (Horsburgh *et al.*, 2002; Kehres *et al.*, 2002b; Patzer and Hantke, 2001; Que and Helmann, 2000). These metal transporter genes are also repressed in the presence of Fe^{2+} due to the activity of the Fur repressor (Guedon *et al.*, 2003; Kehres *et al.*, 2002b; Patzer and Hantke, 2001). Whereas the physiological significance of Fe^{2+} transport by MntH or SitABCD has been questioned (Kehres *et al.*, 2000; Kehres *et al.*, 2002a), the regulation of *mntH* or *sitABCD* by Fe^{2+} is specific in the model systems *E. coli*, *S. enterica* and *B. subtilis*, and underscores the integration of iron and manganese metabolism in those bacteria.

We are interested in understanding manganese homeostasis in *Bradyrhizobium japonicum*, a bacterium that lives as a free-living soil organism, or as the endosymbiont of soybean. *B. japonicum* belongs to the α-Proteobacteria, a diverse taxonomic group of gram negative bacteria containing numerous members that form close or intracellular associations with eukaryotic hosts in a symbiotic or pathogenic context. The *sitABCD* operon has been described in *Sinorhizobium meliloti* (Chao *et al.*, 2004; Davies and Walker, 2007; Platero *et al.*, 2004; Platero *et al.*, 2003). A *sitA* mutant has a growth deficiency in metal-deplete media and other phenotypes that are rescued by manganese supplementation. Thus, SitABCD is likely to be the major Mn^{2+} transporter in those species. Interestingly, Fur mediates Mn^{2+} control of the *sitABCD* operon in *S. meliloti* (Chao *et al.*, 2004; Platero *et al.*, 2004; Platero *et al.*, 2007), *Rhizobium leguminosarum* (Diaz-Mireles *et al.*, 2004) and *Agrobacterium tumefaciens* (Kitphati *et al.*, 2007). The Fur homolog has been renamed Mur in those rhizobial species, but it shows iron responsiveness in *A. tumefaciens* (Kitphati *et al.*, 2007). The Fur homolog in *B. japonicum* has thus far been characterized only as an iron responsive regulator (Friedman and O'Brian, 2004; Yang *et al.*, 2006b), and was originally identified by complementation of an *E. coli* Fur mutant (Hamza *et al.*, 1999).

In the present study, we show the *mntH* gene is essential for Mn^{2+} transport, growth in manganese-deficient media and for maintenance of manganese homeostasis. Furthermore, Fur mediates Mn^{2+}-dependent control of *mntH* gene expression, thus a new role for *B. japonicum* Fur as a Mn^{2+}-responsive regulator is described.

RESULTS

B. japonicum has a manganese-regulated Mn^{2+} transport activity

Most studies that characterize bacterial Mn^{2+} transport directly in detail are carried out in cells in which a transport gene is expressed from on a high copy plasmid (Kehres *et al.*, 2000; Makui *et al.*, 2000). Although informative, that work cannot assess the physiological Mn^{2+} transport activity, its regulation, or the contribution of a particular transporter to that activity. Therefore, we wanted to ask the simple question of whether *B. japonicum* has a high affinity Mn^{2+}

transport activity, and whether it is affected by cellular exposure to manganese. Mn^{2+} uptake was measured in cells grown in high manganese (50 μM $MnCl_2$) or low manganese (1 μM $MnCl_2$) media using 5 nM $^{54}Mn^{2+}$ as the initial substrate concentration (Fig. 1A). Cells grown in high manganese medium had very low Mn^{2+} uptake activity. However, uptake was observed in cells grown in low manganese medium, showing that *B. japonicum* has a manganese-dependent Mn^{2+} uptake activity.

The Mn^{2+} uptake system does not transport Fe^{2+}

Transport systems that take up Mn^{2+} also take up Fe^{2+}, and can be regulated by both metals. We examined whether $^{54}Mn^{2+}$ was taken up by as system that also transports Fe^{2+} by competition experiments with unlabeled metals (Fig. 1B). 1 μM unlabeled Mn^{2+} inhibited uptake of 5 nM $^{54}Mn^{2+}$, but 1 μM Fe^{2+} did not. This suggests that Mn^{2+} and Fe^{2+} are not taken up by a common transport system.

The *mntH* homolog *bll5044* is essential for high affinity Mn^{2+} transport activity

Mn^{2+} is likely to be transported by SitABCD in *Sinorhizobium meliloti* as judged by the rescue of growth and other phenotypes of *sitA* mutants with Mn^{2+} (Chao *et al.*, 2004; Davies and Walker, 2007; Platero *et al.*, 2003). However, *B. japonicum* genome does not contain an obvious *sitABCD* operon homolog (but see Discussion). A BLAST search using *E. coli* MntH identified Bll5044 as a putative protein with 40% identity to it, and Blr6117 and Bll8038 with 25% identity. To further narrow down a candidate Mn^{2+} transporter gene, we examined mRNA levels of *bll5044*, *blr6117* and *bll8038* in cells grown in high or low Mn^{2+} media by quantitative real time PCR (qPCR) (Fig. 2). Whereas *blr6117* and *bll8038* mRNA levels were low independent of the Mn^{2+} status, *bll5044* message was high in cells grown in low manganese media, and very low in the presence of the metal. Thus, *bll5044* is a manganese-regulated gene, and its expression correlated with Mn^{2+} transport activity (Fig. 1A).

We constructed a mutant strain defective in the *bll5044* gene such that the open reading frame was deleted and replaced with an Ω cassette. We measured high affinity Mn^{2+} transport activity in the mutant and the parent strain grown in low manganese media (1 μM $MnCl_2$) (Fig. 3A), since this was the lowest manganese concentration in which the mutant grew (see below). The transport activity observed in the parent strain was almost completely abolished in the mutant. Thus, *bll5044* is required for Mn^{2+} uptake, and is a structural and functional homolog of *mntH.* Accordingly, *bll5044* was designated *mntH*.

The *mntH* gene is required for growth and maintenance of manganese homeostasis under manganese deficiency

The data indicate that MntH is responsible for most of the observed Mn^{2+} transport activity in cells under the growth conditions tested (Fig. 3A), and therefore we predict that the *mntH* strain would be manganese-deficient. To test this, we examined the growth of the parent and mutant strains in manganese-deficient and –replete media (Fig. 4). The *mntH* mutant grew well in media supplemented with 50 μM $MnCl_2$, but did not grow in media with no added manganese (0.2 μM final concentration). The parent strain grew well under either media condition, and thus *mntH* is necessary for growth in manganese-deficient medium. In other bacteria, MntH is reported to transport iron as well as manganese. To verify that that rescue of the *mntH* strain by 50 μM $MnCl_2$ was not due to iron contamination, we carried out the growth studies in low and high iron media (Fig. 4). Iron did not rescue the growth phenotype of the *mntH* strain in low manganese medium, and the mutant grew in high or low iron conditions as long as manganese was present. $FeCl_3$ was used in Fig. 4, and $FeSO_4$ yielded the same results. We conclude that the growth phenotype of the *mntH* strain is due to a manganese deficiency.

The intracellular manganese content was determined for the parent and *mntH* strains grown in high (50 μM) or low (1 μM) manganese media. The manganese content in the parent strain was approximately the same in high or low manganese media (Fig. 5), indicating that *B. japonicum* is able to regulate its cellular manganese content over at least a 50-fold range of extracellular manganese. However, the cellular manganese level of the *mntH* mutant was only about 60% of the wild type when grown in low manganese media. The experimental design, by necessity, underestimates the importance of *mntH* on manganese homeostasis because we must grow the cells at a manganese concentration (1 μM) that allows some MntH-independent uptake of Mn^{2+}. This lower affinity uptake activity is not observed in assays (Fig. 3) because 5 nM of $^{54}Mn^{2+}$ is used as the initial substrate concentration. Nevertheless, these observations, along with the growth phenotype of the mutant, shows that *mntH* is required to maintain manganese homeostasis under low manganese conditions.

Mn^{2+} transport and *mntH* expression are deregulated in a *fur* mutant

Manganese transport genes are regulated by Mn^{2+} via MntR in numerous bacterial species (Horsburgh *et al.*, 2002; Kehres *et al.*, 2002b; Patzer and Hantke, 2001; Que and Helmann, 2000), but *B. japonicum* and most rhizobial species do not have an *mntR* gene homolog in their genomes. However, microarray analysis revealed that *bll5044* (*mntH*) in *B. japonicum* is strongly regulated by Fur (Yang *et al.*, 2006b). Also, the Fur-like protein Mur mediates control of *sitABCD* in *S. meliloti* and *R. leguminosarum* (Chao *et al.*, 2004; Diaz-Mireles *et al.*, 2004; Platero *et al.*, 2004). Thus, we examined control of *mntH* in a *B. japonicum fur* strain by qPCR (Fig. 6). The strong Mn^{2+}-dependent expression of *mntH* transcript observed in the parent strain was lost in the *fur* mutant, showing high levels of transcript in the presence of Mn^{2+}. Thus, Fur normally negatively affects *mntH* expression in the presence of Mn^{2+}.

To further address the role of Fur on manganese metabolism, we examined high affinity Mn^{2+} transport in a *fur* strain (Fig. 3B). Transport of ^{54}Mn was very low in wild type cells grown in high manganese media, but was high in the *fur* strain, comparable to the activity of the parent strain grown in manganese-deficient media. Collectively, the observations show that Mn^{2+} transport is under Fur control via manganese-dependent regulation of the *mntH* gene.

The *mntH* gene is not regulated by iron

Although Fur mediates iron control of numerous *B. japonicum* genes (Friedman and O'Brian, 2004; Yang *et al.*, 2006b), and *mntH* is iron regulated in numerous bacteria (Guedon *et al.*, 2003; Kehres *et al.*, 2002b; Patzer and Hantke, 2001), it was not controlled by iron in the parent strain (Fig. 6). Furthermore, its aberrant expression in the *fur* mutant is not substantially affected by the iron status (Fig. 6). We conclude that *mntH* is not an iron-responsive gene, and that Fur controls this gene only in response to manganese.

Fur binds directly to the *mntH* promoter in vitro with high affinity in a metal-dependent manner

To determine whether Fur is a direct regulator of *mntH*, we determined the transcription start site using 5′ RACE and then analyzed the region upstream of it. DNaseI footprinting analysis showed that recombinant Fur bound the *mntH* promoter in the presence of Mn^{2+}, with maximum occupancy in the −56 to −22 region relative to the transcription start site (+1) (Fig. 7). In addition, a series of double-stranded oligonucleotides that collectively cover 171 bp upstream of the *mntH* transcription start site were analyzed by electrophoretic gel mobility shift assays (EMSA). The smallest DNA fragment tested that bound Fur was a 39 bp fragment that included −56 to −22 region, and no fragments lacking this region bound to Fur (data not shown).

To determine the affinity of Fur for its binding site on the *mntH* promoter, binding reactions containing 0.1 nM radiolabeled DNA were titrated with Fur, and bound DNA was analyzed by EMSA (Fig. 8) The dissociation binding constant (K_d) was 2.2 nM in the presence of Mn^{2+}, which is consistent with a role for Fur as a regulator of *mntH*.

Mn^{2+} transport activity and *mntH* expressions are regulated by manganese. Based on the current findings and the present understanding of Fur family proteins, the observed control is likely due to sensing Mn^{2+} by Fur through direct interactions of the protein with the metal, which affects DNA binding activity. This was addressed in several ways. Using atomic absorption spectroscopy, we established that manganese binds Fur directly, with a stoichiometry of 0.96 ± 0.01 manganese atoms per Fur monomer. The effect of metal on *mntH* promoter occupancy by Fur was assessed by DNase footprinting (Fig. 7). No protection was observed in the absence of metal in the binding reaction, but Fur bound the promoter in the presence of Mn^{2+}, showing that binding is metal-dependent. Furthermore, the affinity of Fur for *mntH* promoter DNA in the absence of metal was approximately 110 nM, over 50-fold weaker than in the presence of Mn^{2+} (Fig. 8). We conclude that Fur mediates Mn^{2+}-dependent control of the *mntH* gene.

The Fur binding site on the *mntH* promoter contains three imperfect direct repeat hexamers

EMSA analysis using 50 nM Fur shows a high mobility complex (HMC) and a low mobility complex (LMC), but an HMC is predominant with 5 nM Fur (Fig. 9). This is similar to what was observed for Fur binding to the *irr* promoter, which corresponded to a dimer and a tetramer or two dimers (Friedman and O'Brian, 2003). Comparison of the *mntH* and irr promoters by EMSA using the DNA probes of equal size (39 bp) revealed that the HMC and LMC ran with the same mobilities, indicating that the *mntH* promoter is also occupied as a dimer at low Fur concentrations and two dimers or tetramer at high Fur concentrations (data not shown).

Examination of the protected region of DNA on the *mntH* promoter or the minimal binding DNA in EMSA shows that the sequence contains three imperfect direct repeat hexamers, similar to that observed within the *irr* promoter (Friedman and O'Brian, 2003). However, the Fur/Mur binding site of the *S. meliloti* or *R. leguminosarum sitA* promoter best fits a palindromic inverted repeat sequence (Diaz-Mireles *et al*., 2004; Platero *et al*., 2007) (Fig. 9), and a bioinformatic search for Fur/Mur binding sites in the rhizobia also defines the consensus as palindrome as well (Rodionov *et al*., 2006). Thus, we determined the effects of mutation of each repeat within the *mntH* promoter on Fur binding using EMSA (Fig. 9). Substitution mutations within direct repeat 1 or 2 (mut1 or mut2) abrogated binding with 5 nM Fur as observed by the loss of the HMC, but DNA was bound with 50 nM Fur. Mutation of the direct repeat 3 (mut3) abrogated Fur binding completely with 5 or 50 nM Fur in the binding reaction. These observations and previous work (Friedman and O'Brian, 2003) support the conclusion that the Fur binding site is best described as a three direct repeat sequence of hexamers in *B. japonicum*.

The *mntH* gene is not required for development of symbiosis with soybean

The parent strain and *mntH* mutant were used to inoculate soybean seedlings, and symbiotic properties of the resultant nodules from plants 26 days post-inoculation. The parent strain elicited an average of 49.3±6.4 nodules per plant, and the mutant 52.2±3.6 nodules per plant (n=5). Acetylene reduction activity, which is a measurement of nitrogen fixation activity, was 10.6±1.2 and 12.8±2.0 μmol ethylene produced/h/g nodule fresh weight for nodules produced from the parent strain and *mntH* mutant, respectively. Thus, *mntH* is not essential for the development of nodules or for symbiotic nitrogen fixation activity.

DISCUSSION

In the present study, we established that *B. japonicum* can maintain manganese homeostasis, as observed by an almost constant intracellular manganese level when grown in 1 μM or 50 μM manganese (Fig. 5). We identified *bll5044* as the *mntH* homolog in *B. japonicum*, and demonstrate that it encodes the primary Mn^{2+} transporter in that organism. MntH contributes to homeostasis as observed by the lower manganese level in the *mntH* strain compared to the wild type (Fig. 5). Consistent with this, the mutant was unviable in media containing 0.2 μM manganese, a concentration sufficient for growth of the parent strain (Fig. 4). Mn^{2+} transport activity was induced under manganese limitation in the parent strain, and this activity was abrogated in the *mntH* strain (Figs. 1A, 3A). Regulation of this activity was found to be due to control by the Fur protein, which binds directly to the *mntH* promoter in the presence of Mn^{2+} to repress gene expression (Figs. 3B, 6, 7). Thus, Fur is a Mn^{2+}-responsive regulator in *B. japonicum.*

Two additional weak *mntH* homologs, *bll6117* and *bll8038*, were also identified based on sequence similarity. We are not aware of any bacterium that has multiple *mntH* genes. It is possible that they transport a different cation, but the genes were expressed at very low levels (Fig. 2), and therefore they were not further characterized.

The strong phenotypes observed for the *B. japonicum mntH* strain are in stark contrast to those described in other bacterial species. In those cases, *mntH* mutants show approximately wild type levels of growth in metal-limited media, cellular manganese content, Mn^{2+} transport activity, manganese toxicity and pathogenicity (Boyer *et al.*, 2002; Domenech *et al.*, 2002; Horsburgh *et al.*, 2002; Kehres *et al.*, 2000; Makui *et al.*, 2000; Que and Helmann, 2000). The lack of phenotypes has made it necessary to study the properties of MntH by overexpression from high copy plasmids (Kehres *et al.*, 2000; Makui *et al.*, 2000). *S. enterica, B. subtilis.* and *Staphylococcus aureus* have *sitABCD* as well as *mntH*, which likely accounts for the lack of phenotypes for the *mntH* strain. However, *sitABCD* does not appear to be present in the *E. coli* K12 genome, and presumably Mn^{2+} can be taken up by a heretofore unidentified transporter.

Among the rhizobia, *sitA* mutants of *S. meliloti* have numerous phenotypes that can be rescued by addition of exogenous manganese (Chao *et al.*, 2004; Davies and Walker, 2007; Platero *et al.*, 2003), strongly supporting a role for SitABCD in manganese acquisition in that organism, and raises the possibility that it is the only Mn^{2+} transporter expressed. *S. meliloti* has a weak *mntH* gene homolog (*sma1115*) based on similarity to *B. japonicum mntH*, but it is not homologous over the entire length, and has not been studied. A modest *sitA* homolog is found in the *B. japonicum* genome (bll7769, 32% identity to *S. meliloti sitA*) adjacent to two additional putative ABC-type transporter genes. However ABC-type transporter proteins are similar to each other at the amino acid sequence level, and thus homology is usually not sufficient to determine the solute substrate. The Mn^{2+} transport defect of the *mntH* strain indicates that *bll7769* is not required for uptake, and it may transport a different solute.

We found that *B. japonicum mntH* is not essential for establishing a symbiosis with soybean. This suggests either that the host provides a sufficiently rich manganese milieu that renders high affinity transport unnecessary, or else the bacterium has another mechanism for manganese acquisition that is not expressed in free living cells. It is intriguing that *B. japonicum* and *S. meliloti* use different systems to transport manganese into cells. In *Salmonella enterica* serovar Typhimurium, MntH activity has a pH optimum of 5.5–6.0, whereas the optimum for SitABCD is greater than 8.0 (Kehres *et al.*, 2002a). This correlates with the optimal growth for soybean, the *B. japonicum* plant host, at moderately acidic soils (www.nsrl.uiuc.edu/aboutsoy/production02.html), whereas *Medicago* hosts of *S. meliloti*

prefer more alkaline soils (Garau *et al.*, 2005). Although *B. japonicum mntH* is not essential for symbiosis with soybean, it is nevertheless plausible that it has adapted to the same niche as its host. Further experiments are needed to test this idea.

Both SitABCD and MntH can utilize Fe^{2+} as well as Mn^{2+} in numerous bacterial species, and the genes that encode them are also regulated by iron at the mRNA level (Bearden and Perry, 1999; Boyer *et al.*, 2002; Ikeda *et al.*, 2005; Kehres *et al.*, 2000; Kehres *et al.*, 2002a, b; Makui *et al.*, 2000; Patzer and Hantke, 2001; Runyen-Janecky *et al.*, 2003). However, *B. japonicum mntH* gene was not regulated by iron (Fig. 6), nor was the growth phenotype of a *mntH* mutant rescued by iron (Fig. 4). In addition, Fe^{2+} did not compete with $^{54}Mn^{2+}$ for transport of wild type cells even at 500-fold excess of the radiolabeled metal (Fig. 1B). These observations indicate that MntH does not have broad substrate specificity, but is specific for Mn^{2+}.

We demonstrate that Fur mediates manganese-dependent expression of *B. japonicum mntH*. Fur also controls *mntH* expression in *E. coli* (Patzer and Hantke, 2001), *S. enterica* (Kehres *et al.*, 2002b) and *B. subtilis* (Guedon *et al.*, 2003), but in those systems Fur mediates regulation by iron, and control by manganese requires MntR. MntR appears to be absent in rhizobia based on homology, with the possible exception of *Mesorhizobium loti. B. japonicum* Fur has been described previously as an iron-responsive regulator (Friedman and O'Brian, 2004; Yang *et al.*, 2006b), and therefore its role in manganese-dependent gene expression is a novel function. However, the Fur protein in *S. meliloti* and *R. leguminosarum* (also called Mur) mediates manganese control of the *sitABCD* operon (Chao *et al.*, 2004; Diaz-Mireles *et al.*, 2004; Platero *et al.*, 2004; Platero *et al.*, 2007), and is both manganese and iron responsive in *Agrobacterium tumefaciens* (Kitphati *et al.*, 2007). In vitro characterization of the *R. leguminosarum* Fur/Mur protein shows that it binds Fe^{2+}, Mn^{2+} and other divalent metals with similar affinities, and that binding of the holo-protein to DNA requires metal, but is not strongly dependent on which divalent metal is bound (Bellini and Hemmings, 2006). Thus, the basis of metal discrimination appears not to be at the level of binding affinity. It is plausible that metal chaperones play a role in metal discrimination, but there is no direct evidence for it. Although this could be unique to the rhizobia, a role for Fur in both Fe^{2+} and Mn^{2+} control of gene expression has been described in *Yersinia pestis* (Bearden *et al.*, 1998). Guedon et al (Guedon *et al.*, 2003) propose that the effects of Mn^{2+} on the Fur regulon in *B. subtilis* is an indirect consequence of disruption of the cellular iron pool. This is clearly not the case in *B. japonicum* with respect to *mntH* since the gene is not regulated by iron (Fig. 6).

B. japonicum Fur protected a three imperfect hexameric repeat sequence in footprinting assays (Fig. 7), and mutation of any of the repeats affected Fur binding (Fig. 9), similar to what was previously observed for the *irr* promoter (Friedman and O'Brian, 2003). Thus, this motif best represents the Fur-binding site in *B. japonicum*, which differs from the inverted repeat described for the *sitA* promoter of *R. leguminosarum* and *S. meliloti* (Diaz-Mireles *et al.*, 2004;Platero *et al.*, 2007), or predicted based on bioinformatic analysis (Rodionov *et al.*, 2006).

EXPERIMENTAL PROCEDURES

Strains and media

B. japonicum USDA110 was the parent strain used in this study. Strains GEM4 (Hamza *et al.*, 1999) and *mntHΩΔ* are mutant derivatives of the parent strain containing a DNA cassette encoding spectinomycin and streptomycin replacing the *fur* and *mntH* genes, respectively. *B. japonicum* strains were routinely grown at 29°C in glycerol-salts-yeast extract (GSY) medium as described previously (Frustaci *et al.*, 1991). Strains GEM4 and *mntHΩΔ* were grown in the presence of 25 μg/ml streptomycin and 100 μg/ml spectinomycin. For low manganese conditions, modified GSY medium was used, containing 0.5 g/L yeast extract instead of 1 g/

l, with either no exogenous manganese or 1 μM $MnCl_2$ added prior to growth. For low iron conditions, the same modified GSY medium was used, with no exogenous iron. The actual concentrations of manganese and iron in the unsupplemented media are 0.2 μM and 0.3 μM, respectively, as determined by atomic absorption using a Perkin-Elmer model 1100B atomic absorption spectrometer. High metal medium was supplemented with either 50 μM $MnCl_2$, 20 μM $FeCl_3$, or both.

Manganese uptake assay

Cells were grown to mid-log phase (OD_{540} 0.4–0.6) in low- or high- manganese media, harvested by centrifugation, washed twice and resuspended in uptake buffer (0.2 M MOPS and 2% (w/v) glycerol, pH 6.8) to a OD_{540} of 0.4. 30 mL of cell solution were placed into a 125-ml Erlenmeyer flask and preincubated 15 minutes at room temperature with shaking. At time zero, 5 nM ^{54}Mn was added to the cell solution. 1-ml aliquots were removed at various time points and added to ice cold quench buffer (0.1 M Tris and 100 μM $MnCl_2$, pH 6.0). The cells were collected immediately after quenching on 0.45μm filters presoaked in quench buffer. Cells were washed with 3 mL ice cold quench buffer and counted using a Wallac 1480 Wizard™ 3″ automatic gamma counter. Internalized ^{54}Mn levels were normalized to protein levels in the cell. ^{54}Mn Uptake Competition Assays were done as described above in the presence or absence of 1 μM $MnCl_2$, or 1 μM $FeSO_4$. The competing metal was added to the cell solution immediately prior to the start of the assay.

Construction of a *B. japonicum mntH* mutant

An *mntH*-deletion strain was constructed for this study. The open reading frame of *mntH* (*bll5044*) 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 described previously (Panek and O'Brian, 2004) and replaced with an Ω-cassette encoding for streptomycin and spectinomycin resistance (Prentki and Krisch, 1984). The construct was introduced into pLO1 (Lenz *et al.*, 1994), mobilized into USDA110, and selected for double recombinant mutants as previously described (Panek and O'Brian, 2004). Mutants were confirmed using PCR and antibiotic resistance.

Bacterial growth studies

The parent strain and *mntHΩΔ* were grown in modified GSY medium under high (50 μM $MnCl_2$) or low (0.2 μM) manganese conditions supplemented with or without 20 μM $FeCl_3$ as described above. Growth rates were analyzed by measuring the optical density of cells at 540 nm every six hours until reaching stationary phase.

Determination of intracellular manganese content

Steady state levels of internalized manganese was determined using Atomic absorption spectroscopy. 40 ml of cultures grown to mid-log phase (OD_{540} 0.4–0.6) were harvested by centrifugation. The pellet was washed twice with 40 ml ice cold 0.1 M Tris and once with double distilled metal free water to remove salts. Cells were lysed by resuspending the pellet in 100 μl of 70% HNO_3 (J.T. Baker, AAS grade), lightly vortexed and incubated at 75°C for 5 minutes. 1 ml metal-free double distilled water was added to the lysed cells and mixed by vortex. Samples were centrifuged at 13 000 r.p.m. for 5 minutes and the supernatant was analyzed for Mn content. Atomic absorption was performed in the furnace mode on a Perkin Elmer Atomic Absorption Spectrometer model 1100B equipped with a model HGA 700 graphite furnace. All samples were diluted with metal free double distilled water to contain 1% HNO_3. A Mn standard curve was set-up by diluting a stock solution of Mn (1mg/ml in 2% HNO_3, Perkin Elmer) ranging from 0.1 ng/10 μl to 1.0 ng/10 μl in 1% HNO_3. 10 μL of sample was used for each measurement, with each sample being performed in triplicate. Samples were

run at 120°C for 40 seconds, 5 second ramp to 1000°C and held for 20 seconds, 2200°C for 5 seconds, and 2300°C for 5 seconds. Mn content was normalized to protein level of the cells. The manganese content of modified GSY medium, containing no exogenous manganese, in 1% HNO_3 was measured on a Perkin Elmer Atomic Absorption Spectrometer model 1100B using the protocol above.

Analysis of RNA

Expression levels of selected genes were determined by quantitative real real time PCR (qPCR) with iQ™ SYBR green supermix (Bio-Rad) using iCycler thermal cycler (Bio-Rad). RNA was isolated from *B. japonicum* cells using a hot phenol method as described previously (Yang *et al.*, 2006a). cDNA was synthesized from 2 μg total RNA using iScript™ cDNA Synthesis Kit (Bio-Rad). Each PCR reaction contained 10 μl 2 × SYBR green supermix, 0.2 μM primers (IDT DNA Technology) and 25ng cDNA in a 20 μl volume. PCR reactions were heated to 95° C for 3 minutes, followed by 40 cycles with steps of 95°C, 56°C, and 72°C for 30 seconds each. The generation of specific PCR products was confirmed using melting curve analysis. RNA samples in which the iScript reverse transcriptase was omitted were used as negative controls. The standard curve method was used for relative quantification of the template, with *gapA* being employed as a housekeeping gene control. Genomic DNA from *B. japonicum* USDA110 was used as PCR templates to generate a standard curve for each gene. Relative starting quantities (SQ) of the mRNAs for the genes of interest and *gapA* were calculated from the corresponding standard curves. Quantity of the interested genes was normalized to the quantity of *gapA* for each respective condition. The results were based on average of triplicates and the standard deviation is shown as the error bar in the results.

The transcriptional start site of *mntH* was determined utilizing 5′ RACE System kit (Invitrogen) as per manufacturer's protocol. RNA was isolated from *B. japonicum* USDA110 as described above. 5 μg total RNA was used for 5′ RACE.

DNase I Footprinting Analysis

DNase I Footprinting analyses examined the DNA region protected by Fur. Fur was incubated for 30 minutes at room temperature in a 50 μl volume of EMSA binding buffer containing 125 ng dI.dC, 5 μg of bovine serum albumin, and 1nM radiolabeled *mntH* probe in the presence or absence of 100 μM $MnCl_2$. The labeled DNA template was synthesized by PCR. The 5′ end of the forward primer was radiolabeled using [γ-^{32}P]dATP (6000Ci/mmol) (Perkin Elmer) and Polynucleotide Kinase (Promega). 50 μl of room temperature solution of 5mM $CaCl_2$ and 10mM $MgCl_2$ was added to the binding reaction and allowed to incubate for 1 min before the addition of 0.45 units of RQ1 RNase-free DNase (Promega) in 5 μl of 40mM Tris-HCl, pH 7.0. Reactions were incubated for 1 minute at room temperature before being stopped with the addition of 90 μl stop solution (200mM NaCl, 30mM EDTA, 1% SDS, 100 μg/ml yeast RNA). DNA was extracted from the reaction using phenol:chloroform (1:1) followed by ethanol precipitation. G + A ladders were produced as described (Maxam and Gilbert, 1980). DNase I digested products were separated on an 8% denaturing polyacrylamide gel containing 7 M urea in Tris borate EDTA electrophoresis buffer. After electrophoresis, gel was exposed on Imaging Screen K, 35 × 45cm (Bio-Rad), and scanned using a Personal Molecular Imager FX (Bio-Rad).

Electrophorectic mobility shift assay

EMSA analysis was employed to study Fur binding to DNA using a protocol modified from de Lorenzo *et al* (de Lorenzo *et al.*, 1988). Fur was incubated 30 minutes at room temperature in a 20-μl volume of EMSA buffer, consisting of 50 ng dI.dC, 2 μg bovine serum albumin, 100 μM $MnCl_2$, and 1 nM radiolabeled DNA probe. Double stranded DNA probes were produced by boiling and slowly cooling synthetic DNA oligonucleotides (Integrated DNA technologies)

in annealing buffer (150 mM $NaCl_2$, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and filled in with [α-^{32}P]dCTP (3000 Ci/mmol) (Perkin Elmer) with the klenow fragment of DNA polymerase (Promega). Following the incubation, EMSA reactions were analyzed on a 5% non-denaturing polyacrylamide gel in electrophoresis buffer (20 mM bis-Tris borate, 100μM $MnCl_2$, pH 7.5) that were prerun for 30 minutes at a constant 200 V at 4°C. After electrophoresis at 4°C for 60 minutes at 200 V, gels were dried and autoradiographed. Autoradiograms were developed on BioMax film (Eastman Kodak Co.) and scanned using a GS-700 densitometer (Bio-Rad). Signal intensities were detected and quantified using Quantity One (Bio-Rad). To determine the dissociation constant (K_d), binding reactions were titrated with a varying amount of Fur. Bound and unbound DNA was quantified by comparing relative signal intensities and analyzed using Graphpad Prism (Graphpad Software Inc.). DNA probes used to represent the Fur binding site in the *mntH* promoter were synthesized as overlapping fragments and compared by EMSA.

Plant growth, infection and assays

Soybeans (Glycine max cv. Essex) were inoculated with the parent strain or *mntHΔΩ* and grown in an environmental growth chamber under a 16 h day/8 h dark regime as described previously (Frustaci and O'Brian, 1992). Nitrogen fixation in root nodules from plants 26 days post-inoculation was assessed as the reduction of acetylene to ethylene as described previously (Frustaci and O'Brian, 1992).

Acknowledgements

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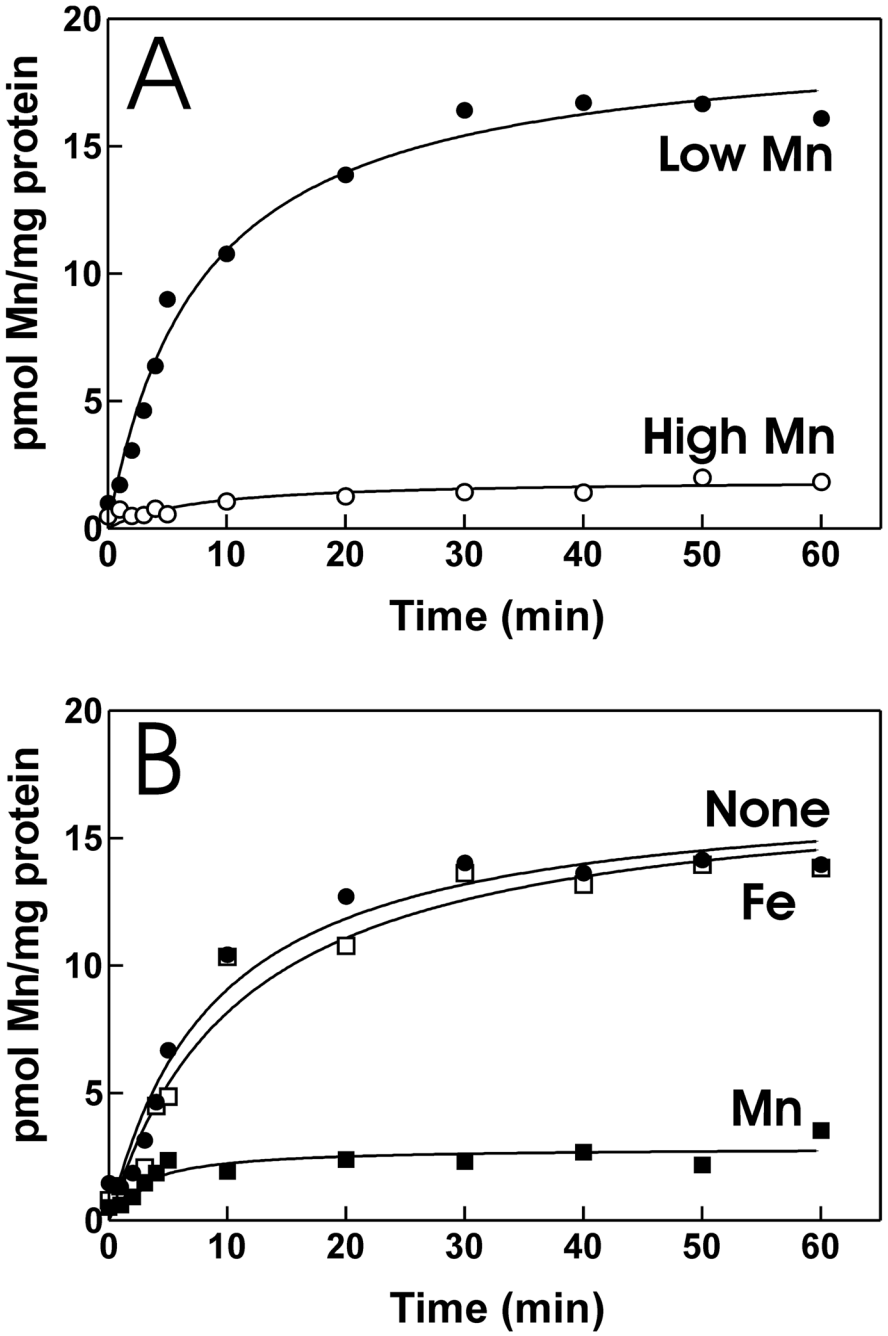

Fig. 1. Mn^{2+} uptake by *B. japonicum.*

A. $^{54}Mn^{2+}$ uptake was measured in parent strain USDA110 in cells grown in media containing 1 μM (closed circles) or 50 μM $MnCl_2$ (open circles). At time zero, 5 nM $^{54}Mn^{2+}$ was added to the cells in assay medium, and aliquots were subsequently taken at various time points and counted. B. Competition of $^{54}Mn^{2+}$ uptake with unlabeled Mn^{2+} or Fe^{2+}. Cells of the parent strain were grown in media containing no added manganese to induce Mn^{2+} transport activity. The assay medium containing cells was supplemented with either no metal (closed circles) or with 1 μM $MnCl_2$ (closed squares) or 1 μM $FeSO_4$ (open squares) immediately prior to addition of 5 nM ^{54}Mn.

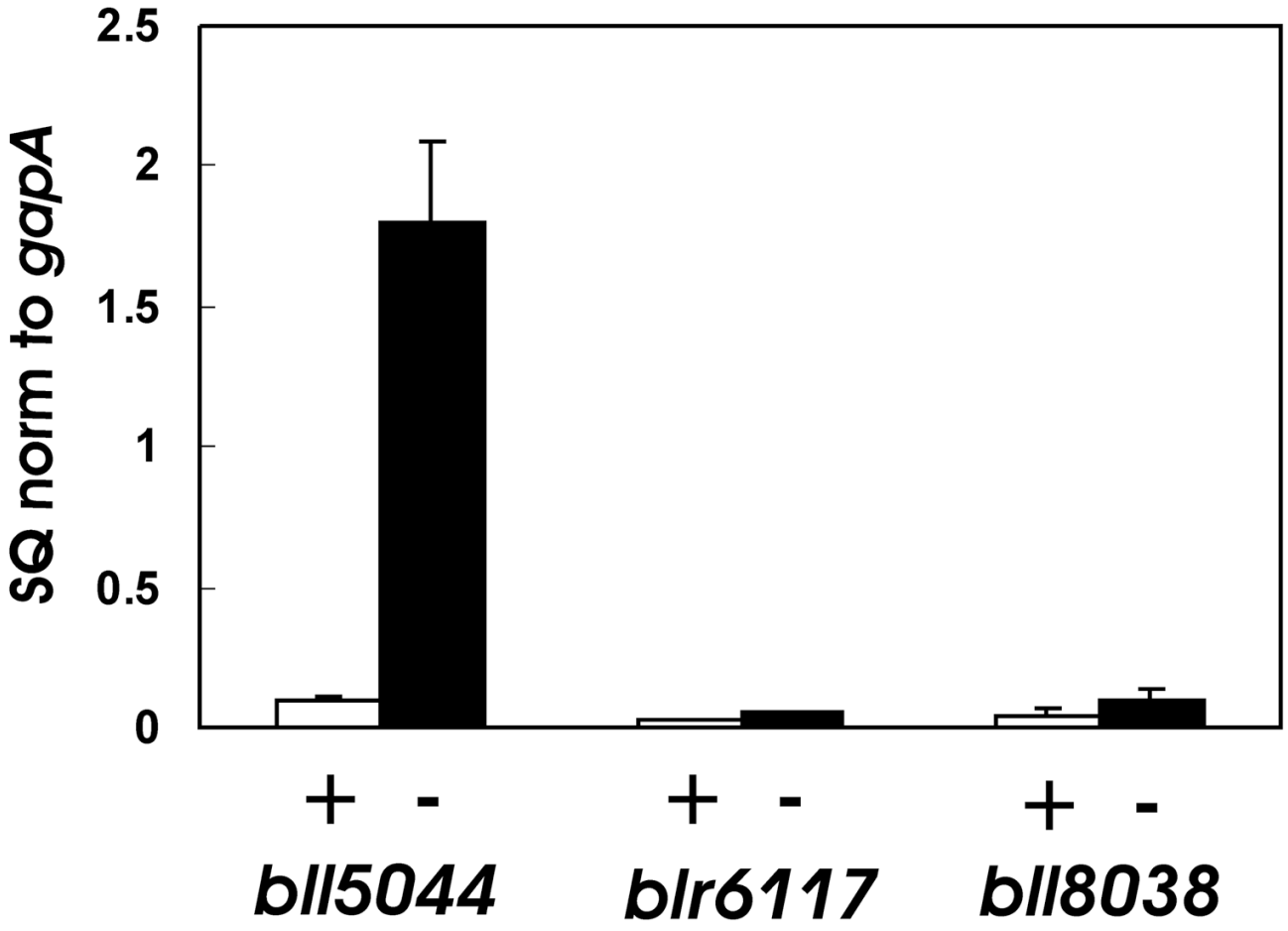

Fig. 2. Manganese-dependent expression of putative *mntH* homologs

mRNAs were analyzed by quantitative real-time PCR from cells grown in media supplemented with no manganese (solid bars) or with 50 μM $MnCl_2$ (open bars). The data are expressed as the relative starting quantity (SQ) of the respective mRNAs normalized to the housekeeping gene *gapA*. The data are expressed as the average of three replicates with error bars representing the standard deviation.

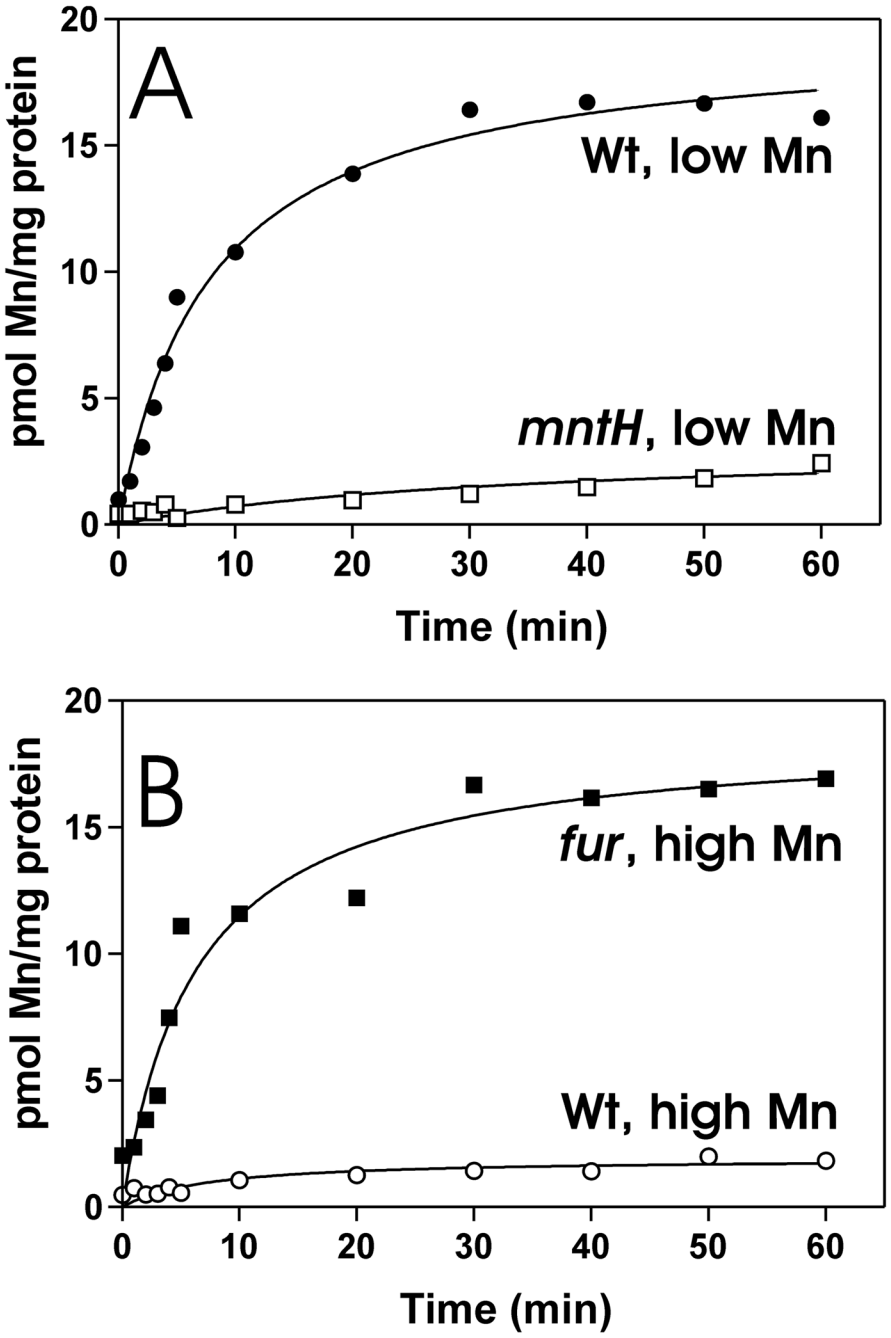

Fig. 3. Mn^{2+} uptake by *B. japonicum* mutants

A. Cells of the parent strain (closed circles) or the *mntH* mutant strain (open squares) were grown in media containing 1 μM $MnCl_2$. At time zero, 5 nM ^{54}Mn was added to the assay medium and aliquots were subsequently taken at various time points and counted. The uptake data of the parent strain cells grown in low manganese are the same as in Fig. 1A, and are shown in two different panels for clarity of presentation.

B. Cells of the parent strain (open circles) or the *fur* mutant (closed squares) were grown in media containing 50 μM $MnCl_2$. At time zero, 5 nM ^{54}Mn was added to the assay medium and aliquots were subsequently taken at various time points and counted. The uptake data of the

parent strain cells grown in high manganese media are the same as in Fig. 1A, and are shown in two different panels for clarity of presentation.

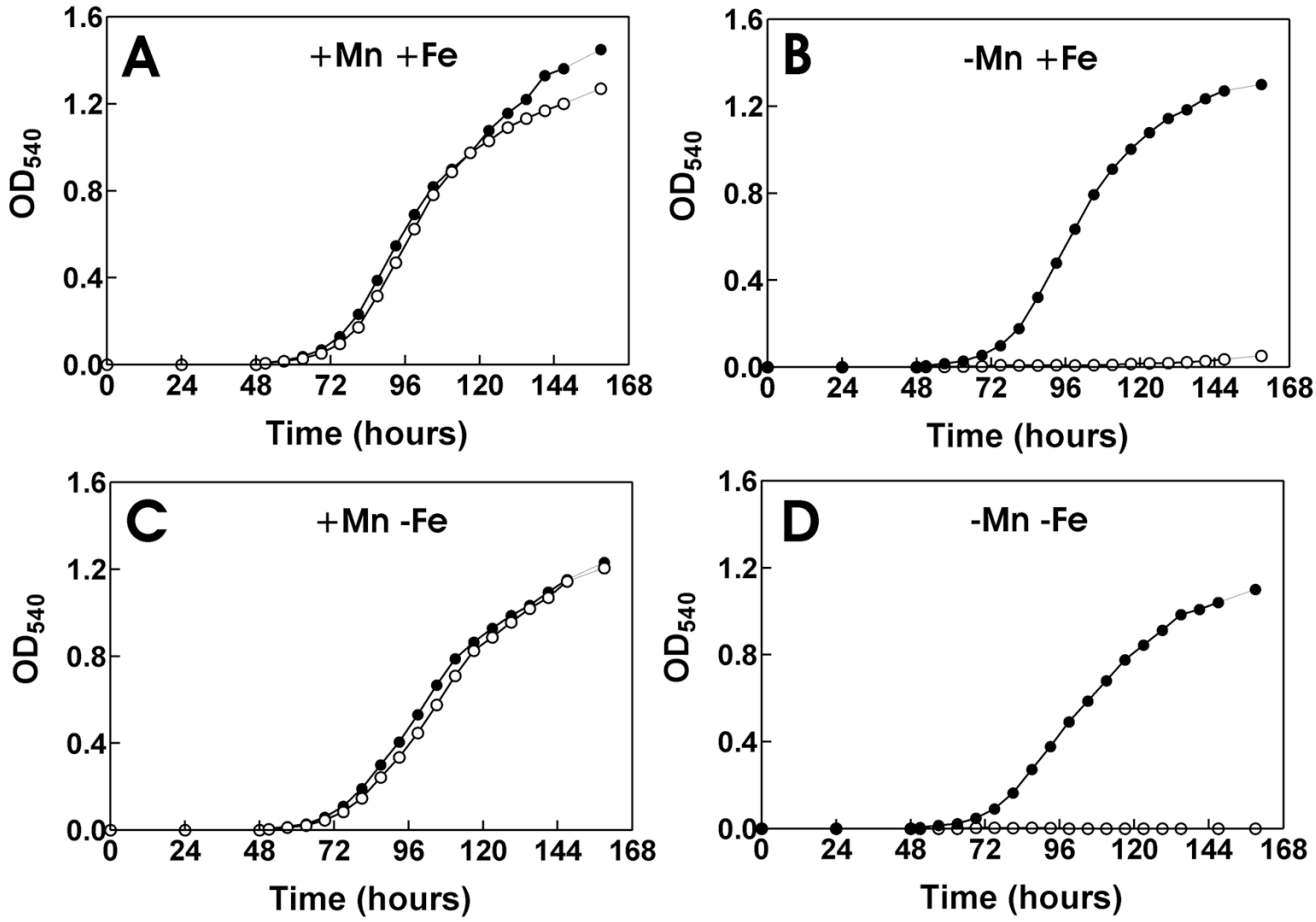

Fig. 4. Dependence on manganese or iron supplementation for growth of *B. japonicum* parent or *mntH* strains

Growth media were inoculated with 5×10^5 cells/ml of parent strain (closed circles) or the *mntH* mutant (open circles) and grown in media containing (A) 50 μM $MnCl_2$ and 20 μM $FeCl_3$, (B) no exogenous manganese and 20 μM $FeCl_3$, (C) 50 μM $MnCl_2$ and no exogenous iron, or (D) no exogenous manganese and no exogenous iron. Unsupplemented media contains 0.2 μM and 0.3 μM manganese and iron, respectively. Aliquots were taken at the indicated time points and the optical density was measured at 540 nm (OD_{540}).

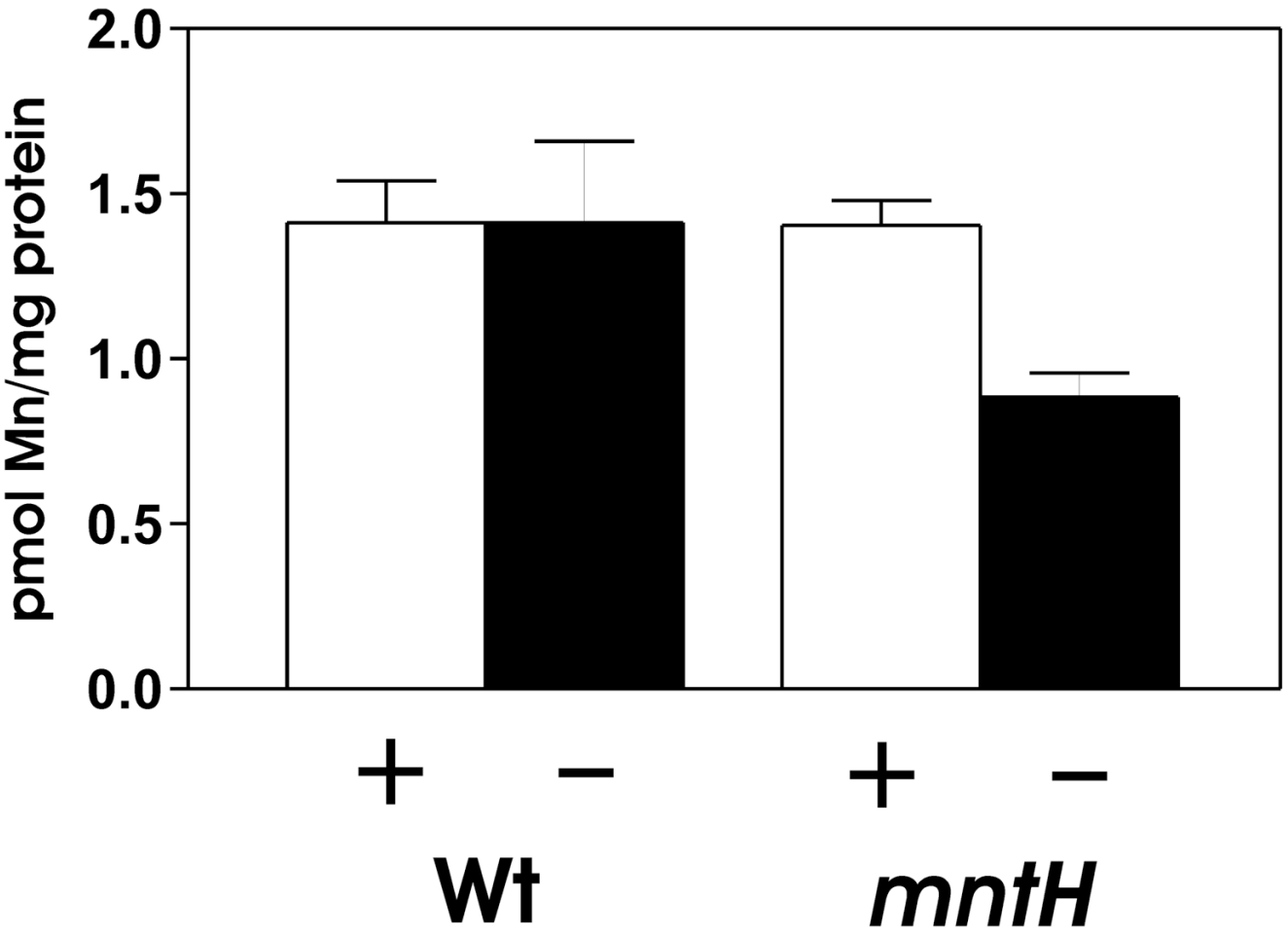

Fig. 5. Cellular manganese content of the parent strain and *mntH* mutant grown in low or high manganese media

Cells were grown in media supplemented with 1 μM $MnCl_2$ (−, solid bars) or 50 μM $MnCl_2$ (+, open bars). Manganese content of whole cells was determined by absorption spectroscopy. The data are based on triplicate samples with error bars representing the standard deviation.

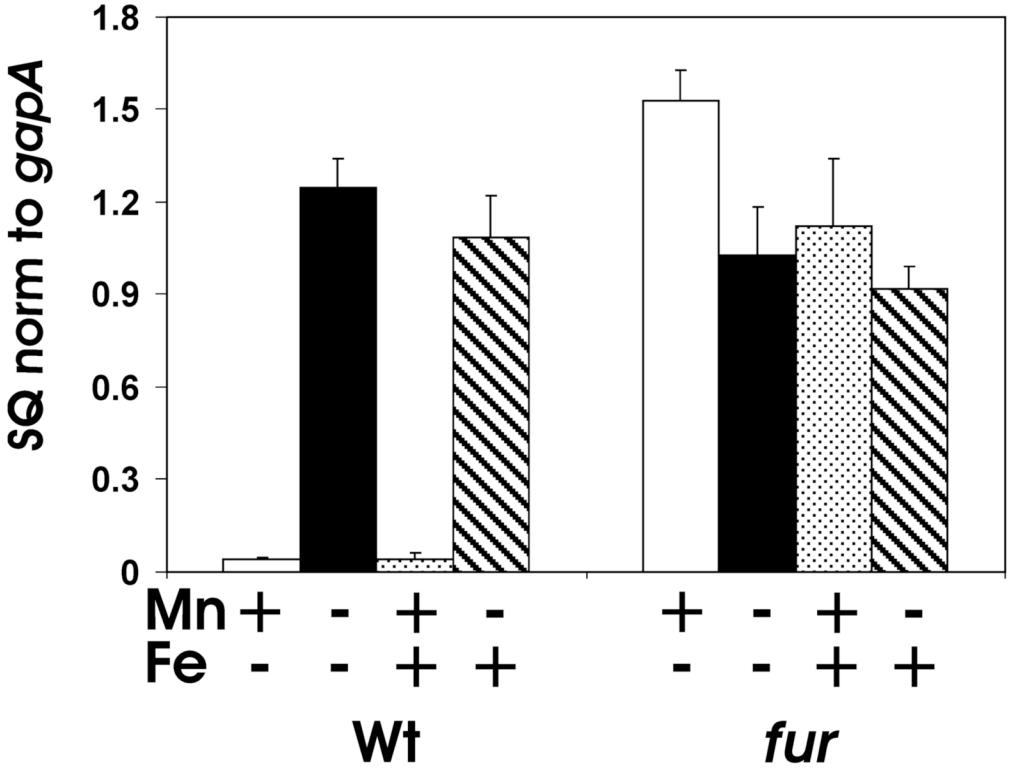

Fig. 6. Regulation of *mntH* gene mRNA by manganese and iron in the parent strain and a *fur* mutant

mRNAs were analyzed by quantitative real-time PCR from cells grown in media supplemented with no metal (black bars), 50 μM $MnCl_2$ (white bars), 20 μM $FeCl_3$ (striped bars) or both $MnCl_2$ and $FeCl_3$ (stippled bars). The data are expressed as the relative starting quantity (SQ) of the respective mRNAs normalized to the housekeeping gene *gapA*, and presented as the average of triplicate samples ± the standard deviation.

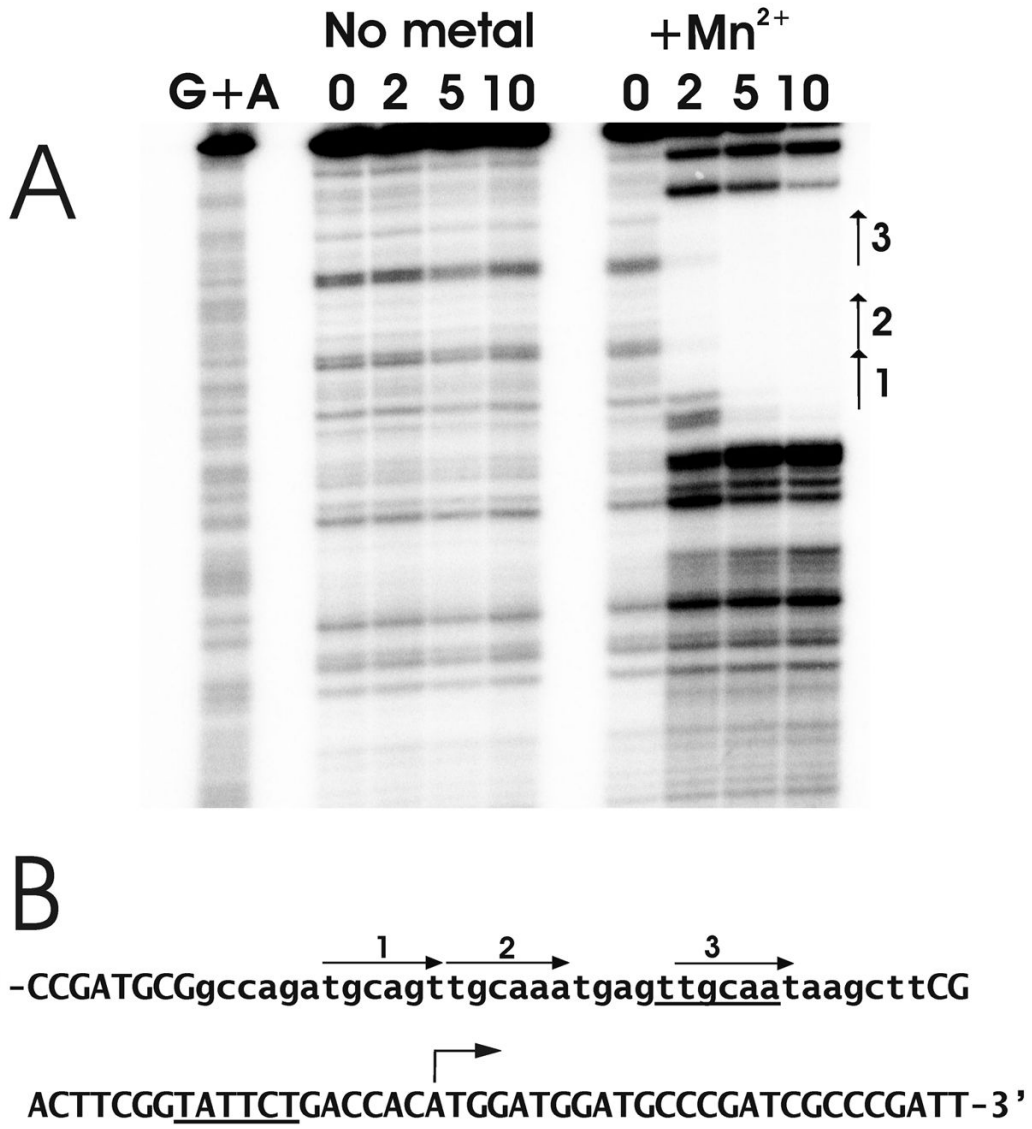

Fig. 7. DNase I Footprinting of the *mntH* promoter with *B. japonicum* Fur in the presence and absence of Mn^{2+}

A. Protection of DNA from DNase I digestion by Fur was carried out in the presence or absence of $MnCl_2$ using 0, 2, 5 or 10 nM Fur. The DNA was radiolabeled at the 5′ end of the non-template strand with respect to the mntH gene, and thus the 3′ end is at the top of the gel. The *arrows* on the *right* denote the sites of the three hexamer repeats.

B. The sequence of the protected region of *mntH* and the direct repeats are shown. The lower case letters show the protected region. The bent arrow represents the transcription start site. The direct repeats are shown by the straight arrows. The underlined sequences show the putative −10 and −35 regions of the promoter.

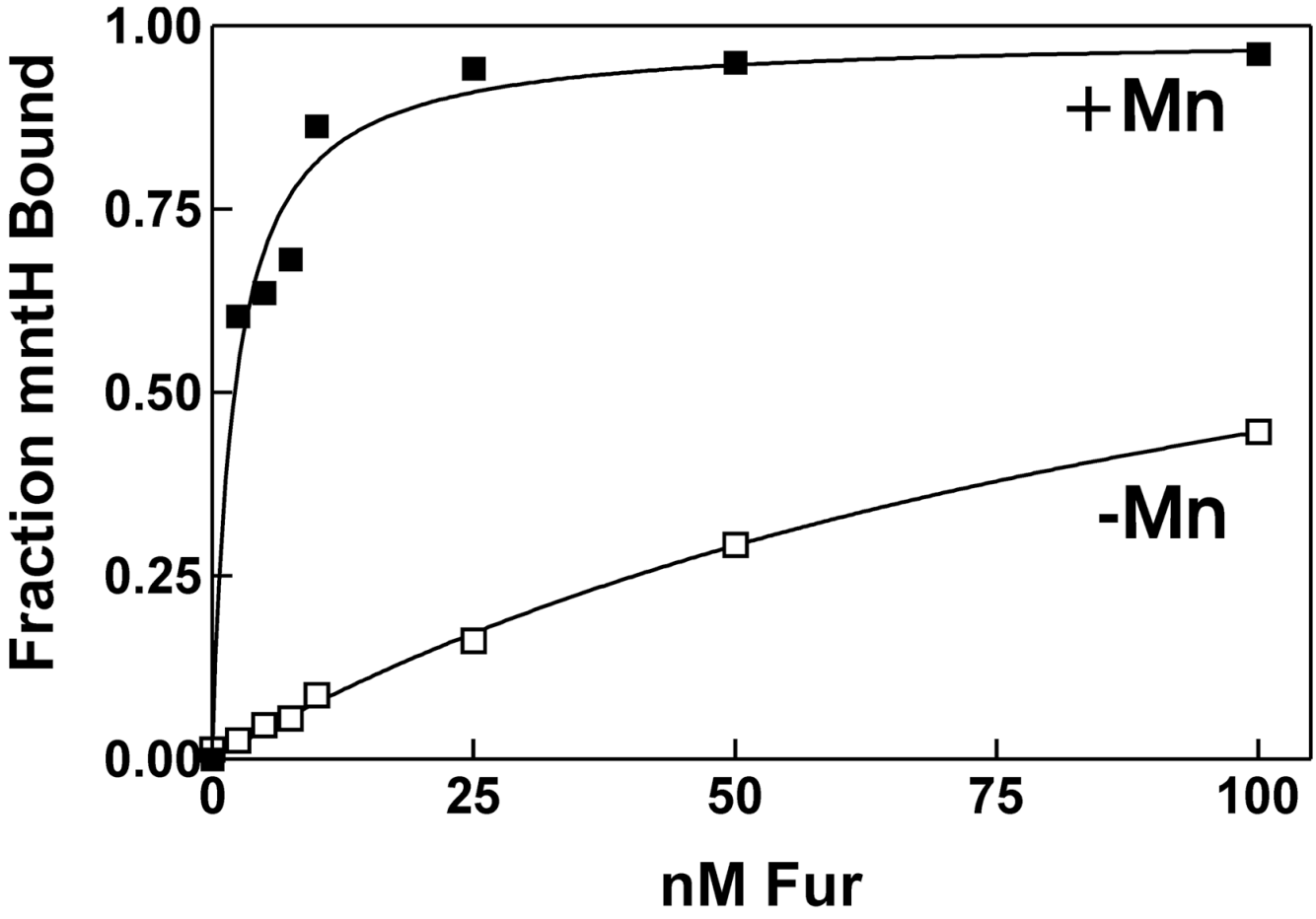

Fig. 8. Effect of manganese on *B. japonicum* Fur binding to the *mntH* promoter

EMSA was carried out using 100 pM ^{32}P-labelled *mntH* promoter DNA titrated with various concentrations of Fur in the presence (closed squares) or absence (open squares) of 100 μM $MnCl_2$. Bound and unbound DNA was resolved on a 5% non-denaturing polyacrylamide gel and visualized by autoradiography. Autoradiograms were scanned, and bands were quantified to determine bound and unbound DNA.

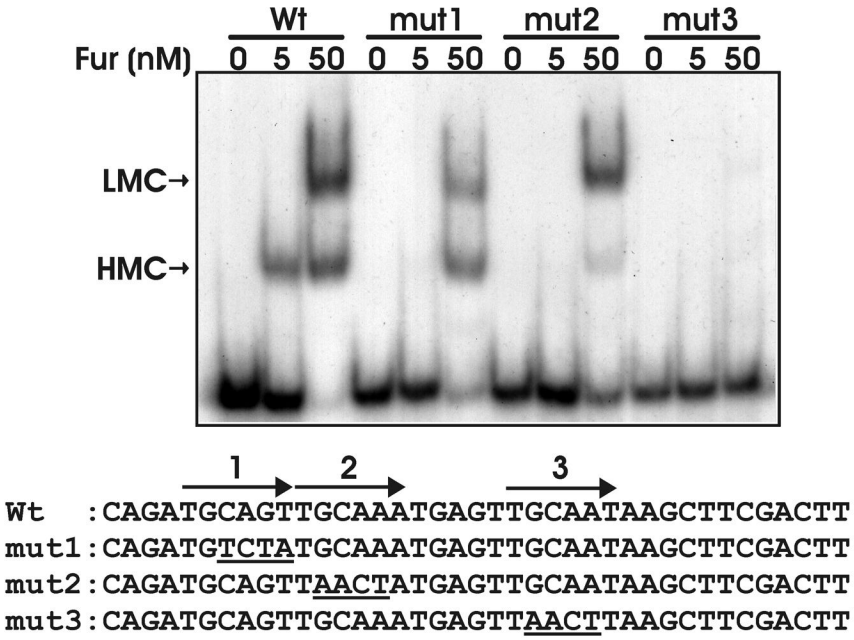

Fig 9. Effect of mutation of direct repeat sequences of the *mntH* promoter on formation of a high mobility complex (HMC) and low mobility complex (LMC) with Fur

EMSAs were carried out using purified recombinant Fur (0, 5, or 50 nM) and either the wild type (Wt) 39-bp *mntH* promoter region DNA or DNA containing substitution mutations in one of the direct repeat sequence DNA. The complexes were resolved on a 5% non-denaturing polyacrylamide gel and visualized by autoradiography.