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A Dominant-Negative *fur* Mutation in *Bradyrhizobium japonicum*

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In many bacteria, the ferric uptake regulator (Fur) protein plays a central role in the regulation of iron uptake genes. Because iron figures prominently in the agriculturally important symbiosis between soybean and its nitrogen-fixing endosymbiont *Bradyrhizobium japonicum*, we wanted to assess the role of Fur in the interaction. We identified a *fur* mutant by selecting for manganese resistance. Manganese interacts with the Fur protein and represses iron uptake genes. In the presence of high levels of manganese, bacteria with a wild-type copy of the *fur* gene repress iron uptake systems and starve for iron, whereas *fur* mutants fail to repress iron uptake systems and survive. The *B. japonicum fur* mutant, as expected, fails to repress iron-regulated outer membrane proteins in the presence of iron. Unexpectedly, a wild-type copy of the *fur* gene cannot complement the *fur* mutant. Expression of the *fur* mutant allele in wild-type cells leads to a *fur* phenotype. Unlike a *B. japonicum fur*-null mutant, the strain carrying the dominant-negative *fur* mutation is unable to form functional, nitrogen-fixing nodules on soybean, mung bean, or cowpea, suggesting a role for a Fur-regulated protein or proteins in the symbiosis.

Rhizobia live in the soil or engage in symbiosis with a suitable legume. Each environment presents unique challenges with respect to iron acquisition. As free-living soil microorganisms, rhizobia must have a way to solubilize iron as well as a way to compete for this nutrient with other organisms present in the rhizosphere. As endosymbionts, rhizobia must have mechanisms for acquiring iron from the host plant.

In many microbes, including various species of rhizobia, iron deficiency induces a variety of high-affinity iron uptake systems that are involved in the solubilization and sequestration of Fe(III) (6). These systems are composed of siderophores, high-affinity Fe(III) chelators that are released by cells to scavenge Fe(III), and their specific uptake systems. In gram-negative bacteria, siderophore uptake requires a TonB-dependent outer membrane protein, a periplasmic binding protein, and a cytoplasmic membrane ATP-binding cassette (ABC) transporter system. Both siderophore biosynthetic genes and the genes for Fe(III)-siderophore uptake systems are only expressed under iron-limiting conditions and have been shown to be negatively controlled by the Fur repressor protein (reviewed in reference 16). Fur regulation appears to be highly conserved among most bacterial species. *fur* genes have been identified in numerous gram-positive or gram-negative bacteria, including *Bradyrhizobium japonicum* (14) and *Rhizobium leguminosarum* (5).

Although originally identified as a repressor of iron transport and siderophore biosynthesis, *fur* has also been reported to regulate genes involved in a wide variety of functions, including oxidative stress, energy metabolism, and virulence, suggesting that defects in Fur regulation could have serious consequences for a microorganism (16). Indeed, attempts to obtain *fur* mutants by gene replacement have been unsuccessful in a number of species, including *Pseudomonas aeruginosa* (33), *Neisseria gonorrhoeae* (4), and *Vibrio anguillarum* (39). However, it is possible to select for *fur* mutants by using manganese (8, 17, 24, 27). Manganese mimics iron by binding to the Fur protein and repressing iron uptake genes. As a result, bacteria with a wild-type copy of the *fur* gene repress iron uptake systems and starve for iron in the presence of manganese, whereas *fur* mutants fail to repress iron uptake systems and survive. The Fur protein from such mutants is thought to retain some function, which is why this particular class of mutations is not lethal.

Here we report on a *fur* mutant of *B. japonicum* selected for resistance to manganese and contrast its symbiotic phenotype with that of a *B. japonicum fur*-null mutant that had previously been shown to be derepressed for iron uptake in culture (14). The *fur*-null mutant forms an effective symbiosis, whereas the manganese-resistant *fur* mutant strain is unable to form functional, nitrogen-fixing nodules on soybean, mung bean, or cowpea, suggesting a role for a Fur-regulated protein or proteins in the symbiosis.

MATERIALS AND METHODS

Materials. Restriction enzymes and the Klenow fragment of DNA polymerase were purchased from New England Biolabs (Beverly, Mass). Ampli *Taq* DNA polymerase was obtained from Perkin-Elmer (Foster City, Calif.). T4 DNA ligase and calf intestinal alkaline phosphatase (CIAP) were purchased from GIBCO-BRL (Gaithersburg, Md.). All other chemicals were purchased from Sigma Chemical Co. (St. Louis. Mo.) unless otherwise stated.

Strains, plasmids, bacteria, phage, and bacterial growth conditions. All strains and plasmids used in this study are listed in Table 1. *Escherichia coli* cultures were grown in Luria-Bertani broth at 37°C supplemented with ampicillin at 50 μg/ml, tetracycline at 20 μg/ml, or kanamycin at 30 μg/ml when necessary. *E. coli* cells grown for phage lambda plating were supplemented with maltose (0.2% final concentration) and $MgSO_4$ (10 mM final concentration), and phage infections were performed by standard procedures (2). *B. japonicum* cells were grown at 30°C in arabinose-gluconate (AG) medium (34), yeast extract-mannitol (YEM) (41), or minimal medium (12). Media were supplemented with 40 mM $MnCl_2$, 200-μg/ml tetracycline, or 30-μg/ml rifampin as needed. The pH of both YEM and minimal medium was adjusted to 6.8 before autoclaving. Cells were cultured initially in YEM or AG medium and then diluted into iron-free minimal medium. After 1 cycle of growth in minimal medium, cells were again diluted into iron-free minimal medium. Precautions were taken to minimize the iron

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TABLE 1. Strains used in this study

Strain or plasmid	Relevant genotype or characteristic(s)	Source or reference
Strains		
E. coli		
DH5α	*hsdR17 endA1 thi-1 gyrA96 relA1 recA1 supE44 ΔlacU169* (*φ80dlacZ*ΔM15)	GIBCO-BRL
XL1-Blue	*recA1 endA1 gyrA96 thi hsdR17*(r_K^- m_K^-) *supE44 relA1* λ^- *lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10*(Tc^r)]	Stratagene
SOLR	e14$^-$ (McrA$^-$) Δ(*mcrCB-hsdSMR-mrr*)*171 sbcC recB recJ uvrC umuC*::Tn*5* (Kan^r) *lac gyrA96 relA1 thi-1 endA1* λ^r [F′ *proAB lacI*^{q}*ZΔM15*] Su$^-$	Stratagene
JP5053	*argH1 metB1 nagA1 rpsL155 rpoB* F$^-$	19
AB9001	*fur* mutant derivative of JP5053	19
B. japonicum		
61A152	Nitrogen-fixing *Glycine max* (soybean) symbiont	Nitragin Co.
MLG100	*B. japonicum* Mn^r strain 61A152	This study
I110	I110 small-colony derivative of USDA 3I1b110	23
GEM4	I110 *fur*::Ω cassette	14
Plasmids		
pBluescript SK+	Ap^r P_{lac} *lacZ′* T7p T3p ColE1 origin, f1 origin	Stratagene
pLAFR3	pLAFR1 with pUC8 polylinker cloned into the *Eco*RI site	36
pwt*fur*	Tet^r, 3.2-kb *Eco*RI genomic clone containing *fur* in pLAFR3	This study
pmr*fur*	Tet^r, 2.4-kb genomic clone containing the Mn^r mutant *fur* gene in pLAFR3	This study

content of both the culture vessels and the medium. Glassware was washed with 1 N HCl and then rinsed with double-distilled water. Plasmids were transferred to *B. japonicum* by using the helper plasmid pRK2013 (7).

Cloning the *fur* gene. Degenerate primers Rfur1 [GA(A/G)GA(T/C)CA(T/C)CCIGA(T/C)GTIGA] and Rfur3rev [TCIATIA(A/G)(A/G)TG(A/G)TC(A/G)TG(A/G)TG] were constructed to conserved regions of the *fur* gene. A fragment of 157 bp was amplified and cloned into pBluescriptSK−. The fragment was used as a probe to screen a Lambda Zap II genomic library of *B. japonicum* 61A152. The library was constructed by digesting genomic DNA with *Tsp*509 and then cloning the DNA fragments into an *Eco*RI site of the Zap II lambda vector (Stratagene). A full-length copy of the *fur* gene was isolated from the library and sequenced. The mutant copy of the *fur* gene was PCR amplified from genomic DNA by using HLPfor (CGTGACTTGTCGTAACATTG) and HLPrev (CGACAGGAGATCACCTCGCTGT) primers. In order to isolate DNA sequence upstream of the *fur* gene carried by the Mn^r mutant, a subgenomic DNA library was constructed. Genomic DNA from the Mn^r mutant was isolated and digested with *Eco*RI and subcloned into CIAP-treated pBluescriptSK+. Probing colony lifts with a wild-type *fur* gene isolated a clone of 2.5 kb that contained the *fur* gene from the Mn^r mutant.

Selection for Mn^r Mutants. Manganese selection was based on the protocol of Hantke (17), with the following modifications. Minimal medium with 15 g of agar per liter was used. Mannitol (0.2%), dipyridyl (0.1 mM), and various concentrations of $MnCl_2$ were added as filter-sterilized stocks after autoclaving. Wild-type *B. japonicum* cells were diluted in 0.1% Tween 80 and spread plated onto selective plates containing 40 mM $MnCl_2$. Growth of wild-type 61A152 cells was completely inhibited by 40 mM $MnCl_2$. Only fresh plates were used as described by Silver et al. (35).

Protein preparation and SDS-PAGE. Outer membrane proteins (OMPs) were isolated as described by LeVier and Guerinot (25). Twenty-five micrograms of protein per lane was run on 8.6% polyacrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels for separation of OMPs. Fifteen percent polyacrylamide SDS-PAGE gels were used to separate total bacterial proteins for Fur Western blots. Total bacterial protein was isolated from mid-log-phase cultures, and 1 ml of culture was pelleted and resuspended in 1× sample buffer and boiled for 5 min. All gels were run with the following running buffer: 3.03 g of Tris, 14.26 g of glycine, and 1 g of SDS per liter (pH 8.3), at 200 V with prestained protein molecular weight standards (Bio-Rad or GIBCO-BRL). After electrophoresis, gels were stained with Coomassie blue, destained, photographed, and stored between sheets of cellophane (Ann Arbor Plastics, Inc., Ann Arbor, Mich.). Protein concentrations were determined with the bicinchoninic acid assay (Pierce, Rockford, Ill.) using bovine serum albumin as a standard.

Immunoblotting. Electrophoretic transfer of proteins to polyvinylidene difluoride membrane (0.45-μm pore size; Gelman Sciences, Ann Arbor, Mich.) was performed according to the manufacturer's instructions. The transfer was performed at constant voltage (20 V) for 25 min, using a semidry electroblotting device (Bio-Rad, Hercules, Calif.). After completion of the transfer, the blots were blocked in phosphate-buffered saline-Tween (PBST) with 5% nonfat dry milk, incubated overnight at 4°C with rabbit immunoglobulin G polyclonal antibodies directed against the *E. coli* Fur protein, and processed for detection with horseradish peroxidase-conjugated goat antirabbit secondary antibodies from the NEN chemiluminescence kit (NEN, Boston, Mass.).

Elemental analysis. Wild-type *B. japonicum* 61A152 and the Mn^r mutant strain were grown in YEM medium and then diluted 1:100 into iron-free minimal medium. After 1 cycle of growth, the cells were again diluted 1:100 into either iron-free minimal medium or minimal medium supplemented with iron. Cells were pelleted, and the protein concentration was determined. Three hundred micrograms of cellular protein was dried and then digested in HNO_3, and elemental analysis by inductively coupled plasma spectrometry (ICP) was performed with an inductively coupled plasma atomic emission spectroscope (Vista; Varian). The analysis was performed at The Scripps Research Institute. Yttrium was used as an internal standard.

H_2O_2 sensitivity assays. Bacteria were grown to mid-log phase in AG medium. Cells (100 μl) were spread plated onto AG plates, AG plates supplemented with 40 mM $MnCl_2$, or AG plates supplemented with 50 μg of tetracycline per ml for the transconjugants. Sterile 0.5-in. filters (no. 740-E; Schleicher & Schuell) were impregnated with 10 μl of 3% H_2O_2 and placed in the center of the plates, and zones of inhibition were recorded after 4 days of growth at 30°C.

Plant assays. *Glycine max* soybeans (yellow butterbeans; Johnny's Selected Seeds, Albion, Maine), mung bean (Vermont Bean Seed Co., Fair Haven, Vt.), and cowpea (Pea Brown Crowder Miss Silver; Vermont Bean Seed Co.) seeds were inoculated with *B. japonicum* strain 61A152, the Mn^r mutant, strain I110, or GEM4 as described by Guerinot and Chlem (11). Soybeans, cowpea, and mung bean plants were grown in modified Leonard jars with N-free medium. Soybeans were placed in a greenhouse with supplemental lighting. Cowpea and mung bean were grown in growth chambers at a temperature range of 25 to 28°C. Soybean plants were harvested 4, 5, 6, 7, and 8 weeks after germination. Mung bean and cowpea plants were harvested 5, 6, and 7 weeks postgermination. At each time point, the shoots and roots were separated, and the fresh weight of the shoots was determined. Acetylene reduction assays were conducted as described by Guerinot and Chelm (11). Chlorophyll extraction assays were performed with fresh leaf tissue. The protocol was adapted from Liscum et al. (26). Briefly, 0.1 g of fresh leaf tissue was collected, ethanol was added, and the tissue was ground, vortexed, and centrifuged. This extraction was repeated, the isolated supernatants were combined with ethanol and acetone, and the A_{664} and A_{647} were measured. Total chlorophyll was determined as described by Grann and Ort (10).

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USDA 110 FUR      MTALKPSSASKASGIEARCAATGMRMTEQRRVIARVLA
61A152 FUR        MTGLKPSSASKATGIEARCAATGMRMTEQRRVIARVLA
61A152 Mn FUR     MTTVKIPPAQKNTGIEARCAATGMRMTEQRRVIARVLA
                     * *** * *

USDA 110 FUR      EAVDHPDVEELYRRCVAVDDKISISTVYRTVKLFEDAG
61A152 FUR        ESVDHPDVEELYRRCVAVDDKISISTVYRTVKLFEDAG
61A152 Mn FUR     EAMDHPDVEELYRRCVAVDDKISISTVYRTVKLFEDAG
                    *

USDA 110 FUR      IIERHDFREGRARYETMRDSHHDHLINLRDGKVIEFTS
61A152 FUR        IIERHDFREGRARYETMRDSHHDHLINLRDGKVIEFTS
61A152 Mn FUR     IIERHDFREGRARYEQMPESHHDHLINLRDGKVIEFTS
                                 * **

USDA 110 FUR      EEIEKLQAEIARKLGYKLVDHRLELYCVPLDDDKPTS
61A152 FUR        EEIEKLQAEIARKLGYKLVDHRLELYCVPLDDDKPTN
61A152 Mn FUR     EEIEKLQAEIARKLGYKLVDHRLELYCVPLDEDGKS
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FIG. 1. Amino acid alignment of Fur proteins from *B. japonicum* USDA I110, 61A152, and the Mn^r 61A152 *fur* mutant. Amino acids that are different in the three strains are highlighted. Asterisks denote amino acid differences between the *fur* mutant and the two wild-type strains. The proposed iron-binding domain is underlined.

Nucleotide sequence accession number. The nucleotide sequence of the *B. japonicum* 61A152 *fur* gene has been deposited in GenBank under accession no. AY357585.

RESULTS AND DISCUSSION

Isolation of the *fur* mutant. An Mn^r strain of *B. japonicum* strain 61A152 was isolated on AG medium supplemented with 40 mM $MnCl_2$. (Wild-type *B. japonicum* is unable to grow on 20 mM $MnCl_2$.) In order to verify that this strain of *B. japonicum* contained a mutation in the *fur* gene, it was necessary to clone and sequence both the wild-type *fur* gene and the *fur* gene from the Mn^r strain. We cloned the *fur* gene of *B. japonicum* strain 61A152 by degenerate PCR. The *fur* DNA fragment was then used as a probe to screen a lambda Zap II genomic library of *B. japonicum* 61A152. The mutant *fur* gene was isolated by creating a subgenomic library of the Mn^r mutant and using the wild-type *fur* gene as a probe.

Figure 1 shows the amino acid alignment of the wild-type *fur* protein from *B. japonicum* 61A152 and the proteins from the manganese-resistant *fur* mutant and *B. japonicum* I110. There are 18 amino acid changes in the mutant *fur* protein relative to wild type; of these, 9 are conserved substitutions. Single point mutations and small insertions have been reported for other manganese-resistant *fur* mutants. Funahashi et al. (8) isolated Mn^r *fur* mutants in *Vibrio parahaemolyticus* and identified four different point mutations that caused amino acid changes and altered protein function. Lam et al. (24) described point mutations and a small insertion in the Mn^r *fur* gene of *V. cholerae*; the mutants contain a single point mutation in either of the conserved regions, the iron-binding domain or the helix-turn-helix domain, resulting in a nonfunctional Fur protein. Our mutant *fur* allele has many amino acid changes, yet the putative iron-binding domain and the helix-turn-helix domains are intact. The majority of the mutations are clustered at the N- and C-terminal regions of the protein. Due to the number and variety of mutations in the *B. japonicum fur* gene carried by the Mn^r mutant, the *rrn* and *sdh* genes from the mutant were PCR amplified and sequenced to determine if other genes were also mutated in this strain. The *rrn* and *sdh* gene sequences were identical to wild-type sequences, suggesting that it is unlikely that we have isolated a mutator strain.

Characterization of a dominant-negative allele of *fur* from *B. japonicum* 61A152. In order to begin characterization of the *fur* mutant, a wild-type clone of the *fur* gene was moved into the *fur* mutant by triparental mating. We anticipated that the plasmid-borne *fur* gene would complement the mutant and restore sensitivity to manganese. However, the resulting colonies were manganese resistant, indicating that the mutation may be dominant negative. To determine if the mutation was indeed dominant negative, the reciprocal experiment was performed. We introduced a copy of the mutant *fur* gene into the wild-type *B. japonicum* 61A152 strain and scored for manganese resistance. The resulting transconjugants were manganese resistant, suggesting that the mutant allele of the *fur* gene is dominant negative. In order to show that the mutated plasmid copy of the *fur* gene had not undergone further changes, the plasmid was prepared from *B. japonicum* 61A152 and used to transform *E. coli* DH5α cells. The plasmid copy of the *fur* gene was then sequenced. There were no additional mutations or reversions in the plasmid copy of the mutant *fur* gene (data not shown).

The fact that the *fur* allele from the mutant is dominant over the wild-type allele suggests that the Fur protein must be expressed. In order to demonstrate this, we performed a Western blot with anti-*E. coli* Fur serum. Total cellular protein was extracted from *B. japonicum* 61A152, the MLG100 *fur* mutant, and wild-type 61A152 carrying the Mn^r *fur* gene on a plasmid, as well as protein from the wild type and a *fur* deletion mutant in *E. coli* as positive and negative controls. As expected, Fur is expressed in the wild-type *E. coli* and is absent in the deletion strain. Fur is also expressed in the Mn^r *fur* mutant (data not shown).

Deregulation of the iron-regulated OMPs. Having verified that the Mn^r mutant did indeed carry a mutant version of the *fur* gene, we went on to examine some of the phenotypes normally associated with *fur* mutants. Wild-type *B. japonicum* and MLG100 were grown under iron-deficient and iron-sufficient conditions, and OMPs were isolated. The wild-type strain expresses certain OMPs only under iron-deficient growth conditions (Fig. 2). In MLG100, however, the OMPs are expressed under iron-sufficient and iron-deficient growth conditions, suggesting that the mutation in *fur* is causing the deregulation of the OMPs. Under iron-sufficient conditions, the wild-type strain of *B. japonicum* carrying the plasmid-borne Mn^r *fur* allele also shows deregulation of the OMPs. One of the iron-regulated OMPs in *B. japonicum* 61A152 is a putative heme receptor with 60% similarity to *hmuR* (Fig. 2, 61A3). In the manganese-resistant *fur* mutant, the putative heme receptor, like the other iron-regulated OMPs, is deregulated, suggesting that *fur* is regulating expression of this gene. Nienaber et al. (30) had previously reported that expression of *hmuR*, the gene encoding the outer membrane receptor for heme, was not deregulated in a *fur*-null mutant. However, their results are based on an *hmuR-lacZ* fusion; protein levels were not examined. Wexler et al. (43) reported that both a *tonB-lacZ* fusion and an *hmuS-lacZ* fusion are iron regulated, but that these genes are not regulated by *fur* in *R. leguminosarum*. Instead, these genes are thought to be regulated by RirA. Interestingly, there is no RirA homolg in *B. japonicum*.

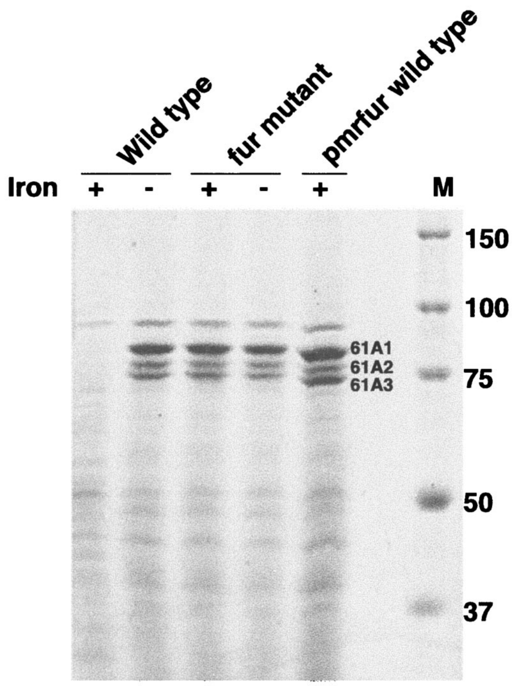

FIG. 2. Iron-regulated OMPs prepared from *B. japonicum* 61A152. Coomassie-stained 8.6% polyacrylamde SDS-PAGE gel of OMPs prepped from cells grown under iron-deficient and iron-sufficient conditions. The three OMPs that are overexpressed under iron-deficient conditions are labeled 61A1 (unknown), 61A2 (FegA, ferrichrome receptor), and 61A3 (putative heme receptor, homolog of *hmuR*). The wild-type strain is 61A152, the *fur* mutant is MLG100, and the pmr*fur* wild type is 61A152, with the Mn^r *fur* gene in *trans*. M (marker) is the protein molecular mass standard, and the sizes (in kilodaltons) are indicated to the right.

Oxidative stress. *E. coli* and *P. aeruginosa fur* mutants have been shown to be more sensitive to oxidative stress, presumably due to an increase in intracellular iron (18, 31). In order to test sensitivity to oxidative stress, cultures of wild-type and mutant *B. japonicum* strains were grown and spread plated, and filters with either sterile, distilled water or 3% H_2O_2 were added to the plates. Zones of inhibition were measured after 4 days. The results of four independent experiments showed that MLG100 (37 ± 1 mm) and 61A152 with pmr*fur* in *trans* (39 ± 0.8 mm) are significantly less sensitive to H_2O_2 than wild-type bacteria (51 ± 2 mm) ($P < 0.05$). There is no statistical difference between the zones of inhibition seen with the transconjugant and the MLG100 *fur* mutant.

Nunoshiba et al. (31) suggest that the increased sensitivity to oxidative stress found in an *E. coli fur* mutant is due to a 2.5-fold increase in the amount of intracellular iron. This excess iron is thought to participate in Fenton chemistry, catalyzing the formation of damaging hydroxyl radicals in the presence of hydrogen peroxide. However, there have been conflicting reports about the levels of iron in *fur* mutants in *E. coli*. Abdul-Tehrani et al. (1) described an *E. coli fur* mutant that has 2.5-fold less iron than the wild-type parental strain. The discrepancies may be due to the form of iron measured in different experiments (40). We wondered if the intracellular levels of iron were increased in the Mn^r *fur* mutant because the siderophore receptors are not repressed under iron-sufficient conditions. However, the mutant is resistant to oxidative stress, suggesting that the intracellular iron levels may be lower than those in the wild type. We examined the intracellular levels of iron in the wild-type, *fur* mutant, and transconjugant strains by ICP analysis. ICP analysis showed that the MLG100 *fur* mutant (1.8-fold increase) and 61A152 with pmr*fur* in *trans* (1.2-fold increase) had modest increases in iron content compared to the wild-type strain. Interestingly, the manganese-resistant *fur* mutant and 61A152 with pmr*fur* in *trans* each contain more manganese than the wild-type strain. Recent studies have suggested that manganese accumulation may play a role in peroxide and superoxide defense in bacteria (20). Perhaps the accumulation of manganese renders these strains more resistant to oxidative stress.

Symbiotic phenotype of the *fur mutant*. Perhaps the most dramatic phenotype of the dominant-negative *fur* mutant is the symbiotic defect. The Mn^r *fur* mutant is not able to form an effective symbiosis with soybean, cowpea, or mung bean plants. Cowpea and mung bean plants did not develop nodules when the plants were inoculated with MLG100. However, plants inoculated with the wild-type bacteria developed functional, effective nodules by week 4 (data not shown). Soybean plants inoculated with MLG100 showed two different phenotypes. Some of the plants developed small, white, ineffective nodules on the lower lateral roots, while other soybean plants did not develop nodules. Six weeks postgermination, the nodules were immature or absent, and there was no nitrogen fixation, as determined by the acetylene reduction assay. Plants inoculated with MLG100 contained less chlorophyll, had fewer nodules, and had a smaller nodule biomass than plants inoculated with wild-type bacteria (Fig. 3). Results with the dominant-negative, manganese-resistant *fur* mutant are in stark contrast to those with the *B. japonicum fur*-null strain GEM4. GEM4 did not show any significant differences from the wild-type strain, I110, in terms of numbers of nodules, nodule weight, shoot weight, or nitrogen fixation when these strains were inoculated on soybeans (data not shown). The *fur*-null strain is able to form an effective symbiosis despite the fact that the Fur protein is not expressed. These data suggest that the mutant Fur protein is either negatively or positively affecting a gene or genes necessary in the symbiosis.

We wondered if strain variations between 61A152 and I110 might explain some of the differences in the *fur* phenotypes. In order to address this concern, we expressed the manganese resistant *fur* allele from strain MLG100 in *trans* in the I110 wild-type strain. The resulting transconjugant strain was able to grow on higher levels of manganese than wild type (50 mM versus 20 mM for wild type). These data suggest that the Mn^r *fur* mutation behaves as a dominant-negative mutation in strain I110 as well as in 61A152. Interestingly, GEM4 is able to grow at 20 mM manganese, but it does not grow at higher levels of manganese. We also tested the I110 *fur*-null mutant GEM4 and GEM4 with the 61A152 *fur* gene in *trans* by using a swarm plate assay. A *Bacillus subtilis fur* mutant was reported to have an altered swarm phenotype on low-agar plates (John Helmann, personal communication). We wondered if GEM4 and the Mn^r *fur* mutant MLG100 also showed altered motility phenotypes relative to the wild type on low-agar plates. Both strains have very small swarms (GEM4, 6.1 ± 0.6 mm; and

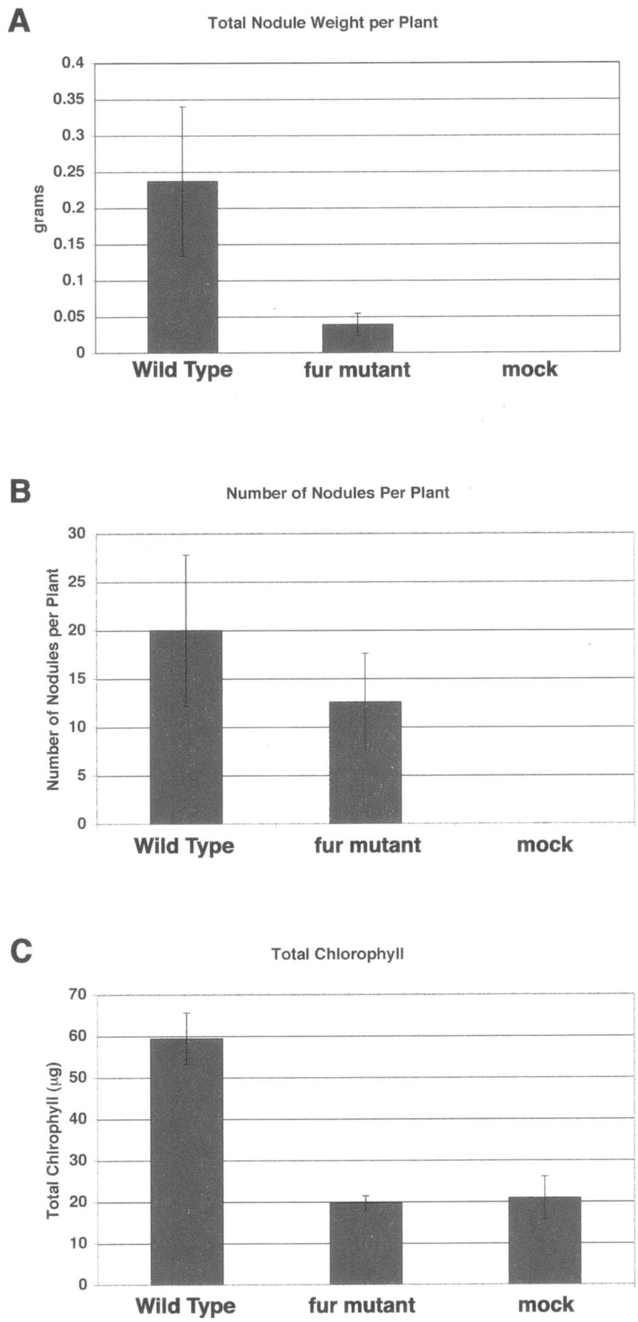

FIG. 3. Soybean plants inoculated with 61A152 or the 61A152 *fur* mutant or mock inoculated without bacteria. Plants were harvested six weeks post germination and assayed for the total nodule weight per soybean plant (A), the total number of nodules per plant (B), and the total chlorophyll extracted from soybean leaves (C). The standard error is shown.

MLG100, 4.9 ± 0.2 mm). Complementing GEM4 with the wild-type 61A152 *fur* gene in *trans* resulted in a strain with a wild-type swarm phenotype (20 ± 2.1 mm compared with 18.1 ± 0.2 mm for 61A152 and 15.3 ± 0.6 for I110). These results suggest that the 61A152 *fur* gene can complement a *fur* mutation in strain USDA I110.

In *E. coli*, more than 90 genes have been found to be regulated by Fur and iron (16). Fur has also been shown to indirectly regulate iron uptake by regulating other regulators, such as AraC-like regulators, two-component signal transduction regulators, and extracytoplasmic function sigma factors (16). Fur has also been shown to repress transcription of a small regulatory RNA that in turn inhibits expression of several genes, including *sodB*, the transcription of which initially appeared to be activated by Fur (28). In *B. japonicum*, Fur has been shown to regulate *irr*, an iron regulator that is involved in regulating the heme biosynthesis pathway (15). Interestingly, *R. leguminosarum*, the pea microsymbiont, has a *fur* gene, a homolog of *irr*, and a third iron regulator, *rirA* (5, 38). The RirA protein, not Fur, appears to be the major iron regulator in *R. leguminosarum* (42). The transcription of iron-responsive genes, such as those involved in the synthesis and uptake of the siderophore vicibactin and in heme uptake, is unaffected in *R. leguminosarum fur* mutants. However, these iron-responsive genes are deregulated in a *rirA* mutant, suggesting that RirA is the primary iron regulator in *R. leguminosarum* (38). Thus, there appear to be significant differences in regulation of iron-responsive genes between *B. japonicum* and *R. leguminosarum*. There is no obvious homolog of *rirA* in the *B. japonicum* genome. There is a homolog of the *fur*-like gene *zur*, which has been shown to be a zinc regulator in a number of bacterial species, including *E. coli* (32) and *B. subtilis* (9).

It is clear from our results that the absence of the Fur protein has a very different effect on downstream targets than does a dominant-negative mutant Fur protein. We do not, however, know all of the gene targets of the Fur protein or how the mutant protein may affect these targets. The *B. japonicum* Fur protein has been shown to regulate the *hemA* gene (13). There are likely to be many other as yet unidentified targets for the Fur protein. Now that the *B. japonicum* genome has been sequenced, it will soon be possible to carry out DNA microarray experiments to determine which genes are regulated by Fur or misregulated in each of the *fur* mutants (21, 22). Several recent studies have used a similar approach to define the *fur* regulon in a number of bacterial species, including *E. coli*, *B. subtilis*, and *Shewanella oneidensis* (3, 29, 37).

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