APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2004, p. 4349–4355 Vol. 70, No. 7
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.7.4349–4355.2004

Fur Is Involved in Manganese-Dependent Regulation of *mntA* (*sitA*) Expression in *Sinorhizobium meliloti*

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Received 29 March 2004/Accepted 2 April 2004

Fur is a transcriptional regulator involved in iron-dependent control of gene expression in many bacteria. In this work we analyzed the phenotype of a *fur* mutant in *Sinorhizobium meliloti*, an α-proteobacterium that fixes N_2 in association with host plants. We demonstrated that some functions involved in high-affinity iron transport, siderophore production, and iron-regulated outer membrane protein expression respond to iron in a Fur-independent manner. However, manganese-dependent expression of the MntABCD manganese transport system was lost in a *fur* strain as discerned by constitutive expression of a *mntA::gfp* fusion reporter gene in the mutant. Thus, Fur directly or indirectly regulates a manganese-dependent function. The data indicate a novel function for a bacterial Fur protein in mediating manganese-dependent regulation of gene expression.

The intracellular concentrations of transition metals such as Fe, Mn, Co, Cd, Ni, and Zn are precisely controlled through the regulation of its transport across the membrane (21, 22). The transport of iron is the best studied transition metal uptake system in bacteria. As no relevant efflux systems are known for iron, it has been hypothesized that core regulation must be at the acquisition level. Bacteria have evolved highly efficient mechanisms to acquire this metal in naturally occurring iron-restricted conditions. Transport of ferric siderophores and heme are two widespread strategies for iron acquisition when the metal is limiting. In gram-negative bacteria, siderophore- or heme-based iron transport systems comprise three main components: iron-repressed outer membrane proteins involved in recognition and transport of the iron-containing compounds; the TonB-ExbB-ExbD complex to couple the proton motive force with outer membrane transport events; and ATP-binding cassette (ABC) transporters present in the inner membrane. ABC transporters involved in high-affinity iron acquisition systems are also present in gram-positive bacteria. Since these bacteria do not possess an outer membrane, lipoproteins analogous to periplasmic binding proteins play a role similar to that of iron-regulated outer membrane proteins (2, 5).

More recently, the physiological significance of manganese homeostasis has received attention (19, 22). Neither specific outer membrane proteins nor specific chelators for manganese (as siderophore for iron) seem to be required for manganese acquisition. Bacterial manganese transport systems identified so far comprise: the natural resistance-associated macrophage protein (NRAMP)-type transporters (*mntH*); the ABC-type transporters *mntABC*, *scaABC*, and *sitABC*; and the P-type ATPase transporter *mntA* (*mntP*) found only in *Lactobacillus plantarum* (23). The SitABC/D operon was originally reported as being involved in iron uptake, but recent studies have clearly defined this system as a manganese transporter (24, 34). Accordingly, Que and Helmann (40) and Kehres and Maguire (25) proposed to rename this operon as MntABC/D.

Fur is a global regulator of iron homeostasis in numerous bacteria (8, 17). This protein senses the intracellular ferrous ion concentration, through the formation of a Fur-Fe^{2+} complex, which in turn interacts with specific DNA targets in the promoters of iron-repressed genes. Presumptive *fur* genes have been found in the genome of some gram-positive bacteria and in almost all gram-negative bacteria.

The Fur family comprises the classical Fur, Zur (a Zn uptake regulator), PerR (an oxidative stress response regulator), and Irr (an iron response regulator involved in heme biosynthesis) (6, 11, 14, 32). In *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella enterica*, a different metalloprotein, MntR, has been described as a central regulator of manganese homeostasis (18, 24, 33, 39). In *B. subtilis* MntR has a dual role: under high-manganese conditions it represses the expression of the *mntH* and *mntABCD* genes, but under low-manganese conditions it acts as a positive regulator of the MntABCD transporter (39). In *E. coli* and *S. enterica*, the expression of *mntH* is repressed by manganese and iron. MntR mediates manganese response in both bacteria, whereas MntR and Fur are involved in iron response of *mntH* gene in *E. coli* (24, 33). To our knowledge no MntR homologs have been found in α-proteobacteria.

One of the most relevant physiological differences between iron and manganese is that while iron is potentially toxic since it is able to catalyze a Fenton-type reaction producing highly noxious reactive oxygen species (O_2^-, H_2O_2, ·OH), manganese contributes to the detoxification of these reactive oxygen species. These facts led to the proposal that cells have to ensure not only specific intracellular levels for each metal but also an accurate balance between these two metals. Therefore, it is not

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
S. meliloti		
1021	Wild type, St^r	29
242	Wild type, St^r	9
Mf1	1021 fur_{Sm}::Ω cassette	This work
Mf2	242 fur_{Sm}::Ω cassette	This work
1021(pShm)	1021 containing plasmid pShm	This work
242 (pShm)	242 containing plasmid pShm	This work
1021 (pMan)	1021 containing plasmid pMan	This work
242 (pMan)	242 containing plasmid pMan	This work
Mf1 (pShm)	Mf1 containing plasmid pShm	This work
Mf2 (pShm)	Mf2 containing plasmid pShm	This work
Mf1 (pMan)	Mf1 containing plasmid pMan	This work
Mf2 (pMan)	Mf2 containing plasmid pMan	This work
1021(pOT)	1021 containing plasmid pOT1	This work
242(pOT)	242 containing plasmid pOT1	This work
Mf1(pLAFur,pMan)	Mf1 containing both plasmids pLAFur and pMan	This work
E. coli DH5α	*supE44 ΔlacU169(ϕ80lacZΔM15)hsdR17 recA1 gyrA96 thi-1 relA1*	15
Plasmids		
pBluescriptSK (pBSK)	Cloning vector, Ap^r	Stratagene
pΩ45	Plasmid containing Ω Sp^r St^r cassette	38
pWS233	Suicide vector in rhizobia, Gm^r	41
pOT1	Wide-host-range gfp-UV promoter-probe plasmid, Gm^r, derivative of pBBR1	1
pOT2	Wide-host-range gfp-UV promoter-probe plasmid, Gm^r, derivative of pBBR1	1
pRK2013	Helper plasmid, Km^r	10
$pFur_{Sm}$	1.25-kb PCR fragment carrying the fur_{Sm} gene and flanking regions cloned in pBSK	This work
$pBSKfur_{Sm}\Omega$	0.8-kb fur_{Sm} ends and flanking regions carrying an Ω cassette in place of fur_{Sm} gene cloned in pBSK	This work
$pWSfur_{Sm}\Omega$	pWS233 carrying the 0.8-kb fragment containing an Ω cassette in place of fur_{sm}	This work
pShmR	pBSK with a 2.8-kb PCR fragment containing *shmR,* its presumptive promoter, and flanking regions	This work
pShm	pOT1 with a 650-bp HindIII, SalI fragment containing the presumptive *shmR* promoter, Gm^r	This work
pMan	pOT2 with a 750-bp KpnI, PstI fragment containing the presumptive *mntA* promoter, Gm^r	This work
pLAFR3	Low-copy-number, broad-host-range cosmid derivative of pRK290 (IncP1) PLAFR3 containing fur_{Sm} and its presumptive promoter	42
pLAFur		This work

surprising to find sophisticated mechanisms of coordination of iron sensing, manganese sensing and oxidative stress response such as the ones reported between PerR/MntR/Fur in *S. aureus* and *B. subtilis* or OxyR/MntR/Fur in *S. enterica* (18, 22, 30).

Sinorhizobium meliloti is an α-proteobacterium that belongs to the Rhizobiaceae family. Bacteria known generically as rhizobia can establish symbiotic associations with host plants to fix nitrogen within the plant. Bona fide Fur homologs have been identified in two genera of rhizobia: *Bradyrhizobium* (Fur_{Bj}) and *Rhizobium* (Fur_{Rl}) (13, 46). Fur_{Bj} is able to complement an *E. coli fur* mutant and binds to a canonical DNA binding element called Fur box (13). Moreover, the rate of Fe uptake is higher in a *Bradyrhizobium japonicum* fur_{Bj} mutant than in the wild-type strain when cells are grown in high-iron medium indicating that iron uptake is normally repressed by Fur (13). Unexpectedly, in *Rhizobium leguminosarum* iron regulation of some iron uptake genes is mediated by RirA rather than by Fur (43, 46). Besides, in silico studies indicate that Fur is absent in the *Mesorhizobium loti* genome. These facts suggest that the pattern of iron uptake regulation is not the same in all genera of rhizobia.

S. meliloti 1021 genome carries three *fur*-like putative genes: *fur*, *irr*, and *zur*. Iron-dependent repression of hemin and rhizobactin 1021 receptors has been described previously, but the regulator involved in this response is not known (3, 28). Concerning Mn acquisition, the presence of one ABC transporter (*sitABC*, renamed here as *mntABC*) and one presumptive NRAMP-type transporter (Sma1115) were identified by physiological or bioinformatic studies (35).

In this work we study the regulation of the expression of the hemin receptor ShmR and manganese transport system MntABCD in wild-type and *fur* mutant backgrounds. We found that *S. meliloti* Fur protein (Fur_{Sm}) is involved in manganese regulation of *mntA* but not in iron regulation of *shmR*. Moreover, *fur* mutants are not affected in siderophore production or in the expression of some iron-regulated outer membrane proteins.

MATERIALS AND METHODS

Bacteria, plasmids, and media. Bacteria and plasmids used in this study are listed in Table 1. *S. meliloti* strains were grown at 30°C in tryptone-yeast extract medium (TY) (4) or in defined minimal medium (M3) (3). Metal chelated

medium was obtained by supplementation with ethylenediamine-di-*o*-hydroxyphenylacetic acid (EDDHA) (TYE or M3E). Micromolar concentrations of added EDDHA are indicated throughout this work as subscripts. $FeCl_3$ and $MnCl_2$ were added at a final concentration of 50 μM as indicated. *E. coli* strains were grown at 37°C in Luria broth. When required kanamycin (50 μg/ml), ampicillin (50 μg/ml), spectinomycin (100 μg/ml), or streptomycin (100 μg/ml) was added to the medium. Gentamicin at 20 or 40 μg/ml was used for *E. coli* or *S. meliloti*, respectively, grown in solid medium.

Phylogenetic analysis. Fur and Irr sequences were obtained from GenBank for all the species considered in this study. Phylogenetic trees based on amino acid sequences of Fur and Irr were inferred with maximum-parsimony and neighbor-joining (NJ) (*p* distance matrix) analyses, using MEGA2 (25). Confidence in topologies was assessed using bootstrapping (100 replicates). Trees were rooted using the Fur-like sequence of *M. loti*.

Primers. For amplification of the 1.25-kb fragment containing the fur_{Sm} gene of *S. meliloti* 1021 and flanking regions, the following pair of primers (F1 and F2) were used: 5′-ATATTCGGGAAGGGCTTCAG-3′ (forward primer) and 5′-TGGAAGAACCTTTCGAACCA-3′ (reverse primer). An inverted PCR was performed to amplify fur_{Sm} ends and flanking regions contained in pBSK using primers P3 and P4 (5′-AACTCGGCTATGACCTGGTG-3′ and 5′-ATCCGAT TCTTGCTCTGGCTC-3′, respectively) as forward primer and as reverse primer.

The 2.8-kb *S. meliloti* 1021 DNA fragment containing *shmR* was amplified by using as forward primer 5′-ATTCGTCTCGCTCCGTAAAA-3′ (S5) and as reverse primer 5′-AGAAACGCGACGATCAAAAT-3′ (S6).

Cloning and mutagenesis. Digestion with restriction endonucleases, DNA ligation, and minipreparation of plasmid DNA were performed essentially as described by Sambrook et al. (40). DNA fragments containing fur_{Sm} or *shmR* genes were obtained from PCR amplification of total DNA isolated from *S. meliloti* strain 1021, and the blunt-end fragments were ligated with EcoRV-digested pBluescript SK. Cloning was confirmed by restriction mapping and sequencing.

To replace the fur_{Sm} gene with an omega cassette, the following strategy was used. An inverted PCR was performed with *Pfu* polymerase and primers complementary to fur_{Sm} extremities (about 20 to 40 bp each) using $pFur_{sm}$ as target DNA. The PCR-amplified fragment contained linear $pFur_{Sm}$ without the internal part (360 bp) of fur_{Sm}. This PCR product was ligated with the cassette obtained by digestion of pΩ45 with SmaI. The resulting $pBSKfur_{Sm\Omega}$ construction was digested to confirm the correct insertion of the cassette. Fragments obtained by EcoRI/SalI double digestion of $pBSKfur_{Sm\Omega}$ and of pWS233, were ligated. The new plasmid was mobilized into *S. meliloti* strains 1021 and 242 by triparental mating by using DH5α(pRK2013) as a helper strain (10). Sp^r Gm^s colonies were selected and genomic DNAs were isolated to check gene replacement by PCR using F1 and F2 primers.

Plasmid pLAFur for complementation of *fur* mutations was generated by cloning the 1,248-bp EcoRI/HindIII fragment of $pFur_{Sm}$ containing wild-type fur_{Sm} gene in pLAFR3 digested with EcoRI and HindIII.

Reporter fusions. A 650-bp HindIII, SalI fragment of pShmR containing the putative promoter of shmR was subcloned in the appropriate orientation, upstream of the *gfp* reporter gene of pOT1, a low-copy-number plasmid able to replicate in rhizobia (1). The new plasmid was named pShm. Plasmid pMan was constructed by ligation of a 750-bp KpnI, PstI fragment of $pFur_{Sm}$ containing the presumptive promoter of *mntA* with KpnI-, PstI-digested pOT2 plasmid. Cloning was confirmed by restriction mapping. Plasmids obtained were transferred into *S. meliloti* wild-type and mutant strains by triparental mating using pRK2013 as a helper plasmid.

GFP-UV expression. Qualitative green fluorescent protein-ultraviolet (GFP-UV) expression of cultures grown on solid M3 or on TY medium was evaluated by visualization of plates under UV light. For quantitative measurements of fluorescence of GFP-UV in cultures, 10 ml of M3, M3 plus 50 μM $FeCl_3$, M3 plus 50 μM $MnCl_2$, or M3 plus 100 μM EDDHA was supplemented with gentamicin at 15 μg/ml, and cultures were inoculated with ca. 10^8 washed cells of 1021(pOT), 1021(pShm), 1021(pMan), Mf1(pShm), Mf1(pMan), 242(pOT), 242(pShm), 242(pMan), Mf2(pShm), and Mf2(pMan) strains. One hundred and fifty microliters of a 3-day-old culture (of about 5×10^8 CFU/ml) were transferred to microtiter plates (four wells per condition), and fluorescence was evaluated with a 960 plate reader (FLUOstar OPTIMA; BMG Labtechnologies) using a 390-nm (10-nm bandwidth) excitation filter and 520-nm emission filter (10-nm bandwidth). Cell optical density at 620 nm (OD_{620}) was measured. Quantitative relative fluorescence was determined according the method of Allaway et al. (1) as fluorescence emission at 520 nm/OD_{620}.

Siderophore production and outer membrane protein expression. Dihydroxamate siderophore production was evaluated by the ferric perchlorate method (7). For these studies, cultures of wild-type and mutant strains were grown in liquid M3 minimal medium with different metal availability. Stationary-phase cultures were centrifuged at 10,000 × *g* for 10 min at 4°C, supernatants were mixed with equal volumes of 10 mM $FeCl_3$ and 0.2 M $HClO_4$, and the absorbance at 450 nm was measured after 10 min of incubation. Deferoxamine mesylate (Desferal; Novartis Co.), a trihydoxamate siderophore, was used as a standard. Values were recorded as micromolar concentrations of rhizobactin 1021 of a supernatant corresponding to a cell culture of OD_{620} equal to 1. Independent assays were performed at least three times. Outer membrane preparations were carried out as previously described (3). Briefly, cells obtained from 50 ml of saturated cultures were disrupted by three passages through a French pressure cell at 25,500 lb/in^2 and incubated for 90 min with a solution containing 0.4 mg of DNase per ml, 0.4 mg of RNase per ml, and 10 mg of lysozyme per ml and for an additional 60 min with 0.75% *N*-laurylsarcosine at room temperature. Cell debris were removed by centrifugation, and the outer membrane enriched supernatant was centrifuged for 2 h at 60,000 × *g*. Outer membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver stained.

Plant assays. *S. meliloti* 1021 and 242 mutants were screened for their symbiotic phenotype on alfalfa plants. Bacteria were grown to early stationary phase on TY-liquid medium. Washed cells were used to inoculate *Medicago sativa* cv. Creola plants at a final concentration of 5×10^6 CFU per plant. Alfalfa plants were grown aseptically in 15 ml of N-free Jensen medium (45) solidified with agar at 15 g/liter. Plants were maintained at 21 ± 2°C in a controlled light room with a photoperiod of 12 h. At least five tubes (two plants per tube) were used for each strain's inoculation. The complete experiment was independently repeated twice. Plant dry weights 2 months after inoculation were determined.

RESULTS

In silico studies. The *S. meliloti* 1021 genome reveals a presumptive *fur* gene upstream of the *mntABC* operon, but in the opposite orientation. We wanted to compare the sequence similarity between the Fur protein deduced from the *S. meliloti* genome and other presumptive proteins that have been assigned in different genomes as classical Fur proteins. A phylogenetic tree of Fur proteins and the related Irr protein, clearly differentiate these proteins in two well-defined groups (Fig. 1). This organization indicates that *S. meliloti* Fur (Fur_{Sm}) protein is more related to Fur proteins from δ-proteobacteria or ε-proteobacteria than to a Fur-like protein from the same α-proteobacterium group. Although only the NJ tree is shown; the tree obtained by maximum parsimony with a heuristic search factor of 2 shows the same overall topology. The tree also shows that α-proteobacterium Fur proteins, with the exception of *M. loti* Fur, cluster separately from the δ-proteobacteria and the ε-proteobacterium examined, which is consistent with the apparent divergent evolutionary trajectories of these bacteria.

Expression of iron-repressed outer membrane proteins and siderophore production respond to iron in a Fur-independent manner. Since Fur has been described as a central iron-sensing metalloregulator, we wanted to test its role in the regulation of some iron-repressed functions in *Sinorhizobium*. For this purpose we constructed a fur_{Sm} knockout mutant in *S. meliloti* by allelic exchange of the fur_{Sm} gene for a spectinomycin resistance cassette. The expected replacement by the omega cassette of the fur_{Sm} genes of *S. meliloti* strains 1021 and 242 was confirmed by PCR using primers F1 and F2, described in Materials and Methods. A unique PCR-amplified fragment of 1,250 bp was obtained for 1021 and 242 wild-type *S. meliloti* strains, and a unique fragment of about 3,100 bp was obtained for Mf1 and Mf2 mutants.

Previously, Battistoni et al. (3) showed that *S. meliloti* strain 242 expresses three outer membrane proteins with estimated molecular masses of 80, 82, and 92 kDa in response to iron

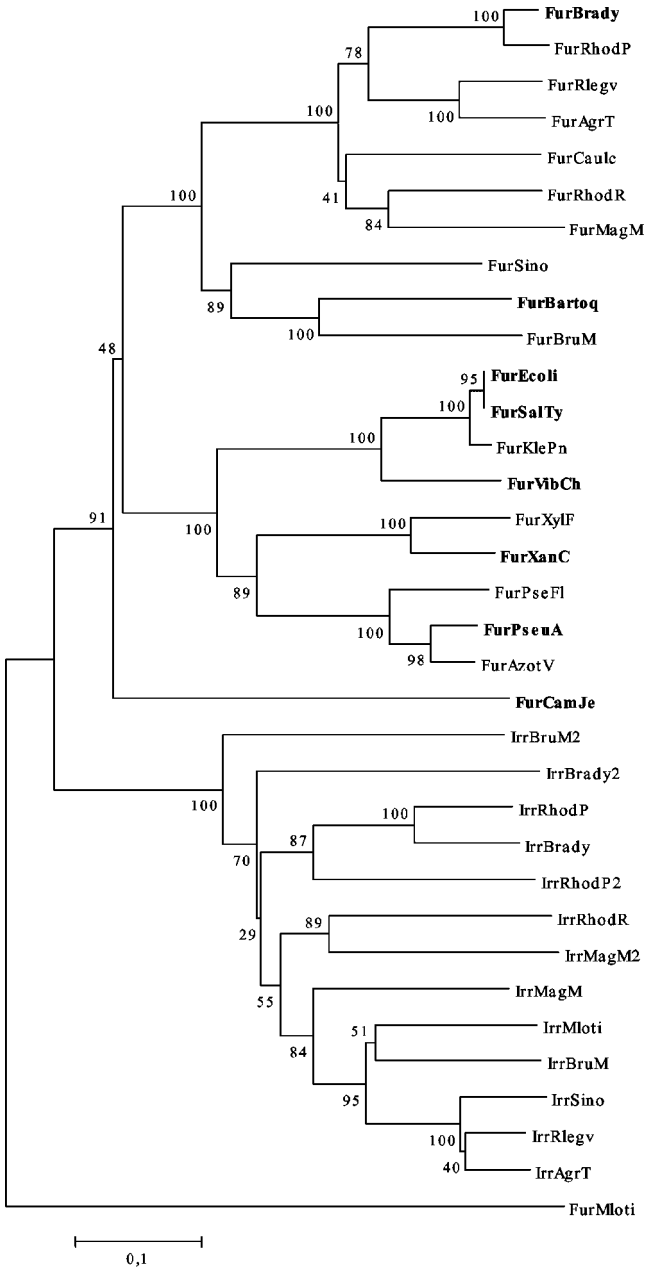

FIG. 1. Phylogenetic analysis of Irr and Fur protein sequences. Trees were constructed by NJ based on *p* distances using MEGA2. The bootstrap values are shown on the branches and indicate the number of times out of 100 replications. *M. loti* Fur sequence (FurMloti NP_104436) was used as an outgroup to root the tree. Fur protein sequences: FurBrady (*B. japonicum* NC_004463.1), FurRhodP (*Rhodopseudomonas palustris* NZ_AAAF01000001.1), FurRlegv (*R. leguminosarum* bv. *viciae* O07315), FurAgrT (*Agrobacterium tumefaciens* NP_531060.1), FurCaulc (*Caulobacter crescentus* NP_418876.1), FurRhodR (*Rhodospirillum rubrum* |ZP_00013164.1), FurMagM (*Magnetospirillum magnetotacticum* ZP_00052636.1), FurSino (*S. meliloti* NP387131), FurBartoq (*Bartonella quintana* AAL04498), FurBruM (*Brucella melitensis* AAB81452), FurEcoli (*E. coli* K-12 P06975), FurSalTy (*S. enterica* serovar Typhimurium NP459678), FurKlePn (*Klebsiella pneumoniae* P45599), Fur VibCh (*Vibrio cholerae* P33087), FurXylF (*Xylella fastidiosa* NP779572), FurXanC (*Xanthomonas campestris* NP_636842), FurPseFl (*Pseudomonas fluorescens* O68563), FurPseAu (*Pseudomonas aeruginosa* Q03456), FurAzotV (*Azotobacter vinelandii* AAN03807), FurCamJe (*Campylobacter jejuni* P48796). Irr protein sequences: IrrBruM2 (*B. melitensis* NP_540872.1), IrrBrady2 (*B. japonicum* NP_767856.1), IrrRhodP (*R. palustris* ZP_00010023.1), IrrBrady (*B. japonicum* NP_767408.1), IrrRhodP2 (*R. palustris* ZP_00011887.1), IrrRhodR (*R. rubrum* ZP_00013142.1), IrrMagM2 (*M. magnetotacticum* ZP_00054559.1), IrrMagM (*M. magnetotacticum* ZP_00052390.1), IrrMloti (*M.loti* NP_106209.1), IrrBruM (*B. melitensis* AAO89498), IrrSino (*S. meliloti* NP384355), IrrRlegv (*R. leguminosarum* bv. *viciae* CAD37806), IrrAgrT (*A. tumefaciens* C58 E97377). Experimentally verified Fur proteins (13, 16, 26, 27, 31, 36, 44) are indicated in boldface type.

TABLE 2. Effect of Mn^{2+} and Fe^{3+} in siderophore production of *S. meliloti* wild-type strains and *fur* mutants

S. meliloti strain	Mean rhizobactin 1021 concn ± SD (nM) of cultures grown in[a]:				
	$M3Fe_{50}$	M3	$M3Mn_{50}$	$M3E_{100}$	$M3E_{100}Mn_{50}$
1021	22 ± 1	61 ± 6	58 ± 4	120 ± 24	144 ± 32
Mf1	24 ± 8	78 ± 8	68 ± 9	135 ± 30	106 ± 18
242	33 ± 4	109 ± 10	95 ± 10	173 ± 18	132 ± 30
Mf2	26 ± 1	94 ± 10	70 ± 20	160 ± 42	160 ± 38

[a] Concentration of rhizobactin 1021 in the supernatant was estimated under the assumption that 1 mol of the trihydroxamate deferoxamine mesylate is equivalent to 1.5 mol of the dihydroxamate rhizobactin 1021. Results were normalized to an optical density of 1. The data shown are based on three separate experiments.

limitation. We compared the induction of these proteins in *S. meliloti* strain 242 and in the *fur* mutant strain Mf2 grown in iron-supplemented or iron-chelated medium. Expression of the outer membrane proteins was iron repressed in both the wild-type and *fur* strains, indicating that the iron responsiveness was Fur independent (data not shown).

Under iron-restricted conditions *S. meliloti* strains 1021 and 242 are able to synthesize rhizobactin 1021, a dihydroxamate-type siderophore (34; E. Fabiano, unpublished data). The presence of this siderophore in culture supernatants was visualized by the development of a reddish color in acidic medium with a ferric perchlorate reagent (7). The rhizobactin 1021 production was estimated for cell cultures of an OD_{620} equal to 1 (micromolar concentration/OD_{620}). As shown in Table 2, when the *fur* mutant strain Mf1 and parent strain *S. meliloti* 1021 were grown in $M3E_{100}$ iron-chelated medium, rhizobactin 1021 production was 135 ± 30 μM and 120 ± 4 μM rhizobactin/OD_{620}, respectively. Siderophore production of cultures grown on 50 μM $FeCl_3$ supplemented M_3 medium were less than 30 μM/OD_{620}. Similar results were obtained for *S. meliloti* 242 and Mf2 strains. These results indicate that Fur_{Sm} is not involved in iron regulation of siderophore production in *S. meliloti*.

ShmR is a hemin-binding outer membrane protein expressed in iron-restricted cultures of *S. meliloti*. In order to study factors involved in the regulation of this protein, we studied the fluorescence produced by *S. meliloti* 1021 and 242 wild-type strains, as well as by the *fur* mutants containing pShm, a pOT1 derived plasmid that carried the presumptive *shmR* promoter upstream the *gfp-UV* reporter gene. As shown in Fig. 2 and Fig. 3, fluorescence corresponding to the expression of the green fluorescent protein was clearly observed in *S. meliloti* wild-type strains containing pShm when they are grown on iron-limited medium. However, the addition of 50 μM $FeCl_3$ repressed GFP-UV expression. This genetic ap-

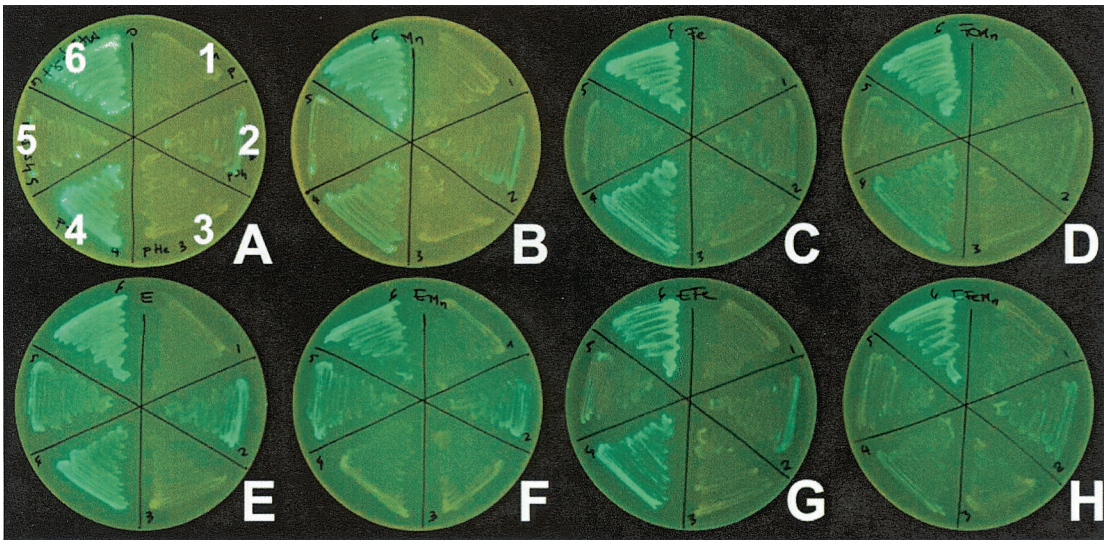

FIG. 2. GFP-UV expression of *shmR-gfp* and *mntA-gfp* transcriptional fusions in *S. meliloti* 242 wild-type strain and in a *fur* mutant (Mf2). Strains used were 242(pOT) (sectors 1), 242(pShm) (sectors 2), 242(pOT) (sectors 3), 242(pMan) (sectors 4), Mf2(pShm) (sectors 5), and Mf2(pMan) (sectors 6). Growth media shown are M3 (A), M3Mn_{50} (B), M3Fe_{50} (C), M3$Fe_{50}Mn_{50}$ (D), M3E_{100} (E), M3$E_{100}Mn_{50}$ (F), M3$E_{100}Fe_{50}$ (G), and M3$E_{100}Mn_{50}Fe_{50}$ (H).

proach confirms previous biochemical observations showing that ShmR expression is iron-regulated (3). Interestingly, the same iron-dependent regulation of GFP-UV expression was also observed with the *fur* mutants containing pShm. No significant differences were detected between *S. meliloti* 1021(pShm) and Mf(pShm) strains, indicating that Fur_{Sm} is not involved in iron regulation of ShmR expression. Similar results were obtained with *S. meliloti* strain 242.

The expression of the *mntA* gene involved in manganese acquisition is regulated by manganese in a Fur-dependent manner. We previously showed that the *mntABC* operon is involved in manganese acquisition in *S. meliloti* 242 (35). This operon is adjacent to the fur_{Sm} gene but in the opposite orientation in the *S. meliloti* 1021 genome. Here, we asked whether *mntA* expression could be regulated by Fur. A fragment containing the presumptive *mntA* promoter was cloned upstream of the *gpf* reporter gene in the plasmid pOT2 generating pMan. As shown in Fig. 2 and 4, *S. meliloti* wild-type strains containing pMan plasmid fluoresced in minimal or rich medium without added manganese. The addition of 50 μM $MnCl_2$ to the medium inhibited, but did not abolish, fluorescence expression. These results indicate that the *mntA* gene responds to manganese and that this control is at the transcriptional level. *S. meliloti fur* mutants, Mf1 and Mf2, containing the reporter plasmid pMan expressed GFP-UV strongly in minimal and rich medium regardless of manganese addition. However, when the wild-type *fur* gene was introduced in *trans* in Mf1(pMan) strain, manganese-dependent expression of MntA was restored

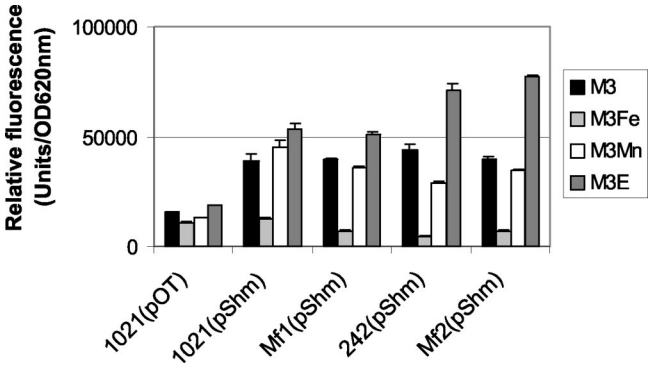

FIG. 3. In vivo expression of *shmR* promoter in *S. meliloti* 1021 and *S. meliloti* 242 and in the *fur* mutants Mf1 and Mf2. Cultures were grown in M3 defined medium supplemented with 50 μM $FeCl_3$ (M3Fe), 50 μM $MnCl_2$ (M3Mn), or 100 μM EDDHA (M3E). Specific fluorescence was determined in arbitrary fluorescence units per units of OD_{620}. The data shown represent the average + standard deviation (error bars) of four measures from one assay. The complete assay was repeated twice.

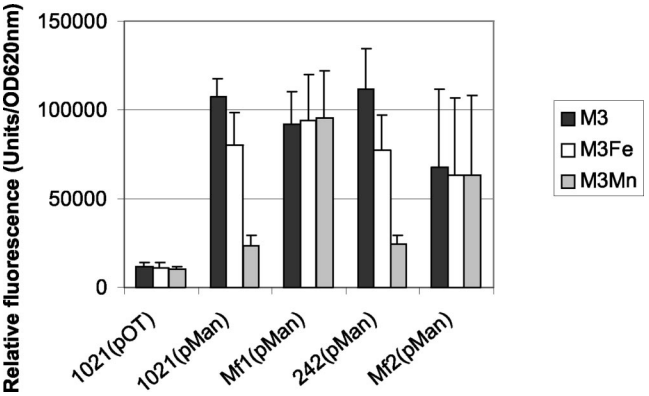

FIG. 4. In vivo expression of *mntA* promoter in *S. meliloti* 1021 and *S. meliloti* 242 and in the *fur* mutants Mf1 and Mf2. Cultures were grown in M3 defined medium supplemented with 50 μM $FeCl_3$ (M3Fe) or 50 μM $MnCl_2$ (M3Mn). Specific fluorescence was determined in arbitrary fluorescence units per units of OD_{620}. The data shown represent the average + standard deviation (error bars) of four measures from three independent assays.

TABLE 3. *fur* gene complements in *trans* the Mn-derepressed phenotype of Mf1

Strain	Relative fluorescence[a] (mean ± SD)	
	M3	M3Mn
1021(pMan)	107 ± 10	23 ± 6
Mf1(pMan)	91 ± 18	95 ± 26
Mf1(pLAFur,pMan)	75 ± 16	24 ± 10

[a] Relative fluorescence is expressed as arbitrary units (10^3) per unit of OD_{620}. Data are based on three separate experiments.

to the same level as in the wild-type strain (Table 3). Thus, the defect in the *fur* gene is the cause of the observed phenotype. Taken together, these results clearly show that the Fur_{Sm} protein regulates manganese-dependent *mntA* repression.

We could not observe a decrease in fluorescence from the *mntA-gfp* fusion on plates supplemented with 50 μM $FeCl_3$. However, quantification of fluorescence in the liquid assay revealed a modest repression upon the addition of $FeCl_3$. This repression was not observed in the *fur* mutants. Thus, Fe has a modest effect on *mntA* expression that is dependent on Fur.

Expression of the iron-regulate ShmR outer membrane protein and siderophore production are not repressed by manganese. In order to evaluate if manganese could functionally mimic the effect of iron in regulation of some iron-repressed function, *shmR* expression and siderophore production were determined in liquid medium supplemented with 50 μM $MnCl_2$. As shown in Table 2 no significant differences were obtained in rhizobactin 1021 concentration in manganese-supplemented medium (M3 versus $M3Mn_{50}$ and $M3E_{100}$ versus $M3E_{100}Mn_{50}$). Conversely, addition of 50 μM $FeCl_3$ to M3 medium produced a clear repression on siderophore production.

In vivo expression of the *shmR* promoter was evaluated, and the results obtained are shown in Fig. 3. Comparison of GFP-UV fluorescence obtained for 1021(pShm) and Mf1(pShm) cultures grown in M3 or $M3Mn_{50}$ demonstrate that this promoter is regulated by iron but not by manganese. Similar results were obtained for 242(pShm) and Mf2(pShm) (data not shown). Therefore, Mn^{2+} cannot substitute for Fe^{2+} in regulation of *shmR*.

Symbiotic phenotype of *fur* mutants. No significant differences could be detected in plant dry weight or visualization of nodules formed between plants inoculated with wild-type strains or *fur* mutant strains (data not shown). These results indicate that Fur_{Sm} expression is not essential for symbiosis with alfalfa in the conditions assayed here.

DISCUSSION

To determine the role of Fur_{Sm} in *S. meliloti*, we have carried out a phenotypic study of *fur* knockout mutants of two *S. meliloti* strains: *S. meliloti* 1021 and *S. meliloti* 242. A Fur homolog was identified from the genome sequence of *S. meliloti* 1021. Sequence alignments and phylogenetic analysis strikingly assign Fur_{Sm} into the group of classical Fur proteins (Fig. 1). Interestingly, we found that *mntA* expression is repressed by manganese and that this repression requires a fur_{Sm} functional gene. Previously we showed that the MntABCD system is involved in manganese uptake in *S. meliloti* 242 (35). Fur-mediated regulation of manganese acquisition has been reported in *E. coli* and *S. enterica* though this regulator represses *mntH* gene in an iron-dependent way (24, 33). Our studies do not reveal whether Fur_{Sm} indirectly regulates manganese-responsive genes or whether it is itself a manganese-sensing regulator. In that sense it is possible that Fur metal specificity would have changed during evolution, as seems to be the case of some DtxR homologs (Tro, Sca, and MntR) (20, 37, 39). Due to the redox properties of Fe, many in vitro DNA-binding studies of Fur are carried out with manganese, indicating that this metal could occupy the iron-binding site. Fur protein sequence comparisons show that the amino acids predicted to be implicated in iron binding are conserved (36). Nonetheless, subtle changes in other positions could lead to relevant changes in metal affinity of the binding site. In view of previous observations of Guedon and Helmann (12), we cannot rule out the possibility that the balance of transition metals differs between bacterial species, and this fact could determine metal regulator selectivity in a particular bacterial background. In that sense, it is interesting that Kehres et al. (24) reported that the intracellular Mn concentration in Enterobacteriaceae could reach levels as high as 10 mM, whereas iron content is usually in the range of some hundred micromolar. Therefore, it is possible that Fur responds to Mn^{2+} even if it has a higher affinity for Fe^{2+}.

Our results indicate that Fur_{Sm} appears not to be a global regulator of iron acquisition in *S. meliloti*. Iron-dependent expression of outer membrane proteins and siderophore production were not affected in *S. meliloti fur* mutants. In a different genus of rhizobia, *Bradyrhizobium*, the Fur_{Bj} protein is involved in the repression of iron uptake under high-iron conditions (13). However, in *R. leguminosarum* high-affinity iron uptake systems are iron regulated in a Fur-independent manner, indicating the existence of a different mechanism of regulation (46). Surprisingly, no typical *fur* homolog could be identified in the *M. loti* genome. Our results together with these previous observations point out that at least in some rhizobial species repression of iron-regulated genes is not exclusively mediated by Fur. In *R. leguminosarum* a new regulator, RirA, has been described as being involved in iron homeostasis. A *rirA* homolog (*aau3*) is also present in the *S. meliloti* genome, although its function has not been studied.

ACKNOWLEDGMENTS

We are very grateful to Federico Battistoni and Francisco Noya for critical suggestions.

E.F. was supported by PEDECIBA and by an ASM International Fellowship to work in the MRO lab. R.P. was supported by CSIC, and M.R.O. was supported by NSF grant MCB-0089928.

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