

# The *Sinorhizobium meliloti fur* Gene Regulates, with Dependence on Mn(II), Transcription of the *sitABCD* Operon, Encoding a Metal-Type Transporter

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*Sinorhizobium meliloti* is an alpha-proteobacterium able to induce nitrogen-fixing nodules on roots of specific legumes. In order to propagate in the soil and for successful symbiotic interaction the bacterium needs to sequester metals like iron and manganese from its environment. The metal uptake has to be in turn tightly regulated to avoid toxic effects. In this report we describe the characterization of a chromosomal region of *S. meliloti* encoding the *sitABCD* operon and the putative regulatory *fur* gene. It is generally assumed that the *sitABCD* operon encodes a metal-type transporter and that the *fur* gene is involved in iron ion uptake regulation. A constructed *S. meliloti sitA* deletion mutant was found to be growth dependent on Mn(II) and to a lesser degree on Fe(II). The *sitA* promoter was strongly repressed by Mn(II), with dependence on Fur, and moderately by Fe(II). Applying a genome-wide *S. meliloti* microarray it was shown that in the *fur* deletion mutant 23 genes were up-regulated and 10 genes were down-regulated when compared to the wild-type strain. Among the up-regulated genes only the *sitABCD* operon could be associated with metal uptake. On the other hand, the complete *rhbABCDEF* operon, which is involved in siderophore synthesis, was identified among the down-regulated genes. Thus, in *S. meliloti* Fur is not a global repressor of iron uptake. Under symbiotic conditions the *sitA* promoter was strongly expressed and the *S. meliloti sitA* mutant exhibited an attenuated nitrogen fixation activity resulting in a decreased fresh weight of the host plant *Medicago sativa*.

The root nodule bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Azorhizobium*, and *Sinorhizobium*, collectively known as rhizobia, are able to establish a nitrogen-fixing symbiosis with their respective leguminous host plants. During this symbiotic interaction the rhizobia first induce the formation of root nodules on their respective hosts, colonize the nodules, and finally, after differentiation into bacteroids, reduce atmospheric nitrogen to ammonia. The fixed nitrogen is then delivered to the host plants in the form of alanine or ammonia. The plant in turn supplies the bacteria with various nutrients. This symbiotic interaction has been described in detail recently (44, 57, 61).

One interesting aspect of the rhizobium-legume symbiosis is the influence of transition metals on nodule formation and nitrogen fixation. A number of these micronutrients are known to be essential for bacterial metabolism (36), yet the mechanisms of metal import and their regulation in rhizobia are largely unknown. One of the most important transition metals is iron, which due to its importance in metabolic electron transport chains is essential for almost all known organisms. Despite the fact that iron is the fourth most abundant element on earth its availability to microorganisms is restricted because of its low solubility under aerobic conditions and physiological pH values. Thus, bacteria have evolved a number of strategies to overcome iron deficiencies (26). Most of these mechanisms involve specialized ABC transporters, which can be classified

into three families, namely the siderophore-type, the ferric-type, and the metal-type transporters (37).

The metal-type transporters appear to be less specific for iron than the other transporter types. The best-characterized metal-type iron ABC transporters are the YfeABCD system of *Yersinia pestis*, the SitABCD system of *Salmonella enterica* serovar Typhimurium, and the more recently characterized SitABCD transporter of *Shigella flexneri*. Metal-type ABC transporters were first shown to be involved in the utilization of chelated iron (6, 72), but evidence also exists for their involvement in Mn(II) acquisition (5, 8, 54). The *S. flexneri* SitABCD and the *Y. pestis* YfeABCD transporters were repressed by iron and manganese (6, 54, 72), whereas for *Salmonella* only iron-dependent repression has been investigated so far. In all cases a Fur (ferric uptake regulator) protein is necessary for iron-dependent repression. Additionally, Fur was also shown to be involved in the repression of the *Y. pestis yfeABCD* operon by Mn(II), whereas in *S. flexneri* Mn(II)-dependent repression of the *sitABCD* operon was attributed to the manganese-binding repressor MntR (54).

In many gram-negative bacteria Fur is the main repressor of iron-responsive genes (27). In the classic model of Fur regulation established for *Escherichia coli* the Fur protein utilizes Fe(II) as a cofactor and binds to specific sequence elements in the promoter regions of these genes, termed Fur boxes, and thereby inhibits gene expression under iron-replete conditions (3, 13). Besides its role as a repressor of iron uptake mechanisms Fur has also been found to be involved in the regulation of alternative sigma factor and activator genes (45, 46), stress response genes (19, 28–31), energy metabolism genes (39, 59, 64), and virulence-associated genes (9, 11, 22, 23, 40, 69). Thus,

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristic(s)	Reference
<i>S. meliloti</i>		
Rm1021	Spontaneous mutant of wild-type strain RU47, Sm <sup>r</sup>	43
Rm1021Δ <i>sitA</i> /Rm1021-02509-01	Rm1021 derivative, Δ <i>sitA</i>	This study
Rm1021Δ <i>fur</i> /Rm1021-02510-01	Rm1021 derivative, Δ <i>fur</i>	This study
Rm1021Δ <i>fur</i> ::pTCB1	Rm1021Δ <i>fur</i> carrying pTCB1 integrated into the chromosome	This study
<i>E. coli</i>		
DH5αMCR	F <sup>-</sup> <i>endA1 supE44 thi-1 λ<sup>-</sup> recA1 gyrA96 relA1 deoR Δ(lacZYA-argF)U169 φ80dlacZΔM15 mcrA Δ(mrr hsdRMS mcrBC)</i>	24
S17-1	<i>E. coli</i> 294::[RP4-2(Tc::Mu)(Km::Tn7)] <i>pro res ΔrecA</i> Tp <sup>r</sup>	60
Plasmids		
pK18mobsacB	pUC18 derivative, <i>sacB lacZα</i> Km <sup>r</sup> , mobilizable	56
pK18mob	pUC18 derivative, <i>lacZα</i> Km <sup>r</sup> mobilizable	56
pTCB1	pK18mob containing a ≈1.5-kb fragment of the <i>S. meliloti</i> chromosome containing the <i>fur</i> gene	This study
pTC02510del	pK18mobsacB carrying a Δ <i>fur</i> construct	This study
pTC02509del	pK18mobsacB carrying a Δ <i>sitA</i> construct	This study
pJP2	pTR102 GUS with artificial multiple cloning site, Ap <sup>r</sup> Tc <sup>r</sup>	50
pTCC1	pJP2 with a 762-bp fragment containing the <i>sitA</i> promoter, Ap <sup>r</sup> Tc <sup>r</sup>	This study

in many bacterial species the role of Fur extends beyond that of a negative regulator of iron acquisition systems.

In *Sinorhizobium meliloti*, the microsymbiont of alfalfa, a putative ferric uptake regulator has been identified in the direct vicinity of a metal-type transporter (12); however, the role of Fur in *S. meliloti* has not been investigated yet. Evidence exists that at least in some rhizobia like *Rhizobium leguminosarum* the Fur protein is not a global regulator of iron metabolism (70). The adjoining metal-type transporter, designated SitABCD, was found to be involved in manganese utilization of *S. meliloti* 2042 grown in the presence of a chelator (48). The SitABCD homologues identified in *Y. pestis*, *S. enterica* serovar Typhimurium, and *S. flexneri* contributed to virulence in their animal hosts (5, 35, 54). The relevance of metal-type transporters to bacterium-plant interactions is unclear though. Platero et al. reported that transposon insertions in the *sitB* and *sitD* genes of *S. meliloti* 2042 do not affect the ability of the bacterium to establish nitrogen-fixing symbiosis with alfalfa (48). In contrast, transcriptome and proteome analysis performed with *S. meliloti* 1021 indicated a possible involvement of the SitABCD transporter in symbiosis (2, 14).

The work of Wexler et al. (70) demonstrated that in rhizobia the Fur protein plays a different role than in other mostly enterobacterial organisms studied so far. Therefore, we applied *S. meliloti* whole-genome microarrays to investigate the role of this regulator on a genome-wide scale. Furthermore, we examined the regulation of the *sitABCD* operon of *S. meliloti*, since previous studies of pathogenic bacteria demonstrated that it is a target of the regulatory Fur protein. Additionally, the roles of the *sitABCD* operon in metal uptake in the free-living state and during symbiosis were analyzed in order to determine the relevance of the SitABCD transporter for symbiosis.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The bacterial strains and plasmids used in this study are shown in Table 1. Strains of *E. coli* were routinely cultured at 37°C in antibiotic medium no. 3 (PA) (Oxoid, Wesel, Germany). *S. meliloti*

strains were cultivated at 30°C either in tryptone yeast (TY) complex medium (7) or in Vincent minimal medium (VMM) (68). For growth assays VMM was prepared without any iron sources and was designated VMM\*. Iron and other metals were added after autoclaving as indicated for the respective bioassays. Fe(III) was added as FeCl<sub>3</sub>, Fe(II) was added as FeSO<sub>4</sub>, and Mn(II) was added as MnSO<sub>4</sub>. Where appropriate, antibiotics were added at the following concentrations: neomycin (100 μg ml<sup>-1</sup>), tetracycline (5 or 10 μg ml<sup>-1</sup>), kanamycin (50 μg ml<sup>-1</sup>), and streptomycin (600 μg ml<sup>-1</sup>). For growth under low-iron conditions glassware was washed with 6 M HCl and rinsed thoroughly with water.

**DNA manipulations.** The protocols of Sambrook et al. (55) were used for routine manipulations of plasmid and chromosomal DNA. Mutated DNA fragments containing either a 261-bp deletion in the *fur* gene or a 585-bp deletion in the *sitA* gene were constructed by gene SOEing (33). In a first PCR step, regions up- and downstream of the desired deletion were amplified and then fused in a second PCR. The obtained deletion constructs were subsequently cloned into the suicide vector pK18mobsacB, which allows sucrose selection for vector loss (56). The resulting plasmids were conjugated into *S. meliloti* via *E. coli* S17-1 to introduce deletions by allelic exchange. Mutants were verified by PCR and Southern hybridizations.

In order to analyze the expression of the *sitABCD* operon and possible regulation by Fur, the *sitA* promoter region (Fig. 1) was amplified and cloned into the promoter test vector pJP2 (50) in front of a *gusA* gene. The resulting plasmid, designated pTCC1, was introduced into the *S. meliloti* 1021 wild-type strain (Rm1021) and the *fur* mutant (Rm1021Δ*fur*) for expression analyses.

**Growth inhibition of *S. meliloti* strains by the compounds H<sub>2</sub>O<sub>2</sub> and MnCl<sub>2</sub>.** For zone inhibition assays 100-μl portions of overnight cultures of *S. meliloti* were added to 2 ml of prewarmed 0.7% top agar and layered onto solid TY plates. After the top agar had hardened a sterile filter disk was placed in the middle of the plate. Fifteen microliters of either H<sub>2</sub>O<sub>2</sub> in various concentrations between 0 and 35 mM or MnCl<sub>2</sub> with a concentration between 0 and 250 μM was applied to the filter. The inhibition zone was measured after 48 h of incubation at 30°C.

**Chromazurol S (CAS) liquid assay to determine siderophore production in *S. meliloti* cultures.** CAS assay solutions for siderophore detection were prepared as described by Schwyn et al. (58). Supernatants containing the siderophores of *S. meliloti* cultures grown in TY medium were mixed 1:1 with CAS assay solution. After equilibrium was reached, the absorbance was measured at 630 nm. Relative siderophore activity was determined as optical density ratios of different cultures.

**Expression analysis of the *S. meliloti sitA* promoter, using a *gus* reporter gene construct.** In order to quantitatively determine the activity of the *sitA* promoter, exponential-phase cultures (optical density at 580 nm of 0.6 to 0.8) of *S. meliloti* strains carrying the promoter test vector pTCC1 were centrifuged and diluted into saline buffer (0.89% [wt/vol] NaCl) to 2 × 10<sup>8</sup> CFU ml<sup>-1</sup>; 310 μl of the culture was mixed with 1,190 μl of buffer (50 mM sodium phosphate, 50 mM dithiothreitol, 1 mM EDTA [pH 7]). Toluene was added, and the mixture was

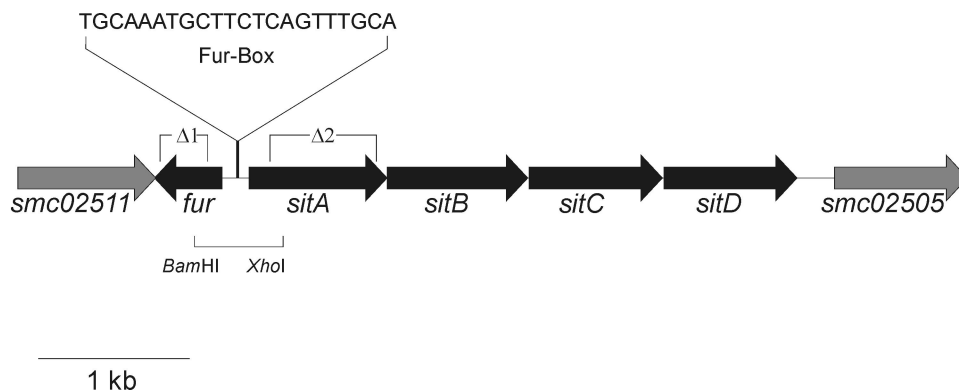


FIG. 1. Genetic organization of a DNA region of the *S. meliloti* chromosome containing the *fur* gene and the *sitABCD* operon. The DNA region of the *S. meliloti* genome containing the *fur* gene, the *sitABCD* operon, and the two neighboring genes, *smc02511* and *smc02505*, of unknown function, is shown. The putative Fur box sequence as identified by an HMM-based search is indicated. The deletions  $\Delta 1$  and  $\Delta 2$  were used to construct mutations in the genes *fur* and *sitA*, respectively. The *fur* deletion  $\Delta 1$  ranges from bases 140 to 400 of the *fur* gene, and the *S. meliloti* strain carrying the deletion  $\Delta 1$  was designated Rm1021 $\Delta fur$ . The *sitA* deletion  $\Delta 2$  ranges from bases 312 to 896 of the *sitA* gene, and the strain carrying the deletion  $\Delta 2$  was named Rm1021 $\Delta sitA$ . In addition, the 760-bp BamHI/XhoI fragment containing most probably the *sitA* promoter was used to construct the promoter test vector pTCC1 for *sitA* promoter studies.

vortexed and then incubated at 37°C for ca. 30 min to remove the toluene. After a short centrifugation to pellet the cell debris, 290  $\mu$ l of the mixture was pipetted into each well of a 96-well suspension culture plate. The reaction was started by adding 10  $\mu$ l of *p*-nitrophenyl  $\beta$ -glucuronide (10.5 mg/ml; Serva, Heidelberg, Germany) to each well. The plates were incubated at 37°C for 2 h in a FLUOstar Galaxy plate reader (BMG Labtechnologies, Offenburg, Germany). The reader was set to measure the optical density at 405 nm every 5 min after an initial incubation of 45 min. The results from 10 time points for six cultures with eight parallel probes each were used to calculate the mean expression level.

In order to visualize  $\beta$ -glucuronidase activity in the nodules, the nodules were harvested, cut with a Vibratome into slices with a thickness of 60  $\mu$ m each, and stained with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid (X-Gluc) (Serva) in a final concentration of 2 mM in Tris/NaCl buffer (100 mM Tris/HCl, 50 mM NaCl [pH 7]) for 2 h at 37°C. Digital images were taken with an Olympus C2000Z camera mounted on an Olympus BH-2 microscope.

**Assays to determine nitrogen-fixation efficiency in the *S. meliloti*-*M. sativa* symbiosis.** Nodulation tests were performed as described by Rolfe et al. (52). Solutions a to c for the plant agar plates (a: 294 g of  $CaCl_2 \cdot 2H_2O$ /liter; b: 50 g of  $KH_2PO_4$ /liter; c: 123 g of  $MgSO_4$ /liter, 87 g of  $K_2SO_4$ /liter, 0.247 g of  $H_3BO_3$ /liter, 0.288 g of  $ZnSO_4$ /liter, 0.1 g of  $CuSO_4 \cdot 5H_2O$ /liter, 0.056 g of  $CoSO_4 \cdot 7H_2O$ /liter, 0.048 g of  $NaMo_4 \cdot 2H_2O$ /liter) were autoclaved separately. One-half milliliter of each solution was added to 1 liter of autoclaved water containing 15 g of agar. Alfalfa (*M. sativa* L. cv. europe) seeds were sterilized with 32% HCl for 30 min and then washed with sterile water. After germination seedlings were placed on nodulation plates and inoculated with equal amounts (around  $6 \times 10^7$  CFU each) of washed wild-type and mutant cells. Plants were weighed after 30 days of growth, and nitrogen fixation activity was tested by the acetylene reduction assay (52).

***S. meliloti* transcript profiling using the genome-wide SM6kPCR microarray.** *S. meliloti* wild-type (Rm1021) and *fur* mutant (Rm1021 $\Delta fur$ ) strains were cultured in VMM in 250-ml Ehrlemeyer flasks with shaking at 150 rpm to an optical density at 580 nm of 0.9 before harvesting. Cells were centrifuged (10,000  $\times g$ , 1 min, 4°C) and afterwards immediately frozen in liquid nitrogen. For total RNA isolation the RNeasy Mini Kit (Qiagen, Hildesheim, Germany) was used. Cells were disrupted in RLT buffer provided in the kit in Fast Protein tubes (Qbiogene, Carlsbad, Calif.), using a Ribolyser (Hybaid, Heidelberg, Germany) (30 s; speed, 6.5) before purification according to the RNeasy Mini Kit RNA purification protocol. Fluorescent labeling of cDNA by amino-allyl dye coupling was performed as described by de Risi et al. (<http://www.microarrays.org/protocols.html>). For this study the Sm6k microarrays described by Rüberg et al. (53) were used. Each microarray contains 6,046 PCR fragments and 161 70-mer oligonucleotides as open reading frame-specific probes, covering the whole *S. meliloti* genome. Each probe was spotted in triplicate. Hybridization and image acquisition of the microarrays were performed as described by Rüberg et al. (53). For acquisition of the mean signal and mean local background intensities for each spot of the microarray ImaGene 5.0 software for spot detection, image segmentation, and signal quantification (Biodiscovery Inc., Los Angeles, Calif.)

was used. Spots were flagged as “empty” if  $R$  was  $\leq 1.5$  in both channels, with  $R = (\text{signal mean} - \text{background mean}) / \text{background standard deviation}$ . The remaining spots were considered for further analysis. The  $\log_2$  value of the intensity ratios was calculated for each spot, with  $M_i = \log_2(R_i/G_i)$  ( $R_i = I_{ch1i} - B_{g, ch1i}$  and  $G_i = I_{ch2i} - B_{g, ch2i}$ , with  $I_{ch1i}$  and  $I_{ch2i}$  being the intensity of a spot in channel 1 or channel 2, respectively, and  $B_{g, ch1i}$  and  $B_{g, ch2i}$  being the background intensity of a spot in channel 1 or channel 2, respectively). The mean intensity was calculated for each spot, with  $A_i = \log_2(R_i/G_i)^{0.5}$  (16). A normalization method based on local regression that accounts for intensity spatial dependence in dye biases was applied. Within a print tip group normalization was performed as described by Yang et al. (71):  $M_i = \log_2(R_i/G_i) \rightarrow \log_2(R_i/G_i) - c_j(A) = \log_2(R_i/[k_j(A)G_i])$ , where  $c_j(A)$  is the lowest fit to the MA plot for the  $j$ th grid (i.e., for the  $j$ th print tip group),  $j = 1, \dots, J$ , with  $J$  denoting the number of print tips. A floor value of 20 was introduced before normalization to be able to use logarithmic values. Genes significantly up- or down-regulated were identified by  $t$  statistics (16). The expression of a gene was considered significantly different if the  $P$  value was  $\leq 0.05$ , the  $\log_2$  ratio of the intensities ( $M$  value) was  $\geq 1$  or  $\leq -1$ , and the mean intensity ( $A$  value) was  $\geq 9$ .

**Bioinformatics tools.** Database searches for homologues to the *S. meliloti* SitABCD and Fur proteins were performed with the BLAST program (1). Potential Fur boxes were identified with the HMMer suite (17). Alignments were performed with the ClustalW program (65). Normalization and  $t$  statistics of microarray data were carried out using the EMMA 1.0 microarray data analysis software (15).

## RESULTS

**Genetic organization of the *S. meliloti* DNA region carrying the *fur* gene and the *sitABCD* operon.** The *S. meliloti* 1021 *sitABCD* locus (Fig. 1) is located on the chromosome of the tripartite genome of this bacterium. According to BLAST searches of available databases the *S. meliloti* SitA, -B, -C, and -D proteins exhibit high homologies to metal-type ABC transporter proteins. Most of them were identified in a variety of animal pathogen  $\gamma$ -proteobacteria, with the notable exceptions of the plant pathogen *Agrobacterium tumefaciens*, an  $\alpha$ -proteobacterium, closely related to *S. meliloti*. Other sequenced rhizobial genomes like *Bradyrhizobium japonicum* and *Mesorhizobium loti* do not possess *sitABCD* homologues. The *S. meliloti* SitA, -B, -C, and -D proteins showed highest similarities to the proteins of the *S. enterica* serovar Typhimurium SitABCD transporter (between 65 and 78% identity) (72), to the *Y. pestis* YfeABCD transporter (between 54 and 66% iden-

tity) (6), and to those of an uncharacterized transporter of *A. tumefaciens* (AGR\_L\_798, AGR\_L\_799, AGR\_L\_801, and AGR\_L\_803, between 64 and 80% identity). Based on sequence similarities it can be assumed that in *S. meliloti* *sitA* encodes the periplasmic binding protein, *sitB* encodes the ATPase, and *sitC* and *sitD* encode the transmembrane domains of the ABC transporter. The genes *sitA*, *-B*, *-C*, and *-D* in the *S. meliloti* chromosome appear to be translationally coupled, which is a strong indicator that they represent an operon. Adjacent to the *S. meliloti* *sitABCD* operon a *fur* gene is located in the opposite orientation. The *S. meliloti* Fur protein showed the highest similarities to the Fur proteins of *Bartonella* species (60% identity) (47).

To analyze the role of the *sitABCD* operon in metal acquisition and to investigate the regulatory role of the *fur* gene in *S. meliloti* 1021, the marker-free deletion mutants Rm1021 $\Delta$ *sitA* and Rm1021 $\Delta$ *fur*, respectively, were constructed. The construction of the deletion mutants is described in Materials and Methods. The deleted fragments are indicated in Fig. 1.

**Roles of the *S. meliloti* *sitABCD* operon in Mn(II) and Fe(II) utilization.** For investigation of the involvement of the *S. meliloti* *sitABCD* operon in both manganese and iron utilization, we analyzed the abilities of strain Rm1021 $\Delta$ *sitA* to grow under iron- and manganese-limiting conditions. First we used defined VMM\* in order to be able to test different metals in defined concentrations (Fig. 2). When grown in VMM\* supplemented with Mn(II) but not with any iron source, the wild type and the *sitA* mutant displayed limited growth with no significant differences between each other. Under these conditions, obviously both strains suffer from iron deficiency. In VMM\* supplemented with 30  $\mu$ M Fe(II) as the sole iron source the *sitA* mutant exhibited a strongly reduced growth compared to the wild type. Growth was less than during incubation in VMM\* without added iron sources. Addition of 1  $\mu$ M Mn(II) to the medium fully restored growth to wild-type levels, which was not the case when up to 60  $\mu$ M Fe(II) was added. The manganese-dependent growth defect of Rm1021 $\Delta$ *sitA* suggests that at least during growth in VMM the *sitABCD* operon is needed for the high affinity transport of Mn(II).

It has been reported that SitABCD homologues are also involved in the acquisition of chelated iron (6). Since the Mn(II) growth deficiency in VMM\* might obscure any iron-dependent growth phenotypes, we also tested the growth of the *sitA* mutant in TY complex medium. Platero et al. demonstrated that the iron chelator EDDHA (ethylenediamine di-*o*-hydroxyphenylacetic acid) also appears to heavily affect manganese availability in TY medium (48). Therefore, we tested different chelators that might reduce iron availability while not causing manganese deficiency. Under sufficiently high conalbumin (Con) concentrations (>240 mg/liter) the *S. meliloti* *sitA* mutant Rm1021 $\Delta$ *sitA* exhibited a prolonged lag phase when compared to growth of the wild type. This growth deficit could be alleviated by the addition of Fe(II) but not by the addition of Mn(II) (Fig. 3), indicating that iron deficiency is responsible for this growth phenotype. The prolonged lag phase might be explained by the assumption that the growth of Rm1021 $\Delta$ *sitA* is dependent on the expression of alternative iron ion transport systems. The results therefore suggest that the *sitABCD* system

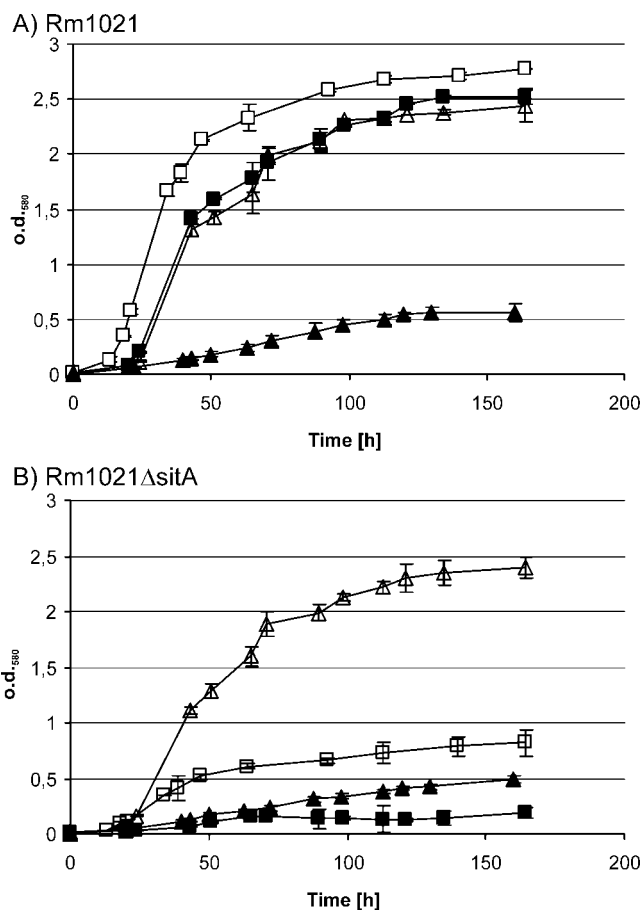


FIG. 2. Growth of the *S. meliloti* wild type and the *S. meliloti* *sitA* mutant strain with dependence on Mn(II) and/or Fe(II) supplementation of the medium. Overnight cultures of the *S. meliloti* wild-type strain Rm1021 (A) and the *S. meliloti* *sitA* mutant Rm1021 $\Delta$ *sitA* (B) were washed and diluted in fresh VMM\* containing 30  $\mu$ M Fe(II) (■), 60  $\mu$ M Fe(II) (□), 1  $\mu$ M Mn(II) (▲), or 30  $\mu$ M Fe(II) + 1  $\mu$ M Mn(II) (△). Growth was monitored via optical density at 580 nm (o.d.<sub>580</sub>). The results are derived from five independent replicates, and the error bars indicate the standard deviations.

contributes to acquisition of chelated iron during early growth stages.

**Phenotypic characterization of the *S. meliloti* *fur* mutant Rm1021 $\Delta$ *fur*.** In many gram-negative bacteria the Fur protein acts as a global repressor, and therefore a defect in Fur often results in pleiotropic phenotypes. Common traits in *fur* mutants include siderophore overproduction, enhanced manganese resistance, and H<sub>2</sub>O<sub>2</sub> sensitivity (27). Therefore, we tested the inhibitory capacity of Mn(II) and H<sub>2</sub>O<sub>2</sub> on the growth of the *S. meliloti* wild-type Rm1021 and the *fur* mutant Rm1021 $\Delta$ *fur* on TY agar plates as described in Materials and Methods. The inhibition zone was monitored after 48 h of growth, but no difference between the wild-type and mutant strains were observed (data not shown). Additionally, in a CAS assay the amounts of produced siderophores in the supernatants of the wild-type and the Rm1021 $\Delta$ *fur* culture grown in TY medium were measured, but no differences between the wild type and the *fur* mutant were found (data not shown).

We further tested whether Rm1021 $\Delta$ *fur* exhibited iron-de-

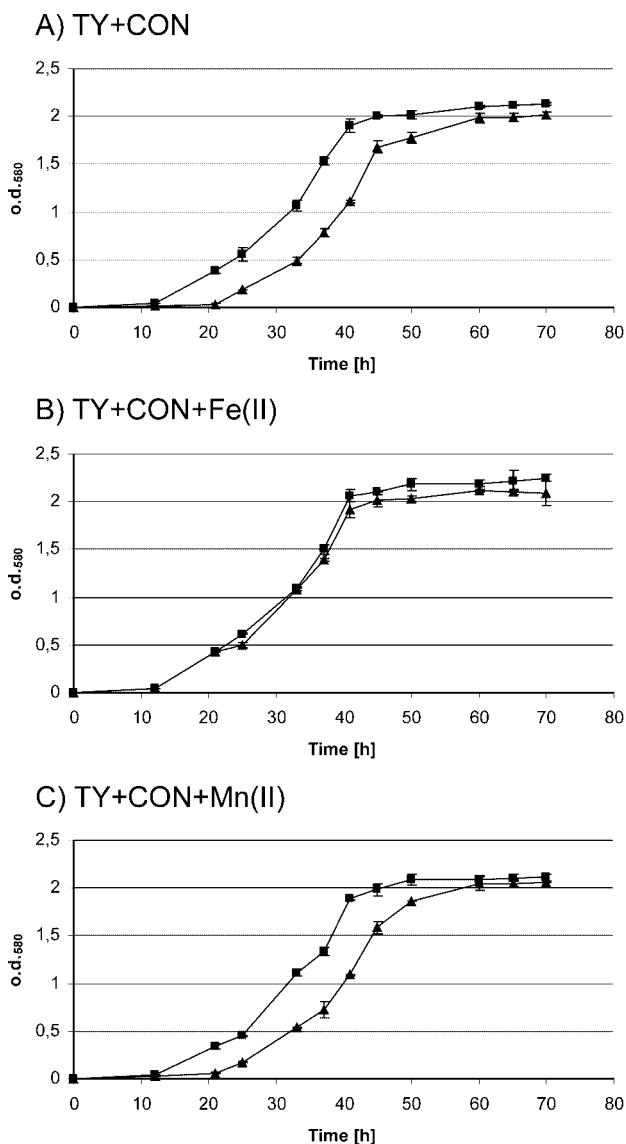


FIG. 3. Effects of the iron chelator Con on the growth of the *S. meliloti* wild-type strain and the *S. meliloti sitA* mutant strain. Strains Rm1021 (■) and Rm1021Δ*sitA* (▲) were grown in TY medium containing 240 mg of Con/liter (A), 240 mg of Con/liter and 25 μM Fe(II) (B), or 240 mg of Con/liter and 25 μM Mn(II) (C). The depicted results were obtained from five independent replicates, and the error bars indicate the standard deviations. o.d.<sub>580</sub>, optical density at 580 nm.

pendent growth phenotypes. For this purpose overnight cultures of the *S. meliloti* wild type (Rm1021) and the *S. meliloti fur* mutant (Rm1021Δ*fur*) were washed and diluted into fresh VMM\* containing either no added iron or 30 μM Fe(II). Growth was then monitored by measuring the optical density (Fig. 4). The mutant strain Rm1021Δ*fur* showed no growth differences compared to the wild type when grown in medium containing 30 μM Fe(II). During growth under low-iron conditions in VMM\*, both the wild type and *fur* mutant exhibited a longer lag phase and reduced overall growth than under iron-replete conditions, likely caused by iron deficiency. Surprisingly though, the *fur* mutant showed a higher initial growth and reached a higher cell density than the wild type.

**Regulation of the *S. meliloti sitA* promoter, with dependence on the *fur* gene and metal ions.** Since the growth experiments suggested that the *sitABCD* operon is involved in Mn(II) and Fe(II) acquisition, we investigated whether its expression is regulated by those metals. The *S. meliloti* wild-type strain Rm1021 carrying the reporter plasmid pTCC1 was grown in VMM\*, to which various amounts of different metals were added. Since pTCC1 carried the *sitA* promoter fused to a *gusA* codon region, *sitA* expression could be measured by determining the β-glucuronidase activity during logarithmic growth (Fig. 5).

The expression of the *sitA* promoter in wild-type *S. meliloti* cells grown in VMM\* containing 30 μM Fe(III) (Fig. 5B) was only marginally lower than in cells incubated in medium containing no added iron (Fig. 5A). Doubling the amount of Fe(III) to 60 μM did not result in a significant change of *sitA* expression. In contrast, addition of 30 μM Fe(II) reduced the expression of the *sitABCD* operon to half of the level ( $P < 0.01$ , *t* test) of that in cells grown in medium with no added iron (Fig. 5C). This indicates that the *sitA* promoter is only weakly affected by Fe(III) and moderately by Fe(II). Addition of 1 μM Mn(II) on the other hand repressed *sitA* promoter activity to background levels, regardless of the concentration of other metals. Addition of Zn(II) or Ca<sup>2+</sup> to the medium had no effect on the expression of *sitA* (data not shown), indicating that the moderate repression by Fe(II) and the strong repression by Mn(II) are specific.

We further investigated whether repression by Fe(II) and Mn(II) is mediated by Fur. The *sitA* promoter test vector pTCC1 was introduced into the *fur* mutant strain Rm1021Δ*fur*. *sitA* expression was determined as described above. The *sitA* promoter activity in Rm1021Δ*fur* was under all tested conditions higher than in the wild type (Fig. 5), indicating that the *fur* gene regulates the *sitABCD* operon as a repressor. The *sitA* promoter activity of the *fur* mutant was totally unaffected by supplementation with Mn(II); thus, it can be concluded that the *fur* gene is needed for Mn(II)-mediated repression of the *sitA* promoter. In contrast, *sitA* expression in the *fur* mutant background was still repressed by Fe(II), indicating that Fur is not essential for the repression of the *sitA* promoter by Fe(II). Since Fe(II)-dependent repression of the *sitA* promoter appears to be stronger in the wild type than in the *fur* mutant, it is possible that Fur enhances the repression. Altogether these results strongly suggest that the *S. meliloti* Fur protein is an Mn(II)-dependent repressor of the *sitABCD* operon.

To verify that the observations for Rm1021Δ*fur* were indeed solely caused by the mutation in the *fur* gene, we integrated plasmid pTCB1, carrying the *S. meliloti fur* gene, into the chromosome of the *fur* mutant. The *sitA* promoter activity in the resulting strain, Rm1021Δ*fur*(pTCB1), was under all conditions similar to that of the wild type (Fig. 5). This demonstrates that only the *fur* gene is needed for the Mn(II)-dependent repression of the *sitA* promoter.

**Micorarray-based analysis of transcription of *S. meliloti* genes with dependence on the *fur* gene.** In order to identify which genes beside the *sitABCD* operon are affected by the *fur* mutation, a genome-wide expression profiling of the *fur*-defective *S. meliloti* strain Rm1021Δ*fur* compared to the wild-type, Rm1021, using the *S. meliloti* SM6kPCR microarrays (53), was performed. Cultures of both strains were grown in VMM to a

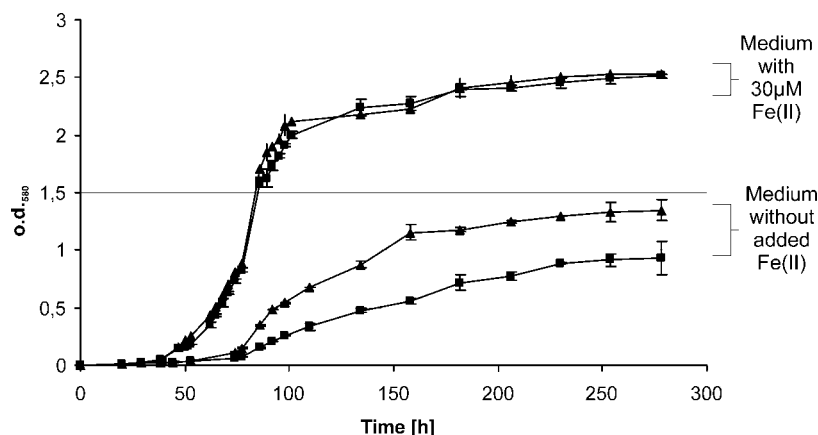


FIG. 4. Growth of the *S. meliloti* wild-type strain Rm1021 and the *fur* mutant strain Rm1021 $\Delta$ *fur* under high and low iron concentrations. Overnight cultures of Rm1021 (■) and Rm1021 $\Delta$ *fur* (▲) were washed and diluted into fresh VMM\* containing 30  $\mu$ M Fe(II) or no added iron sources and incubated at 30°C for 180 h. The growth was monitored by measuring the optical density at 580 nm (o.d.<sub>580</sub>). The error bars indicate the standard deviations, which were calculated from six independent experiments.

cell density of about  $8 \times 10^8$  CFU ml<sup>-1</sup>. RNA isolation, labeling, microarray hybridization, and data analysis were performed as described in Materials and Methods. The expression of a gene was considered significantly different if the *M* value was  $\geq 1$  or  $\leq -1$ , the *A* value was  $\geq 9$ , and *P* was  $\leq 0.05$ . The results depicted in the scatter plot (Fig. 6) and summarized in Table 2 were obtained from two independent biological replicates. Altogether, 33 genes displayed an altered expression in the *fur* mutant when compared to expression in the wild-type.

Of these genes, 23 were induced and 10 were repressed in the *fur* mutant.

In accordance with *gus* reporter gene analyses, the genes of the *sitABCD* operon were found to be highly induced in the *fur* mutant. Surprisingly, none of the other up-regulated genes showed homologies to genes of known metal utilization systems. Instead, the up-regulated genes were found to be involved in the transport of sugar (*smc02474*) or peptides (*smc03269* and *smc02025*) or to encode efflux transporters

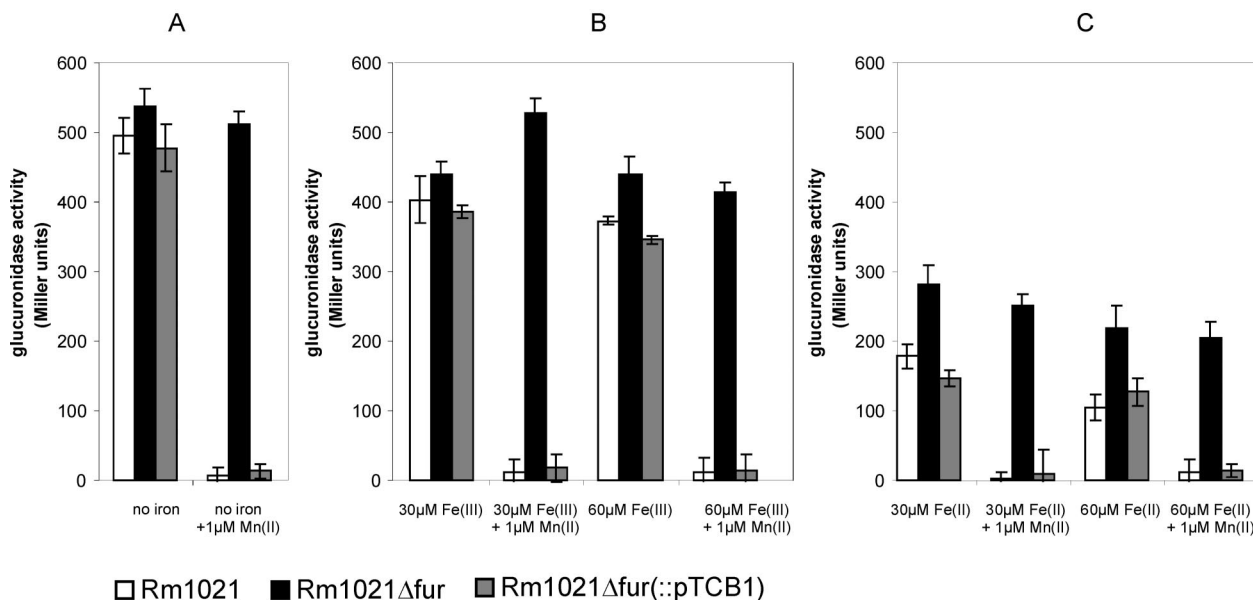


FIG. 5. Effects of different metal sources on the expression of the *S. meliloti* *sitA* promoter. A promoter test plasmid containing a *sitA* promoter-*gusA* fusion (pTCC1) was introduced into the *S. meliloti* wild-type strain Rm1021 (white bars), the *S. meliloti* *fur* mutant strain Rm1021 $\Delta$ *fur* (black bars), and the *fur* mutant strain Rm1021 $\Delta$ *fur* complemented with plasmid pTCB1 carrying an intact *fur* gene (grey bars). The strains were grown in VMM\* under the following conditions: (A) no added iron, (B) addition of Fe(III), (C) addition of Fe(II) as the sole iron source. Additionally, Mn(II) was added as indicated. The different strains were grown to an optical density of 0.6 before the assays for glucuronidase activity were carried out. The  $\beta$ -glucuronidase levels are expressed in Miller units, and the error bars indicate the standard deviations. In each case the depicted results were derived from six independent cultures.

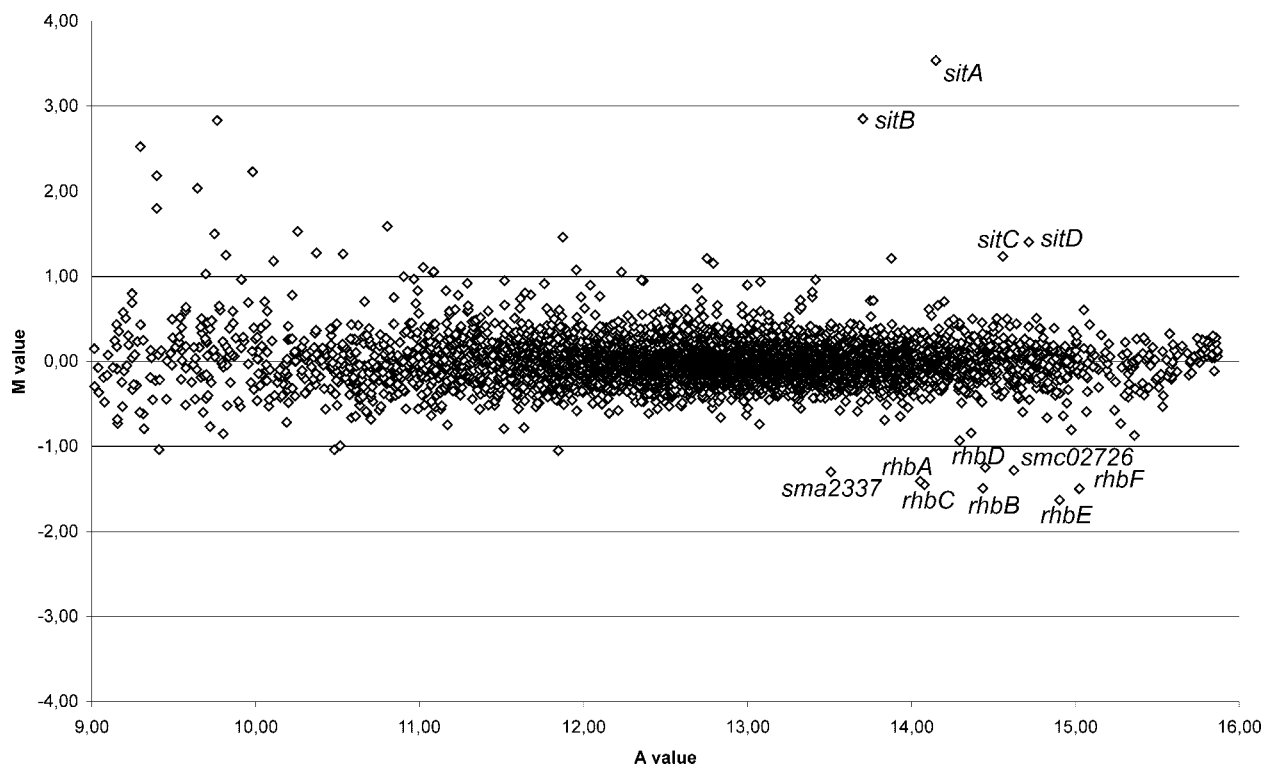


FIG. 6. Scatter plot for the microarray-based analysis of *S. meliloti* genes affected by deletion of the *fur* gene. The scatter plot shows the logarithmic mean signal ratios (*M* values) versus the logarithmic mean signal intensities (*A* values) obtained for comparison of the *S. meliloti* wild-type strain Rm1021 to the *fur* mutant Rm1021Δ*fur* by microarray hybridization. Indicated are the differentially expressed genes that are involved in metal acquisition. Genes were only regarded as differentially expressed when the *M* values were  $\geq 1.0$  or  $\leq -1.0$ , the *A* values were  $\geq 9.0$ , and the *P* values  $\leq 0.05$ .

(*phaF* and *nolG*), a regulator (*smc01664*), five probable enzymatic proteins (*mdeA*, *glnD*, *sma2137*, *smc01821*, and *smc02416*), and seven proteins which have been annotated as hypothetical proteins (*smc01637*, *sma1413*, *smc04431*, *smb20335*, *smc01701*, *smb21403*, and *smb20532*). An updated annotation of these hypothetical proteins did not give any evidence that they might be involved in metal utilization.

Ten genes were moderately (between 2.05- and 3.09-fold) less expressed in the *fur* mutant Rm1021Δ*fur* than in the wild-type strain. It is noteworthy that except for the genes *smb20728* and *sma0629*, all of them seem to be involved in iron acquisition. Six of the genes comprise the complete *rhbABCDE* operon (Table 2), which is involved in the synthesis of rhizobactin 1021, the siderophore of *S. meliloti* 1021 (41). Directly upstream of the siderophore synthesis operon a gene encoding a putative transmembrane transporter (*sma2337*) is located, which is also down-regulated in the *fur* mutant. Finally, the gene *smc02726*, which was found to be repressed in the *fur* mutant Rm1021Δ*fur*, was annotated to encode a putative outer membrane iron receptor.

In order to identify putative Fur boxes in the *S. meliloti* genome, we performed a genome-wide hidden Markov model-based search (17) for putative Fur binding regions on the basis of the alignments of known Fur boxes. Only in front of the *sitABCD* operon, but not in front of the other up-regulated genes, a significant Fur-box motif (GCAAATGCTTCTCATT

TGC) could be detected. This motif exhibits only moderate homologies to either the classical Fur box motif or the 7-1-7 motif as proposed by Baichoo and Helmann (4), but it could be interpreted as an F-F-x-R motif (GCAAAT GCTTCT C ATT TGC) (18, 38) with two imperfect direct repeats and an inverted repeat.

**Roles of the *S. meliloti fur* gene and the *sitABCD* operon in nodule symbiosis.** Up to this point we investigated the role of the *fur* and *sitA* genes during free-living growth of *S. meliloti*. To assess whether defects in the *sitABCD* operon and the *fur* gene also affect the symbiotic abilities of *S. meliloti*, we inoculated the *S. meliloti* mutant strains Rm1021Δ*fur* and Rm1021Δ*sitA* and the wild-type strain Rm1021 onto *M. sativa* seedlings. After 4 weeks of growth the fresh weight of the plants was measured and the ability of the nodules to fix nitrogen was assessed by acetylene reduction tests. Both mutants were able to nodulate normally, and the number of nodules varied between 5 and 18 per plant, with no significant difference between the mutants and the wild type. Moreover, *M. sativa* plants inoculated with the *fur*-defective *S. meliloti* strain showed no differences in fresh weight and nitrogen fixation rate compared to plants inoculated with the wild-type strain (Table 3). However, *M. sativa* plants which were inoculated with the *S. meliloti sitA* mutant strain showed a slight but significant decrease in wet weight as well as in the acetylene reduction rate ( $P < 0.001$ , *t* test). Thus, the *sitA* mutation

TABLE 2. Genes induced or repressed more than twofold in the *S. meliloti fur* mutant Rm1021 $\Delta$ fur as compared to the wild type, Rm1021

Gene	Expression ratio	M value	A value	Function
<i>smc04431</i>	2.38(+)	1.25	9.82	Conserved hypothetical protein
<i>smc03269</i>	3.00(+)	1.59	10.80	Putative dipeptide periplasmic ABC transporter
<i>smc03183 (phaF1)</i>	2.15(+)	1.11	11.02	pH adaptation potassium efflux system
<i>smc02509 (sitA)</i>	11.62(+)	3.54	14.15	Manganese/iron transporter periplasmic binding protein
<i>smc02508 (sitB)</i>	7.22(+)	2.85	13.70	Manganese/iron transporter ATPase
<i>smc02507 (sitC)</i>	2.35(+)	1.23	14.56	Manganese/iron transporter membrane protein
<i>smc02506 (sitD)</i>	2.64(+)	1.40	14.72	Manganese/iron transporter membrane protein
<i>smc02474</i>	4.69(+)	2.23	9.98	Putative sugar ATP binding ABC transporter protein
<i>smc02416</i>	4.54(+)	2.18	9.40	Putative hydrolase
<i>smc02025</i>	2.32(+)	1.21	13.88	Putative dipeptide periplasmic binding protein
<i>smc01821</i>	2.11(+)	1.07	11.96	Putative dihydropyrimidinase
<i>smc01701</i>	2.08(+)	1.05	11.09	Conserved hypothetical protein
<i>smc01666 (mdeA)</i>	5.75(+)	2.52	9.30	Putative methionine gamma lyase
<i>smc01664</i>	7.12(+)	2.83	9.76	Putative transcription regulator
<i>smc01637</i>	4.09(+)	2.03	9.64	Conserved hypothetical protein
<i>smc01124 (glnD)</i>	2.42(+)	1.28	10.37	Putative uridyltransferase
<i>smb21403</i>	2.06(+)	1.04	11.08	Hypothetical protein
<i>smb20532</i>	2.00(+)	1.00	10.90	Hypothetical protein
<i>smb20335</i>	2.26(+)	1.18	10.11	Conserved hypothetical protein
<i>sma2137</i>	2.32(+)	1.21	12.75	Putative glycerate dehydrogenase
<i>sma1413</i>	2.83(+)	1.50	9.75	Hypothetical protein
<i>sma1016</i>	2.23(+)	1.15	12.79	Putative acetyltransferase
<i>sma0875 (nolG)</i>	2.75(+)	1.46	11.87	Putative efflux transporter
<i>sma0629</i>	2.05(-)	-1.04	10.48	Hypothetical protein
<i>sma2337</i>	2.46(-)	-1.30	13.51	Putative transmembrane transport protein
<i>sma2400 (rhbA)</i>	2.66(-)	-1.41	14.05	Rhizobactin 1021 siderophore biosynthesis
<i>sma2402 (rhbB)</i>	2.81(-)	-1.49	14.44	Rhizobactin 1021 siderophore biosynthesis
<i>sma2404 (rhbC)</i>	2.74(-)	-1.45	14.08	Rhizobactin 1021 siderophore biosynthesis
<i>sma2406 (rhbD)</i>	2.37(-)	-1.25	14.45	Rhizobactin 1021 siderophore biosynthesis
<i>sma2408 (rhbE)</i>	3.09(-)	-1.63	14.90	Rhizobactin 1021 siderophore biosynthesis
<i>sma2410 (rhbF)</i>	2.82(-)	-1.50	15.02	Rhizobactin 1021 siderophore biosynthesis
<i>smb20728</i>	2.07(-)	-1.05	11.85	Hypothetical protein
<i>smc02726</i>	2.43(-)	-1.28	14.63	Putative iron transport protein

seems to attenuate the nitrogen fixation capability of the microsymbiont. Increasing the Fe(II) concentration of the plant medium to 30  $\mu$ M abolished the differences between the wild type and *sitA* mutant, whereas addition of up to 30  $\mu$ M Mn(II) had no effect (Table 3).

Furthermore, we analyzed the expression of the *sitABCD* operon during symbiosis by inoculating *M. sativa* plants with the wild-type strain Rm1021 and the *fur* mutant Rm1021 $\Delta$ fur, both carrying the *sitA* promoter *gusA* reporter construct on the plasmid pTCC1. As a negative control the *S. meliloti* wild type carrying plasmid pJP2 without inserts was used. By X-Gluc staining of the nodules, the activity of the *sitA* promoter was visualized. As expected, no  $\beta$ -glucuronidase activity was detected in the negative control (Fig.

7A). Since we demonstrated earlier that the *sitABCD* operon is derepressed in the *fur* mutant, it could be expected that this is also the case in the nodule. Indeed, *M. sativa* nodules that were inoculated with the *fur* mutant carrying pTCC1 showed a strong blue staining throughout all zones of the nodule with the exception of the meristem (Fig. 7B). This indicates that in the *S. meliloti fur* mutant background the *sitABCD* operon is highly expressed in the nodule. Nodules that were induced by the wild-type strain carrying the *sitA* promoter test plasmid (Fig. 7C) exhibited a  $\beta$ -glucuronidase pattern similar to that of nodules induced by the *fur* mutant. One difference, however, was that the infection zone of nodules induced by the wild type was generally more weakly colored than the nitrogen fixation zone. This may

TABLE 3. Nitrogenase activity and wet weight of *M. sativa* plants inoculated with the *S. meliloti* wild type (Rm1021), the *fur* mutant (Rm1021 $\Delta$ fur), and the *sitA* mutant (Rm1021 $\Delta$ sitA) after 4 weeks of growth on plant medium containing the indicated amounts of Fe(II) and Mn(II)

Strain	Nitrogenase activity (nmol of ethylene plant <sup>-1</sup> h <sup>-1</sup> )			Foliage fresh wt (mg plant <sup>-1</sup> )		
	10 $\mu$ M Fe(II)	30 $\mu$ M Fe(II)	10 $\mu$ M Fe(II) + 30 $\mu$ M Mn(II)	10 $\mu$ M Fe(II)	30 $\mu$ M Fe(II)	10 $\mu$ M Fe(II) + 30 $\mu$ M Mn(II)
Rm1021	3.1 $\pm$ 0.8	2.4 $\pm$ 0.4	3.1 $\pm$ 0.7	152 $\pm$ 10	134 $\pm$ 11	141 $\pm$ 13
Rm1021 $\Delta$ fur	3.1 $\pm$ 0.2	2.1 $\pm$ 0.6	2.9 $\pm$ 0.9	132 $\pm$ 6	138 $\pm$ 29	148 $\pm$ 26
Rm1021 $\Delta$ sitA	1.6 $\pm$ 0.3	2.3 $\pm$ 0.3	1.4 $\pm$ 0.5	71 $\pm$ 8	131 $\pm$ 37	87 $\pm$ 11



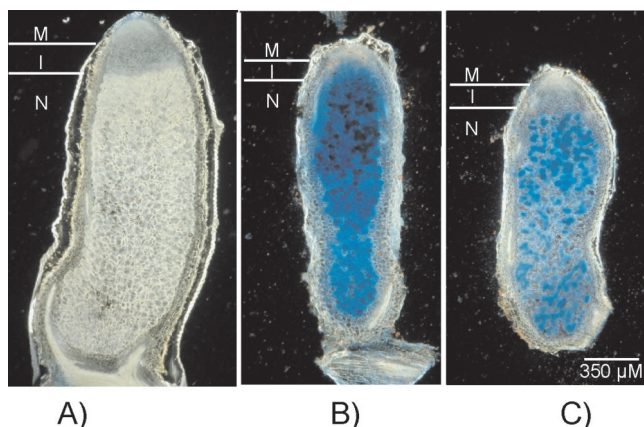


FIG. 7. Visualization of *S. meliloti sitA* promoter activity in *M. sativa* nodules. Nodules of *M. sativa* were harvested, thin sectioned, and stained with the dye X-Gluc. (A) *M. sativa* nodule induced by the *S. meliloti* wild-type strain carrying pJP2. (B) *M. sativa* nodule induced by the *S. meliloti fur* mutant carrying *sitA* promoter-*gusA* fusion plasmid pTCC1. (C) *M. sativa* nodule induced by the *S. meliloti* wild-type strain carrying pTCC1. Abbreviations: M, meristem; I, infection zone; N, nitrogen fixation zone.

indicate that in the wild-type strain the *sitABCD* operon is generally more strongly expressed in the nitrogen-fixing zone than in the infection zone.

## DISCUSSION

**The *S. meliloti sitABCD* operon is involved in Mn(II) and Fe(II) utilization.** In this study we analyzed *S. meliloti* mutants to investigate the involvement of the *sitABCD* operon and the *fur* gene in metal acquisition and regulation. The manganese-deficient growth of the *sitA* mutant clearly demonstrated that the *sitABCD* operon is involved in Mn(II) utilization. Moreover, the strength of the growth inhibition implies that SitABCD is, at least under the tested conditions, the main transporter for Mn(II) in *S. meliloti*. Since addition of Mn(II) restored the growth of Rm1021Δ*sitA*, it is probable that *S. meliloti* possesses alternative transporters with a lower affinity for Mn(II). In the genome of *S. meliloti* a hitherto uncharacterized, putative manganese transporter is located (*sma1115*), which is a possible candidate for the low-affinity transporter of Mn(II).

In contrast to the manganese-dependent growth, the iron deficiency of the *S. meliloti sitA* mutant was far less apparent. Only in the presence of high concentrations of the chelator Con did growth experiments reveal an extended lag phase for the *sitA* mutant when compared to the wild type. This indicates that other iron transport systems that have been annotated for *S. meliloti* (12) might be able to compensate for the missing iron transport of SitABCD. Nonetheless, the growth experiment showed that the *sitABCD* operon does promote growth of *S. meliloti* in the presence of chelated iron; however, the physiological relevance is not clear. The contribution of metal-type ABC transporters to either iron or manganese uptake varies in different species. In *Yersinia* and *Shigella* the Sit homologues appear to be primarily involved in iron uptake (5, 54), while in

*Salmonella* and, according to our results, in *S. meliloti* the manganese uptake capabilities are of more importance.

**The *S. meliloti fur* gene is required for Mn(II)-dependent repression of the *sitABCD* operon.** The expression analyses showed that the *S. meliloti sitABCD* operon is repressed by Mn(II) and Fe(II), but not by Fe(III). These results support the observation that the *sitABCD* operon is involved in the acquisition of Mn(II) and Fe(II). The repression by Mn(II) was completely eliminated in the *S. meliloti fur* mutant. However, the *fur* deletion did not negate the repression of *sitA* by Fe(II). Thus, the Mn(II)-dependent repression of the *S. meliloti sitABCD* operon is strongly dependent on the *fur* gene, while Fe(II)-dependent repression is only weakly affected by the *fur* mutation. Probably another, hitherto uncharacterized regulator is involved in Fe(II)-dependent repression of the *sitABCD* operon.

The orthologous *yfeABCD* operon of *Y. pestis* and the *sitABCD* operon of *S. enterica* serovar Typhimurium were both reported to be repressed by iron, and at least the *yfeABCD* operon was also repressed in the presence of Mn(II) (6, 72), which is in accordance with our results. One difference is that in *S. enterica* serovar Typhimurium and *Y. pestis* a *fur* gene is needed for the repression of the *sitABCD* operon by Fe(II), whereas Fur in *S. meliloti* plays only a subordinate role, if any, in the Fe(II)-dependent repression of the *sitA* promoter. Our results indicate that Fur is mainly an Mn(II)-dependent repressor of the *S. meliloti sitABCD* operon.

**The *S. meliloti fur* gene does not encode a global regulator of iron uptake.** In many gram-negative bacteria the *fur* gene has been well established as a central repressor of iron uptake and other iron-responsive genes (27). Our microarray experiments showed that altogether only 23 genes were significantly up-regulated in a *fur* mutant. In similar global analyses of iron-responsive genes in *E. coli* and *Neisseria meningitidis* (25, 42), a larger number of genes was found to be repressed by Fur. It is of special interest that in our experiments the genes of the *sitABCD* operon were the only ones known to be involved in metal uptake. The other up-regulated genes have no similarities to those for known iron utilization systems. Additionally, only in front of the *sitABCD* genes could a significant Fur box motif be found. This supports our reporter fusion-based expression analyses, with which we demonstrated that *S. meliloti* Fur is a manganese-dependent repressor of the *sitABCD* system. The lack of putative Fur boxes in front of the other genes identified in the microarray experiment might indicate that those genes are not directly controlled by Fur. On the other hand, in *B. japonicum* an atypical Fur-binding site was identified (20). Whether this is also the case in *S. meliloti* remains to be determined.

While we cannot state with absolute certainty whether some of the hypothetical proteins might represent completely novel metal utilization systems, it is intriguing that none of the known iron uptake systems of *S. meliloti* are up-regulated in the *fur* mutant. It is noteworthy in this context that a putative iron transport gene (*smc02726*) and the complete rhizobactin 1021 synthesis operon are, in contrast, repressed in the *S. meliloti fur* mutant, instead of being induced. The simplest explanation is that the derepression of the *sitABCD* operon led to an increase in intracellular Mn(II) and/or Fe(II) concentration, which in turn caused the observed repression of the iron utilization

systems. This would also explain the higher initial growth of the *S. meliloti fur* mutant compared to the wild type during incubation in iron-limited medium. The enhanced expression of the *S. meliloti sitABCD* operon caused by the *fur* mutation might thus improve the ability of *S. meliloti* to scavenge iron from the medium. The altered intracellular metal content caused by the derepression of the *sitABCD* operon might also be responsible for the differential expression of other genes identified in our microarray experiments. These results clearly demonstrate that, unlike in other gram-negative bacteria, in *S. meliloti* the protein encoded by the *fur* gene does not function as the main repressor of iron uptake. This finding is also supported by the phenotypic analyses of the *S. meliloti fur* mutant, in which we found that this mutant did not exhibit common phenotypes of other known *fur*-defective strains like H<sub>2</sub>O<sub>2</sub> sensitivity, manganese resistance, siderophore overproduction (31, 62), and impaired growth in the presence of iron (19, 31, 39, 62, 63, 67). Additionally, the *S. meliloti fur* mutant nodulated *M. sativa* plants and fixed nitrogen with the same efficiency as the wild type. These results thus verify that the role of the *fur* gene in *S. meliloti* differs substantially from that in other bacterial species. This is in accordance with experiments performed by Wexler et al. in which it was demonstrated that the *fur* gene in *R. leguminosarum* is not involved in the regulation of operons involved in iron metabolism and that an *R. leguminosarum fur* mutant did not exhibit any phenotypes generally associated with a *fur* mutation (70). In fact, in *R. leguminosarum* a different protein termed RirA appears to fulfill most functions that are usually attributed to Fur (66).

Given the fact that the Fur homologue in *S. meliloti* is indeed not a ferric uptake regulator, but rather an Mn(II)-dependent repressor, renaming the *S. meliloti fur* gene *mur* (manganese uptake regulator) should be considered. To date only a few regulators specific for manganese uptake systems have been described for bacteria (32). Most of these regulators belong to the group of DtxR-like proteins such as TroR in *Treponema pallidum* (49), ScaR in *Streptococcus gordonii* (34), and MntR in *Bacillus subtilis* (51), none of which exhibits significant homologies to the *S. meliloti* Fur protein. Another metal-dependent repressor is PerR of *Bacillus subtilis* (10). PerR represses genes in the presence of either Fe(II) or Mn(II); however, the regulation of *fur* by PerR in *B. subtilis* appears to be solely Mn(II) dependent (21). The results of our work now indicate that the *S. meliloti* Fur homologue is the first characterized member of the Fur family which is mainly involved in manganese regulation in gram-negative bacteria.

**The *sitABCD* operon is induced during nodule symbiosis and contributes to symbiotic nitrogen fixation.** In plant tests we found that the mutation in the *S. meliloti sitA* gene led to an attenuated nitrogen fixation activity. Additionally, the *sitA* promoter showed strong expression in the nodule, indicating that the *sitABCD* operon contributes to successful symbiosis. This is in accordance with macroarray experiments performed by Ampe et al. who found an induction of the *S. meliloti sitA* gene under symbiotic conditions (2). Additionally, Dvordjevic et al. identified the SitA and SitB protein among proteins isolated from nodules which were induced by *S. meliloti* (14).

Since the nitrogen fixation efficiency of the *sitA* mutant could be restored to wild-type levels by supplementing the plant medium with Fe(II), but not with Mn(II), it appears unlikely

that the altered symbiotic properties were caused by manganese deficiency. A possible explanation for this observation might be that different sets of transport systems are expressed during free-living growth and in symbiosis. In liquid medium iron transporters other than the SitABCD system (e.g., siderophore-dependent iron transporters) might be the main importers of iron. This would be in agreement with the weak iron-dependent growth phenotype of the *S. meliloti sitA* mutant in VMM. During symbiosis, however, the genes involved in siderophore synthesis (*rhbABCDE*) and transport (*rhtA*) in *S. meliloti* are inactive (41). Under these conditions the SitABCD transporter may have an increased importance for iron acquisition. Our data thus suggest that the relevance of the SitABCD transporter for manganese and iron uptake might be different during free-living growth than under symbiotic conditions. It is obvious however that in any case the *sitABCD* operon is not essential for metal acquisition during symbiosis since the nitrogen fixation rate was only moderately lower in the *sitA* mutant than in the wild type.

It is interesting that the metal-type ABC transporters encoded by *yfeABCD* of *Y. pestis* and *sitABCD* of *S. enterica* serovar Typhimurium and *S. flexneri* contribute significantly to the virulence of the respective pathogens (5, 35, 54). Moreover other (uncharacterized) homologues of these metal-type transporters are mainly found in pathogens like *Haemophilus* or enteroinvasive *E. coli* isolates. It thus appears that the highly conserved *sitABCD* system generally contributes to the supply of the respective bacteria with metals in their animal or plant host organisms but is not always essential.

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