

Glutathione Plays a Fundamental Role in Growth and Symbiotic Capacity of *Sinorhizobium meliloti*

Judith Harrison,¹† Alexandre Jamet,¹ Cecilia I. Muglia,² Ghislaine Van de Sype,¹
O. Mario Aguilar,² Alain Puppo,^{1*} and Pierre Frendo¹

Interactions Plantes-Microorganismes et Santé Végétale, UMR CNRS-INRA-Université de Nice-Sophia Antipolis, Sophia Antipolis, France,¹ and Instituto de Bioquímica y Biología Molecular, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina²

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Rhizobia form a symbiotic relationship with plants of the legume family to produce nitrogen-fixing root nodules under nitrogen-limiting conditions. We have examined the importance of glutathione (GSH) during free-living growth and symbiosis of *Sinorhizobium meliloti*. An *S. meliloti* mutant strain (SmgshA) which is unable to synthesize GSH due to a gene disruption in *gshA*, encoding the enzyme for the first step in the biosynthesis of GSH, was unable to grow under nonstress conditions, precluding any nodulation. In contrast, an *S. meliloti* strain (SmgshB) with *gshB*, encoding the enzyme involved in the second step in GSH synthesis, deleted was able to grow, indicating that γ -glutamylcysteine, the dipeptide intermediate, can partially substitute for GSH. However, the SmgshB strain showed a delayed-nodulation phenotype coupled to a 75% reduction in the nitrogen fixation capacity. This phenotype was linked to abnormal nodule development. Both the SmgshA and SmgshB mutant strains exhibited higher catalase activity than the wild-type *S. meliloti* strain, suggesting that both mutant strains are under oxidative stress. Taken together, these results show that GSH plays a critical role in the growth of *S. meliloti* and during its interaction with the plant partner.

All aerobic organisms are exposed to reactive oxygen species (ROS), such as the superoxide anion, hydrogen peroxide (H₂O₂), and the hydroxyl radical, during normal aerobic metabolism or after exposure to stress conditions. ROS can cause irreversible damage to cellular components if they are not rapidly detoxified by antioxidant defense systems (15). To protect themselves against oxidant damage, cells contain effective defense mechanisms, including scavenging enzymes, such as catalases, superoxide dismutases, and glutathione peroxidases, and antioxidants, such as the tripeptide glutathione γ -glutamyl-L-cysteinylglycine (GSH).

GSH is synthesized by a two-step process. In the first step, glutamate and cysteine are conjugated by γ -glutamyl cysteine synthetase (γ ECS) to form γ -glutamyl cysteine (γ EC). In a second step, glycine is added to γ EC to form GSH in a reaction catalyzed by glutathione synthetase (GSHS). The redox-active sulfhydryl group of GSH protects cells from ROS by directly scavenging free radicals and acting as a cofactor for antioxidant enzymes such as glutathione peroxidases. GSH oxidized in this manner forms GSH disulfide, which is recycled back to its reduced form by glutathione reductase.

Initial studies of the requirement for GSH and its role in protection against oxidative stress were performed on bacteria. *E. coli* mutants devoid of GSH were isolated by means of a

transposon insertion in *gshA*, which encodes γ ECS (13). From these studies, it was found that not only was GSH nonessential under normal growth conditions, but also, rather surprisingly, these mutants were unaffected in their resistance to compounds which cause oxidative damage. These results contrast with the situation found in mammalian cells, where GSH deficiency results in cellular damage, suggesting an important difference in GSH function between prokaryotes and eukaryotes. Yeast mutants lacking GSH through transposon insertion into the *gsh1* gene, which encodes γ ECS, showed a severely retarded growth phenotype and enhanced sensitivity to oxidative stress (10, 11).

Sinorhizobium meliloti is an aerobic gram-negative bacterium from the rhizosphere able to infect the roots of *Medicago* species and develop symbiosis. During this process, an exchange of recognition signals occurs between the two partners (20), leading to the development of infection threads and finally to the formation of the root nodule (23). Inside the root nodule, the bacteria differentiate into their symbiotic forms, which are capable of reducing atmospheric nitrogen to ammonium. Soil rhizobia are constantly challenged by a variety of stresses in their natural environments, including elevated temperature, acidity, and osmotic and oxidative shock. Moreover, it has been suggested that during the symbiotic interaction, the bacteria are potentially exposed to oxidative stress during the oxidative burst that follows the infection process (28). In addition, the functional nodule is prone to high levels of ROS due to the high rate of respiration necessary to supply energy required by the nitrogen reduction process. Thus, it appears to be important to understand the roles played by the various antioxidants in both free-living and symbiotic rhizobia.

In this study, we investigated the requirement for GSH in

* Corresponding author. Mailing address: Interactions Plantes-Microorganismes et Santé Végétale, UMR CNRS-INRA-Université de Nice-Sophia Antipolis, 400 Route des Chappes, BP167, 06903 Sophia-Antipolis Cedex, France. Phone: (33) 492 38 66 31. Fax: (33) 492 38 66 40. E-mail: puppo@unice.fr.

† Present address: Faculty of Applied Sciences, Frenchay Campus, University of the West of England, Bristol BS32 1QY, United Kingdom.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Reference
Strains		
<i>S. meliloti</i>		
RCR2011	SU47; wild type; Nod ⁺ Fix ⁺	25
Rm1021	RCR2011 derivative; Sm ^r	21
SmgshA	Same as Rm1021 but with <i>gshA</i> insertion mutation; Sm ^r Nm ^r	This study
SmgshB	Same as Rm1021 but with <i>gshB</i> insertion mutation; Sm ^r Tc ^r	This study
<i>E. coli</i>		
DH5 α	F ⁻ <i>supE44</i> Δ <i>lacU169</i> (Φ 80 <i>dlacZDM15</i>) <i>hsdR17</i> (r _K ⁻ m _K ⁺) <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Bethesda Research Laboratories
MT607	<i>pro-82</i> <i>thi-1</i> <i>hsdR17</i> <i>supE44</i> <i>recA56</i>	5
MT616	MT607(pRK600)	5
Plasmids		
pLAFR1	IncP1 cosmid cloning vector; Tc ^r	7
pRK600	ColE1 replicon with RK2 transfer region; Cm ^r	5
pBluescript KS(+)	Derivative of pUC19 with fl(+) <i>oriR</i> ; Ap ^r	Stratagene
pGEM-T	T vector with fl <i>oriR</i> ; Ap ^r	Promega Corp.
pGEM-T easy	T vector with fl <i>oriR</i> ; Ap ^r	Promega Corp.
pSUP202	ColE1; Mob ⁺ ; Tc ^r Ap ^r Cm ^r	30
pKOK4	Ap ^r Km ^r ; pSUP202 derivative; source of Km ^r	18
pBBR1MCS-5	Derivate of pBBR1-MCS; Gm ^r	19
pGEMgshA	pGEM-T, with 800-bp PCR-amplified fragment of <i>gshA</i>	This study
pSUPgshA	pSUP202 with 800-bp PCR-amplified fragment of <i>gshA</i>	This study
pJHgshA	pSUP202 with 800-bp PCR-amplified fragment of <i>gshA</i> and Km ^r from pKOK4	This study
pGEMcgshA	pGEM-T with 1,500-bp PCR-amplified fragment of <i>gshA</i>	This study
pgshAc	pBBR1MCS-5 with 1,500-bp PCR-amplified fragment of <i>gshA</i>	This study
pGMgshB	pGEM-T with 784-bp PCR-amplified fragment of <i>gshB</i>	This study
pCMgshB	pSUP202 with 800-bp PCR-amplified fragment of <i>gshB</i>	This study

^a Tc, tetracycline; Sm, streptomycin; Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol; Sp, spectinomycin; Gm, gentamycin; Nm, neomycin.

free-living *Sinorhizobium meliloti* and the effects of depletion of GSH on the ability of the bacteria to produce nitrogen-fixing nodules with their symbiotic partner, *Medicago sativa*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37°C, and *S. meliloti* strains were grown in LB medium supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ (LB_{MC}) at 30°C. Antibiotics were added as required at the following concentrations (micrograms per milliliter): ampicillin, 100; chloramphenicol, 10; kanamycin, 100; neomycin, 100; spectinomycin, 100; tetracycline, 10; and gentamicin, 10. For growth curve analysis, high-performance liquid chromatography (HPLC) analysis, and catalase activity, cultures of *S. meliloti* grown on LB_{MC} were inoculated at an initial optical density at 600 nm (OD₆₀₀) of 0.1 onto M9 minimal medium.

General molecular biology techniques. Molecular cloning techniques, restriction analysis, ligation, and transformation were performed essentially according to the standard methods (26). DNA probes were labeled using the Prime-a-gene random-priming system (Promega, Charbonnières, France) and [α -³²P]dCTP (Amersham Biosciences, Orsay, France). Plasmids and DNA fragments were purified using Nucleospin (Macherey Nagel EURL, Hoert, France) and QIAGEN (Courtaboeuf, France) kits.

Construction of the SmgshA and SmgshB strains and of the pgshAc plasmid. *S. meliloti* Rm1021 *gshA* and *gshB* DNA fragments were amplified by PCR using oligonucleotides designed against the predicted *gshA* sequence (5'-GTTCTG ATCCGACCGAAC-3' and 5'-GACGGACGTGAAAGAGG-3') and the *gshB* sequence (5'-ACCATGTTTCGGGCATCACCATT-3' and 5'-ATGCATGTTG GAGCGAGAATCG-3'). The 800- (*gshA*) and 784-bp (*gshB*) fragments amplified were cloned into pGEM-T and pGEM-T Easy (Promega), respectively. The *gshA* DNA fragment was subsequently subcloned into the vector pSUP202 using BamHI-HindIII sites. A kanamycin resistance cassette from pKOK4 was added in the PstI site of the *gshA* DNA fragment. The resulting recombinant plasmid was named pJHgshA. The *gshB* DNA fragment was subcloned into the vector pSUP202 using an EcoRI site. The resulting recombinant plasmid was named pCMgshB. The recombinant suicide plasmids were transferred to the Rm1021 recipient strain by triparental mating using the MT616 *E. coli* strain as a helper,

as described previously (9). Simple recombinant clones were selected on LB_{MC} media containing spectinomycin and neomycin to select the *gshA* mutant strain and spectinomycin and tetracycline to select the *gshB* mutant strain. Recombination at the correct location was checked by Southern hybridization. One *S. meliloti* mutant clone in which the *gshA* gene was interrupted and one *S. meliloti* mutant clone in which the *gshB* gene was interrupted were selected for further analysis. These two strains were named SmgshA and SmgshB, respectively. The *S. meliloti* Rm1021 *gshA* gene containing the promoter region was amplified by PCR using oligonucleotides designed against the predicted *gshA* sequence (5'-AAGGATCCCGCGCGCTCCGTCCG-3' and 5'-AAAAGCTTGGCGATG AGCCGTCCATCC-3'). The 1,500-bp amplified DNA fragment was cloned into pGEM-T and sequenced. The *gshA* gene was subsequently subcloned into the vector pBBR1MCS-5 using BamHI and HindIII sites (underlined in the oligonucleotide sequences). The resulting recombinant plasmid was named pgshAc. The recombinant plasmid was transferred to the SmgshA recipient strain by triparental mating using the MT616 *E. coli* strain as a helper. SmgshA(pgshAc) exconjugants were selected on LB_{MC} medium containing neomycin, spectinomycin, and gentamicin. The strains and plasmids used during the study are shown in Table 1.

HPLC analysis of low-molecular-weight thiols. Bacterial cells (10⁹) were centrifuged at 5,000 \times g for 10 min. The resulting pellet was resuspended in 150 μ l of 0.1 N HCl, derivatized using monochlorobimane, and quantified following separation on reverse-phase HPLC according to the protocol described previously (6). Commercial GSH (Sigma, Saint Quentin Fallavier, France) and γ EC (Promochem, Molsheim, France) were used as standards.

Catalase activity and in-gel assays. Crude bacterial extracts were prepared from free-living bacteria by sonication in a phosphate buffer as described previously (14). Catalase was visualized on nondenaturing polyacrylamide gels by inhibition of diaminobenzidine oxidation (3). Total catalase activity was measured spectrophotometrically by following the decomposition of H₂O₂ at 240 nm (17). The protein concentration was determined with the Bio-Rad (Marnes-la-Coquette, France) protein assay kit using bovine serum albumin as a standard.

Plant nodulation assays. *M. sativa* L. var. Europe (alfalfa) was used as a host plant to test nodulation of the *S. meliloti* strains. Surface-sterilized germinating seedlings were grown in test tubes on modified Fahraeus medium (2) containing 1.5% agarose. Five days after germination, the roots were inoculated with 200 μ l of the appropriate strain of bacterial suspension culture/plant. The bacterial

TABLE 2. Growth-dependent accumulation of γ EC and GSH in *S. meliloti*

Strains	Accumulation ^a							
	12 h		24 h		48 h		72 h	
	γ -EC	GSH	γ -EC	GSH	γ -EC	GSH	γ -EC	GSH
1021	— ^b	6 ± 1	—	7 ± 1	—	7 ± 1	—	3 ± 0.3
SmsgshA	—	—	—	—	—	—	—	—
SmsgshB	17 ± 3	—	19 ± 2	—	19 ± 2	—	14 ± 3	—
SmsgshA (pgshAc)	0.4 ± 0.2	14 ± 2	0.5 ± 0.1	14 ± 3	0.5 ± 0.1	14 ± 3	—	8 ± 2

^a γ EC and GSH were measured by HPLC as described in Materials and Methods. The data represent the means of three experiments ± standard errors.

^b —, not detected.

suspension culture was prepared from overnight LB_{MC} cultures of the bacteria (containing the appropriate antibiotics), washed three times, and diluted to the desired concentration (5×10^7 /ml) with sterile distilled water. The nitrogen fixation capacity of the nodules was determined by the acetylene reduction assay as described previously (14).

Microscopy studies. For microscopy, nodules were harvested 4 weeks after inoculation. The nodules were fixed, postfixed, and embedded as previously described (27). Sections were performed with a diamond knife in a Reichert Ultracut Ultramicrotome (Leica, Rueil-Malmaison, France). After being stained with uranyl acetate and Reynold's lead citrate, ultrathin sections were examined in a transmission electron microscope (CM12; Philips, Cambridge, United Kingdom).

Statistical analysis. All the data presented for thiol concentrations, catalase activity, acetylene reduction assays, and nodule numbers are given as means with standard errors. The significance of the results was assessed using Student's *t* test.

RESULTS

The GSH synthesis pathway is essential for *S. meliloti* growth. In a first step in studying the role of GSH in the growth of *S. meliloti*, in silico analysis of the bacterial genome (8) was performed to find the genes involved in GSH synthesis. The deduced amino acid sequence of the open reading frame SMc00825 showed ~75% identity with putative *Rhizobiales* γ ECS (*Agrobacterium tumefaciens*, *Brucella melitensis* biovar Suis1330, and *Mesorhizobium* sp. strain BNC1) and ~55% identity with plant γ ECS (*Arabidopsis thaliana* and *Medicago truncatula*). Similarly, the translational product of the open reading frame SMc00419 was found to display 85% protein identity with the GSHS from *Rhizobium tropici* (AAL91575). In order to investigate the importance of endogenous GSH production by *S. meliloti* in free-living growth and during interaction with the host plant, alfalfa, the γ ECS- and GSHS-defective mutant strains (SmsgshA and SmsgshB, respectively) derived from wild-type (wt) *S. meliloti* Rm1021 were constructed (see Materials and Methods).

The GSH and γ EC contents of the wild type and the glutathione-deficient mutants were determined during growth on M9 minimal medium (Table 2). γ EC was not detectable in wild-type Rm1021 under our assay conditions. The GSH concentration was found to be higher during the exponential growth phase and early stationary phase, reaching a maximum of 7 nmol/10⁹ cells after 24 h of growth, which was followed by a decrease in GSH content. As expected, both γ EC and GSH were found to be undetectable in the SmsgshA strain. Analysis of the thiol content of the mutant strain SmsgshA expressing the *gshA* gene constitutively, SmsgshA(pgshAc), revealed the accumulation of both γ EC and GSH, which showed growth phase-dependent accumulation similar to that found with the wt Rm1021. The presence of a multicopy plasmid, however,

resulted in concentrations of GSH that were twice those detected in the wt Rm1021. GSH was undetectable in the SmsgshB mutant, which showed, in contrast, a marked accumulation of γ EC (up to 19 nmol/10⁹ cells).

The growth curves of free-living wt *S. meliloti* Rm1021 and mutant strains SmsgshA and SmsgshB on M9 following overnight culture on LB_{MC} are shown in Fig. 1A. In comparison to the wt Rm1021, both mutant strains showed a reduced growth rate, with that of SmsgshA being the most severely affected. The growth of the wt Rm1021 and the mutant strains on M9 following overnight adaptation to M9 is shown in Fig. 1B. All

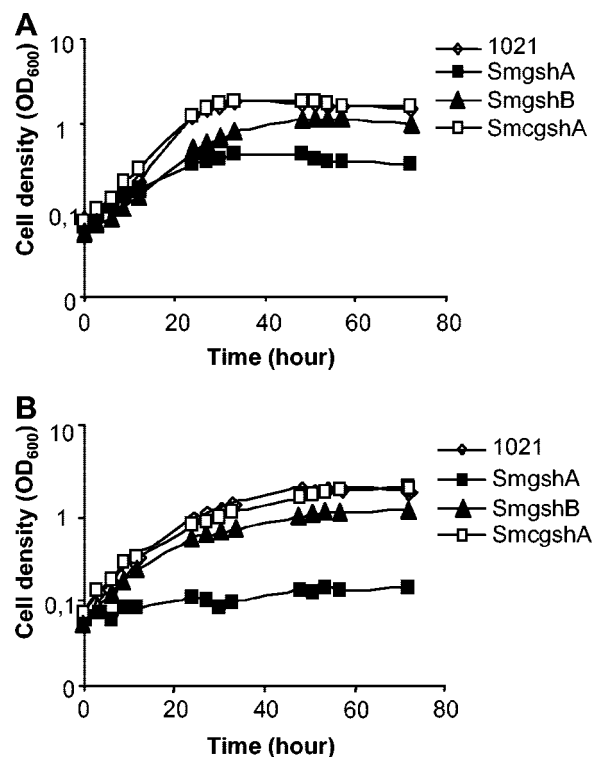


FIG. 1. Growth curves of *Sinorhizobium meliloti* strains. At time zero, rhizobial cells were diluted with minimal medium (M9) to an OD₆₀₀ of 0.1. The growth of wild-type Rm1021, mutant strain SmsgshA, mutant strain SmsgshB, and mutant strain SmsgshA complemented with *pgshAc* was monitored by measuring the OD₆₀₀. (A) Growth curves of free-living *S. meliloti* strains on M9 medium following overnight culture on LB_{MC}. (B) Growth curves of free-living *S. meliloti* strains on M9 medium following overnight adaptation to M9. The data are the means from three experiments.

though the exponential growth phases of all cultures were slightly slower than those shown in Fig. 1A, the most striking result was the complete growth arrest of the SmgshA mutant. Under both culture conditions, growth of the SmgshA strain was fully restored by complementation with a plasmid containing the *S. meliloti* γ ECS gene (pgshAc) or by the addition of 1 mM GSH or γ EC to the growth medium (data not shown). In parallel, growth of the SmgshB strain was restored by the addition of 100 μ M GSH to the growth medium (data not shown). Taken together, these results show that the GSH synthesis pathway is essential for the free-living growth of *S. meliloti*. However, γ EC, which is present at a high concentration in the SmgshB strain, is able to complement the lack of GSH.

Since it has been proposed that the oxidized/reduced ratio of GSH in the cells is essential for proper scavenging of ROS, we examined whether low levels of thiol resulted in a change in the GSH redox state. Due to the severely retarded growth phenotype following overnight adaptation to M9, all experiments were performed on cultures grown overnight on LB_{MC} and subsequently subcultured into M9. Both reduced and oxidized GSH and γ EC pools were determined during the exponential growth phase. The percentage of the oxidized γ EC pool was found to be 34% \pm 4% in the SmgshB mutant strain, whereas the oxidized GSH and γ EC pools represented just 10% \pm 6% in the wt Rm1021 and the complemented SmgshA(pgshAc) strains. This suggests that the SmgshB strain is under oxidative stress.

Catalase activity is modulated by GSH deficiency in *S. meliloti*. It has been demonstrated that catalases are induced by oxidative stress in *S. meliloti* (14, 29), and therefore, we concluded that catalase activity represents a good physiological marker to assess whether the bacteria are under oxidative stress. It had been reported that three distinct hydroperoxidases of *S. meliloti* are involved in the dismutation of H₂O₂, two monofunctional catalases (KatA and KatC) and one bifunctional catalase-peroxidase (KatB). Each of these catalases has a distinctive expression profile during growth of the bacteria under free-living conditions and in response to particular stresses (14, 16, 29). *katA* is expressed during the exponential growth phase and is induced by H₂O₂. *katB* encodes a house-keeping catalase-peroxidase that is expressed constitutively. *katC* is expressed during the stationary phase and is induced by environmental stresses.

In order to examine whether catalase activity was higher in mutant strains than in the wt Rm1021 strain, the level of total catalase activity was determined in cells during the late exponential growth phase (Fig. 2A). The mutant SmgshA was found to have a sevenfold-higher total catalase activity than the wt Rm1021. The total catalase activity of the SmgshB mutant also exhibited a three- to fourfold increase compared to that of the wt Rm1021. These results showed that catalase activity is enhanced in GSH-deficient mutant strains. In order to extend this analysis, we evaluated the effect of GSH deficiency on the catalase activity profile by using catalase activity gel (Fig. 2B). As previously reported (29), only KatA and KatB are expressed in *S. meliloti* during the late exponential phase. An increase in KatA and KatB, and in addition the detection of KatC, could be observed in the SmgshA strain compared to wt Rm1021. The SmgshB strain showed an intermediate phenotype in which the activities of the three catalases were found to

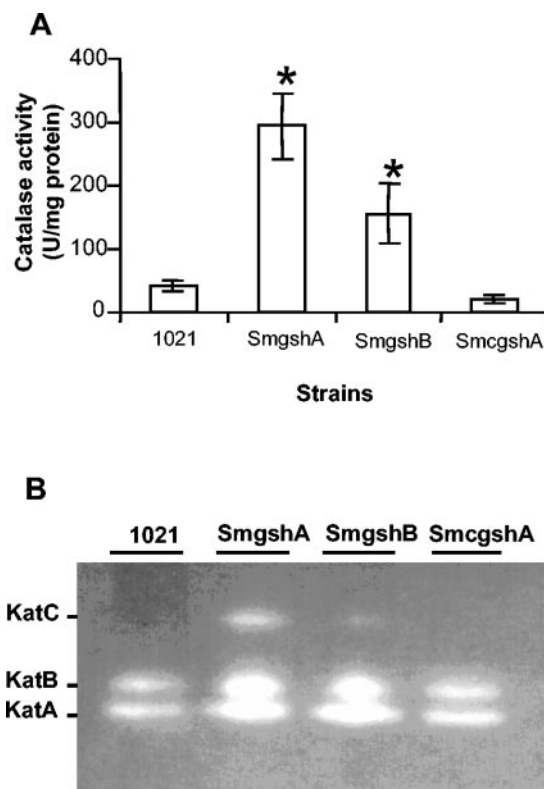


FIG. 2. Catalase activities of *S. meliloti* strains. At time zero, rhizobial cells were diluted with M9 medium to an OD₆₀₀ of 0.1. Cell extracts were prepared from the Rm1021 (1021), SmgshA, and SmgshB strains and the SmgshA strain complemented with pgshAc (SmcgsA) after 24 h. (A) Total catalase activities for the different strains. The data are the means (\pm standard errors) of triplicates from three experiments.* indicates significant difference ($P < 0.05$). (B) Catalase activity patterns. Proteins (20 μ g) were submitted to electrophoresis on a native polyacrylamide gel (7%) that had been stained for catalase activity. The positions of KatA, KatB, and KatC are indicated according to Sigaud et al. (29).

be high, albeit at lower levels than in the SmgshA strain. Complementation of the SmgshA strain by the plasmid pgshAc resulted in the recovery of a wild-type phenotype in terms of the total catalase activity and catalase profile. The higher level of oxidation of the γ EC pool in the SmgshB strain, which correlated with the higher level of catalase activity observed in strains SmgshA and SmgshB, demonstrates that GSH deficiency causes oxidative stress in *S. meliloti*.

Glutathione deficiency strongly affects the nodulation process. In order to test the implication of GSH in the symbiotic process, the capacities of the SmgshA and SmgshB mutant strains to nodulate and produce nitrogen-fixing nodules were examined by alfalfa inoculation experiments. No nodules were produced by plants inoculated with the SmgshA mutant strain. The nodulation efficiencies following inoculation with Rm1021 and SmgshB are shown in Fig. 3. Seven days postinoculation, significantly fewer nodules were observed on plants inoculated with *S. meliloti* strain SmgshB than on those inoculated with strain Rm1021. However, this difference was no longer significant 11 and 14 days postinoculation, suggesting that although

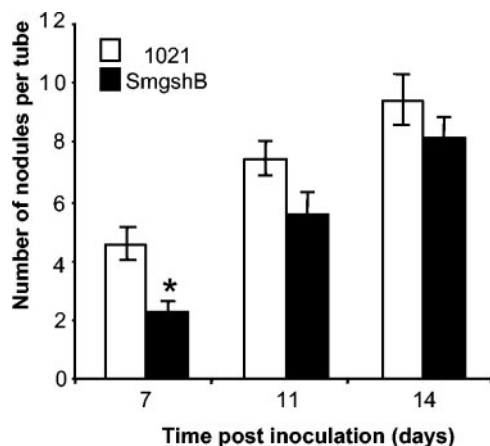


FIG. 3. Plant nodulation efficiency. Plants were inoculated with the *S. meliloti* Rm1021 (1021) and SmgshB strains. The total numbers of nodules per tube were assessed 7, 11, and 14 days postinoculation. Each tube contained three plants. The data are the means (\pm standard errors) of 48 tubes from two experiments; * indicates a significant difference ($P < 0.05$).

the nodulation capacity itself was unaffected, the timing of nodule formation was delayed in the SmgshB mutant.

To test the functionality of nodules, their nitrogen-fixing capacities were also determined 4 weeks after inoculation with the Rm1021 and SmgshB strains. Using the acetylene reduction assay, it was found that the nitrogen fixation capacity was severely affected in the SmgshB mutant, with a reduction from 11.0 ± 2 nmol of C_2H_4 /mg of nodules/h in wt Rm1021 to 2.7 ± 0.7 nmol of C_2H_4 /mg of nodules/h in the mutant.

Microscopic analysis of the nodules was performed to visualize the nodule structure (Fig. 4). All the steps in cell differentiation were observed in nodules after infection with Rm1021 (31). The different zones observed in a wt Rm1021 nodule are presented in Fig. 4A. The meristematic zone I was bacterium free. Bacteria released from infection threads in plant cells were surrounded by a peribacteroid membrane in the distal infection zone II (type 1 bacteroids). Bacteroids elongated without dividing in the proximal infection zone II (type 2 bacteroids). In interzone II-III (IZ), bacteroids stopped their elongation and displayed cytoplasmic heterogeneity (type 3 bacteroids). In zone III, the bacteroids showed higher cytoplasmic heterogeneity and corresponded to nitrogen-fixing rhizobia (type 4 bacteroids) (Fig. 4C and E). Finally, symbionts degenerated in the senescent zone IV. In contrast, SmgshB nodules exhibited a large senescent zone IV (Fig. 4B). The fixing zone III was not clearly defined, and amyloplast-rich cells, which are characteristic of the IZ, appeared to be adjacent to senescent zone IV (Fig. 4D). Ultrastructural studies (Fig. 4F) showed that SmgshB bacteria were released but rapidly underwent senescence, often before differentiating into fixing bacteroids. Both nonfixing bacteroids (types 1 to 3) and senescent bacteroids could be observed in the same plant cell (Fig. 4F). Taken together, these results demonstrated that *S. meliloti* requires GSH in order to achieve an efficient symbiotic interaction with alfalfa, particularly in preventing early senescence.

DISCUSSION

During this study, the synthesis of glutathione and the importance of this low-molecular-weight thiol were examined in free-living and symbiotic *S. meliloti*. The functions of *gshA* and *gshB* were confirmed by HPLC analysis of the low-molecular-weight thiol contents of the strains with mutations in the *gshA* and *gshB* genes, respectively. A mutant strain defective in synthesis of γ ECS (strain SmgshA) does not accumulate γ EC and GSH, and a strain lacking glutathione synthetase (SmgshB strain) accumulates γ EC. Therefore, there are no other genes coding for functions able to replace those sequences altered in our mutants. In order to test the importance of the GSH synthesis pathway in *S. meliloti*, the growth, antioxidant status, and nodulation capacities of the mutant strains SmgshA and SmgshB were compared to those of the wild-type Rm1021. Our study demonstrates that the growth of an *S. meliloti* strain with the *gshA* gene mutated was severely impaired, whereas only a slightly reduced growth phenotype was observed in the SmgshB strain. These results indicate that γ EC is able to compensate for the absence of glutathione during growth under free-living conditions, and they are remarkably different from the results observed in *E. coli*. Mutation of *gshA* and the subsequent deficiency in glutathione or γ EC did not modify the growth rate of *E. coli*, indicating a nonessential role for the GSH synthesis pathway under nonstress conditions (13). In contrast, it was demonstrated that a yeast mutant with γ ECS deleted was unable to grow, while mutation in GSHS resulted in an impaired growth phenotype (10, 11). Thus, our study demonstrated that *S. meliloti* behaves in a manner similar to yeast, while it diverges from the behavior of *E. coli*.

Hydroperoxidases are implicated in the detoxification of H_2O_2 . Our studies showed that a lack of glutathione is associated with an increase in total catalase activity. Gel assays revealed that this increase is due to augmentation in KatA and KatB and the appearance of KatC. The fact that a decrease in GSH content induces both KatA and KatC activities suggests that the mutant strains SmgshA and SmgshB are facing stress conditions. Moreover, the highly oxidized γ EC pool present in the SmgshB strain suggests that the threefold-higher γ EC concentration does not suffice for the maintenance of the redox state found in the Rm1021 strain. Previous studies have shown that although mutation in the *gshA* gene of *E. coli* did not result in a growth phenotype, the bacteria were found to be more sensitive to H_2O_2 and had a higher level of catalase activity (22). The authors suggested that glutathione may play a role in the regulation of catalases, possibly through its regulation of intracellular H_2O_2 or through its interaction with redox-sensitive sites in the OxyR regulatory protein. GSH-deficient yeast mutants, as well as *R. tropici* *gshB* mutants, showed dramatic sensitivity to H_2O_2 (12, 24). Altogether, these data suggest that GSH and catalases provide overlapping defenses for protection against H_2O_2 in both prokaryotic and eukaryotic cells.

The nodulation efficiencies of *S. meliloti* glutathione pathway-deficient mutants were investigated by inoculating *M. sativa* plants with the SmgshA and SmgshB strains. Under our experimental procedures, we found no evidence that the mutant SmgshA was capable of nodulating *M. sativa*. Since the growth of the mutant rhizobium was severely affected, it

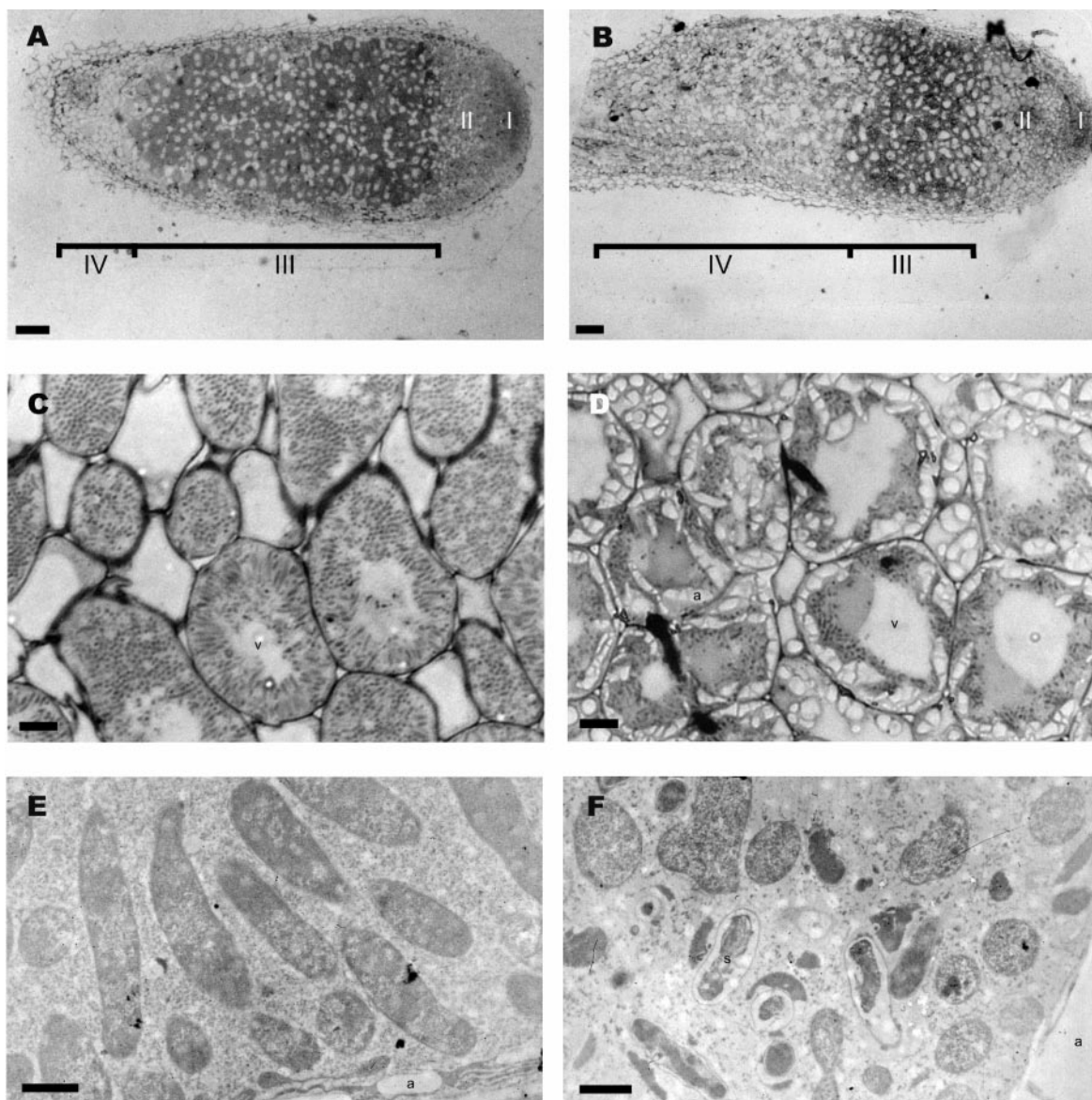


FIG. 4. Structure and ultrastructure of 4-week-old nodules. Nodules were incited by Rm1021 (A, C, and E) and SmgshB (B, D, and F) strains. The meristematic (I), infection (II), fixing (III), and senescent (IV) zones and II-III IZ are represented in A and B. a, amyloplast, and s, senescing bacteroid. Scale bars = 100 (A and B), 10 (C and D) and 1 μ m (E and F).

seemed logical to expect that its nodulation ability would also be negatively affected. However, the stage of early interaction between the mutant strain and alfalfa, such as attachment, curling, and infection thread formation, that might be affected remains to be determined. The emergence of nodules on *M. sativa* following inoculation with the mutant strain SmgshB was delayed in comparison to wt Rm1021, although the final nodule number was unaffected. Similarly, the number of nodules on *Phaseolus vulgaris* and *Leucaena leucocephala* 4 weeks postinoculation with the *gshB*-deficient strain was found to be similar to that on plants inoculated with the wild-type strain (24). A highly significant feature of our study was the large reduction in the nitrogen fixation capacity of nodules. This phenotype is consistent with the microscopy studies, in which early senescence was observed in the mutant strain SmgshB. The

studies do suggest that redox balance is an important factor in nodulation. Superoxide anions and H_2O_2 are produced during the initial stages of the plant-microbial interaction (28). To protect itself from this oxidative burst, *S. meliloti* possesses three catalases and two superoxide dismutases (SodA and SodC). Transcriptome analysis of *S. meliloti* has shown that *sodC* is induced during the infection process (1), and Sod activity appears to play an important role during the early steps of symbiosis (27). In addition, catalases present a differential pattern of expression during nodule development. *katB* is constitutively expressed in the nodules, *katA* is expressed in zone III, and *katC* is expressed in zones II and IV (16). While single null mutants of *katA*, *katB*, and *katC* have a wild-type nodulation phenotype, *katB katC* and *katA katC* double mutants have reduced infection efficiencies and decreased nitrogen fixation

capacities, showing that degradation of H₂O₂ in planta is essential to allow an efficient nodulation process (14, 16, 29). The reduction of nitrogen fixation capacity observed in the *gshB* mutant strain could be associated with an alteration of the bacterial antioxidant defense, which promotes early senescence. It could also be suggested that the γ EC produced in the *gshB* strain may not be able to fully substitute for the specific GSH function during cell differentiation occurring in the symbiotic process. This deficiency may cause early senescence of nodules, which is reflected by lower nitrogenase activity.

The requirement for rhizobia in the GSH synthesis pathway during growth in free-living conditions seems to be analogous to that in yeast and higher eukaryotes and unlike the situation in *E. coli*. This dissimilarity in physiology may be paralleled by phylogenetic differences between *S. meliloti* and *E. coli* γ ECS. Indeed, the sequences of *gshA* from γ -proteobacteria (*E. coli*) and α -proteobacteria (*S. meliloti*) fall into two distinct groups (4). The latter group consists primarily of α -proteobacteria, such as *Mesorhizobium loti*, *Agrobacterium tumefaciens*, and *S. meliloti*, and flowering plants, such as *A. thaliana*, *M. truncatula*, and *Glycine max*. The different influences of GSH deficiency on cell growth observed in *S. meliloti* and *E. coli* may arise from divergent evolutionary processes of these two bacterial genera, which is demonstrated by γ ECS gene evolution.

In *S. meliloti*, it appears that GSH may be replaced by γ EC under free-living conditions but not during the symbiotic association with *M. sativa*. This differential behavior may also be linked to the evolution of the GSH synthesis pathway. Copely and Dhillon (4) suggested that *gshB* was recruited later during evolution than *gshA*. In this scenario, bacterial γ EC may have served multiple functions, including those carried on by GSH before the recruitment of the *gshB* gene. However, our study also shows that glutathione itself is important for optimal nitrogen fixation, showing that endogenous γ EC could not fully restore some essential functions of GSH which may have been acquired after the recruitment of the *gshB* gene.

Taken together, our results indicate that the GSH synthesis pathway plays a crucial role in the growth and symbiotic capacity of *S. meliloti*. The transcriptome analysis of the SmgshA and SmgshB strains should bring significant insights into the different roles of γ EC and GSH.

REFERENCES

- Ampe, F., E. Kiss, F. Sabourdy, and J. Batut. 2003. Transcriptome analysis of *Sinorhizobium meliloti* during symbiosis. *Genome Biol.* **4**:R15.
- Boisson-Dernier, A., M. Chabaud, F. Garcia, G. Bécard, C. Rosenberg, and D. G. Barker. 2001. *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. *Mol. Plant-Microbe Interact.* **14**:695–700.
- Clare, D. A., M. N. Duong, D. Darr, F. Archibald, and I. Fridovich. 1984. Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. *Anal. Biochem.* **140**:532–537.
- Copely, S. D., and J. K. Dhillon. 2002. Lateral gene transfer and parallel evolution in the history of the glutathione biosynthesis genes. *Genome Biol.* **3**:1–16.
- Finan, T. M., B. Kunkel, G. F. De Vos, and E. R. Signer. 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *J. Bacteriol.* **167**:66–72.
- Frendo, P., D. Gallesi, T. Turnbull, G. Van de Sype, D. Hérouart, and A. Puppo. 1999. Localization of glutathione and homogluthathione in *Medicago truncatula* is correlated to a differential expression of genes involved in their synthesis. *Plant J.* **17**:215–219.
- Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel. 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. *Gene* **18**:289–296.
- Galibert, F., T. M. Finan, S. R. Long, A. Puhler, P. Abola, F. Ampe, F. Barloy-Hubler, M. J. Barnett, A. Becker, P. Boistard, G. Bothe, M. Boutry, L. Bowser, J. Buhrmester, E. Cadieu, D. Capela, P. Chain, A. Cowie, R. W. Davis, S. Dreano, N. A. Federspiel, R. F. Fisher, S. Gloux, T. Godrie, A. Goffeau, B. Golding, J. Gouzy, M. Gurjal, I. Hernandez-Lucas, A. Hong, L. Huizar, R. W. Hyman, T. Jones, D. Kahn, M. L. Kahn, S. Kalman, D. H. Keating, E. Kiss, C. Komp, V. Lelaure, D. Masuy, C. Palm, M. C. Peck, T. M. Pohl, D. Portetelle, B. Purnelle, U. Ramsperger, R. Surzycki, P. Thebault, M. Vandenbol, F. J. Vorholter, S. Weidner, D. H. Wells, K. Wong, K. C. Yeh, and J. Batut. 2001. The composite genome of the legume symbiont *Sinorhizobium meliloti*. *Science* **293**:668–672.
- Glazebrook, J., and G. C. Walker. 1991. Genetic techniques in *Rhizobium meliloti*. *Methods Enzymol.* **204**:398–418.
- Grant, C. M., F. H. MacIver, and I. W. Dawes. 1996. Glutathione is an essential metabolite required for resistance to oxidative stress in the yeast *Saccharomyces cerevisiae*. *Curr. Genet.* **29**:511–515.
- Grant, C. M., F. H. MacIver, and I. W. Dawes. 1997. Glutathione synthetase is dispensable for growth under both normal and oxidative stress conditions in the yeast *Saccharomyces cerevisiae* due to the accumulation of the dipeptide gamma-glutamylcysteine. *Mol. Biol. Cell* **8**:1699–1707.
- Grant, C. M., G. Perrone, and I. W. Dawes. 1998. Glutathione and catalase provide overlapping defenses for protection against hydrogen peroxide in the yeast *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **253**:893–898.
- Greenberg, J. T., and B. Demple. 1986. Glutathione in *Escherichia coli* is dispensable for resistance to H₂O₂ and gamma radiation. *J. Bacteriol.* **168**:1026–1029.
- Hérouart, D., S. Sigaud, S. Moreau, P. Frendo, D. Touati, and A. Puppo. 1996. Cloning and characterization of the *kata* gene of *Rhizobium meliloti* encoding a hydrogen peroxide-inducible catalase. *J. Bacteriol.* **178**:6802–6809.
- Imlay, J. A. 2003. Pathway of oxidative damage. *Annu. Rev. Microbiol.* **57**:395–418.
- Jamet, A., S. Sigaud, G. Van de Sype, A. Puppo, and D. Hérouart. 2003. Expression of the bacterial catalase genes during *Sinorhizobium meliloti*-*Medicago sativa* symbiosis and their crucial role during the infection process. *Mol. Plant-Microbe Interact.* **16**:217–225.
- Jones, D. P. 1982. Intercellular catalase function: analysis of the catalytic activity by product formation in isolated liver cells. *Arch. Biochem. Biophys.* **214**:806–814.
- Kokotek, W., and W. Lotz. 1989. Construction of a *lacZ*-kanamycin-resistance cassette, useful for site-mutagenesis and as promoter probe. *Gene* **84**:467–471.
- Kovach, M. E., R. W. Phillips, P. H. Elzer, R. M. Roop II, and K. M. Peterson. 1994. pBBRMCS: a broad-host-range cloning vector. *BioTechniques* **16**:800–802.
- Long, S. R. 1996. Rhizobium symbiosis: nod factors in perspective. *Plant Cell* **8**:1885–1898.
- Niel, C., J. B. Guillaume, and M. Bechet. 1977. Mise en évidence de deux enzymes présentant une activité β -galactosidase chez *Rhizobium meliloti*. *Can. J. Microbiol.* **23**:1178–1181.
- Oktyabrsky, O. N., G. V. Smirnova, and N. G. Muzyka. 2001. Role of glutathione in regulation of hydroperoxidase 1 in growing *Escherichia coli*. *Free Radic. Biol. Med.* **31**:250–255.
- Perret, X., C. Staehelin, and W. J. Broughton. 2000. Molecular basis of symbiotic promiscuity. *Microbiol. Mol. Biol. Rev.* **64**:180–201.
- Riccillo, P. M., C. I. Muglia, F. J. de Bruijn, A. J. Roe, I. R. Booth, and O. M. Aguilar. 2000. Glutathione is involved in environmental stress responses in *Rhizobium tropici*, including acid tolerance. *J. Bacteriol.* **182**:1748–1753.
- Rosenberg, C., P. Boistard, J. Denarie, and F. Casse-Delbart. 1981. Genes controlling early and late functions in symbiosis are located on a megaplasmid in *Rhizobium meliloti*. *Mol. Gen. Genet.* **184**:326–333.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press Cold Spring Harbor, N.Y.
- Santos, R., D. Hérouart, A. Puppo, and D. Touati. 2000. Critical protective role of bacterial superoxide dismutase in *Rhizobium*-legume symbiosis. *Mol. Microbiol.* **38**:750–759.
- Santos, R., D. Hérouart, S. Sigaud, D. Touati, and A. Puppo. 2001. Oxidative burst in alfalfa-*Sinorhizobium meliloti* symbiotic interaction. *Mol. Plant-Microbe Interact.* **14**:86–89.
- Sigaud, S., V. Becquet, P. Frendo, A. Puppo, and D. Hérouart. 1999. Differential regulation of two divergent *Sinorhizobium meliloti* genes for HPII-like catalases during free-living growth and protective role of both catalases during symbiosis. *J. Bacteriol.* **181**:2634–2639.
- Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. *Bio/Technology* **1**:784–791.
- Vasse, J., F. de Billy, S. Camut, and G. Truchet. 1990. Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. *J. Bacteriol.* **172**:4295–4306.