Sinorhizobium meliloti Glutathione Reductase Is Required for both Redox Homeostasis and Symbiosis

Guirong Tang,[a,b] Ningning Li,[a] Yumin Liu,[a] Liangliang Yu,[a] Junhui Yan,[a] Li Luo[a]

[a]Shanghai Key Laboratory of Bio-energy Crops, School of Life Sciences, Plant Science Center, Shanghai University, Shanghai, China
[b]School of Communication and Information Engineering, Shanghai University, Shanghai, China

ABSTRACT Glutathione (L-γ-glutamyl-L-cysteinylglycine) (GSH), one of the key antioxidants in *Sinorhizobium meliloti*, is required for the development of alfalfa (*Medicago sativa*) nitrogen-fixing nodules. Glutathione exists as either reduced glutathione (GSH) or oxidized glutathione (GSSG), and its content is regulated by two pathways in *S. meliloti*. The first pathway is the *de novo* synthesis of glutathione from its constituent amino acids, namely, Glu, Cys, and Gly, catalyzed by γ-glutamylcysteine synthetase (GshA) and glutathione synthetase (GshB). The second pathway is the recycling of GSSG via glutathione reductase (GR). However, whether the *S. meliloti* GR functions similarly to GshA and GshB1 during symbiotic interactions with alfalfa remains unknown. In this study, a plasmid insertion mutation of the *S. meliloti gor* gene, which encodes GR, was constructed, and the mutant exhibited delayed alfalfa nodulation, with 75% reduction in nitrogen-fixing capacity. The *gor* mutant demonstrated increased accumulation of GSSG and a decreased GSH/GSSG ratio in cells. The mutant also showed defective growth in rich broth and minimal broth and was more sensitive to the oxidants H_2O_2 and sodium nitroprusside. Interestingly, the expression of *gshA*, *gshB1*, *katA*, and *katB* was induced in the mutant. These findings reveal that the recycling of glutathione is important for *S. meliloti* to maintain redox homeostasis and to interact symbiotically with alfalfa.

IMPORTANCE The antioxidant glutathione is regulated by its synthetase and reductase in cells. In the symbiotic bacterium *S. meliloti*, the *de novo* synthesis of glutathione is essential for alfalfa nodulation and nitrogen fixation. In this study, we observed that the recycling of glutathione from GSSG not only was required for redox homeostasis and oxidative stress protection in *S. meliloti* cells but also contributed to alfalfa nodule development and competition capacity. Our findings demonstrate that the recycling of glutathione plays a key role in nitrogen fixation symbiosis.

KEYWORDS *Sinorhizobium meliloti*, glutathione reductase, redox homeostasis, symbiosis, reactive oxygen species

Most organisms contain high concentrations of low-molecular-weight (LMW) thiols, which serve as redox buffers to protect cells against a variety of reactive chemical species, such as reactive oxygen species (ROS) (1, 2). ROS include hydrogen peroxide (H_2O_2), superoxide anions, and hydroxyl radicals. Glutathione is considered the most ubiquitous and potent LMW thiol antioxidant. Glutathione is present at millimolar concentrations in nearly all eukaryotic cells and many bacteria (including most Gram-negative bacteria) (3, 4). In cells, the content of glutathione is regulated by two pathways. The first pathway is the *de novo* synthesis pathway, in which two ATP-dependent enzymes, γ-glutamylcysteine synthetase (GshA) and glutathione synthetase (GshB), which are encoded by the *gshA* (GSH1) and *gshB* (GSH2) genes, respectively, sequentially catalyze the synthesis of glutathione from glutamate, cysteine, and glycine
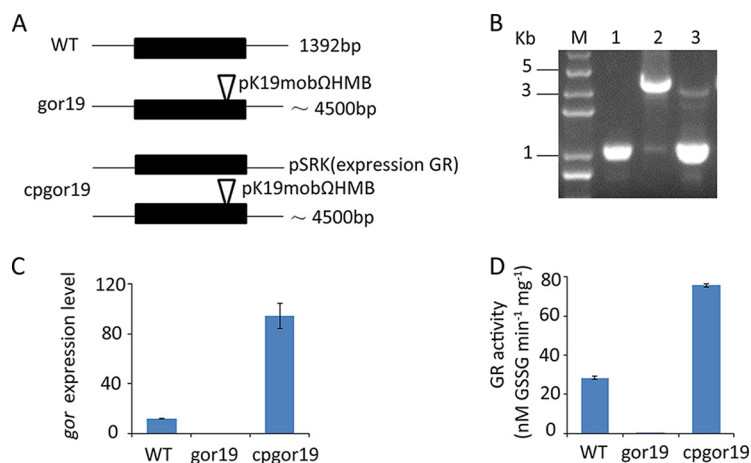
FIG 1 Strategies for plasmid insertion and characterization of the gor19 mutant. (A) Schematic representation of the location of the genomic locus before and after integration of the plasmid into the genome in the WT, gor19, and cpgor19 strains. WT, *S. meliloti* 1021; gor19, mutant with a plasmid insertion in *gor*; cpgor19, strain containing pSRK19 in the gor19 mutant. (B) Agarose gel with a DNA size marker and DNA fragments amplified with different genomic DNA. Lane M, DNA marker 2K Plus; lane 1, amplified *gor* PCR fragment from the *S. meliloti* 1021 (WT) strain; lane 2, amplified *gor* PCR fragment from the gor19 mutant; lane 3, amplified *gor* PCR fragment from the cpgor19 strain. (C) Real-time PCR analysis of *gor* expression levels in the WT, gor19, and cpgor19 strains. (D) GR activity of total protein in the WT, gor19, and cpgor19 strains. The data shown are the means of three experiments, with the error bars indicating the standard errors.

(5). Free glutathione exists in two different forms in cells, i.e., reduced glutathione (GSH), with a free thiol group, and oxidized glutathione (GSSG), with a disulfide bond between two identical molecules. Two GSH molecules are oxidized to form a GSSG molecule via a disulfide bond under oxidative stress, while glutathione reductase (GR) catalyzes the reduction of GSSG to GSH. This reduction represents the recycling synthesis pathway. In the yeast *Saccharomyces cerevisiae*, GSH is required as a reductant and may function to remove endogenously derived toxic metabolites. Yeast mutants lacking GSH showed a severely retarded growth phenotype and enhanced sensitivity to oxidative stress (6–8). Interestingly, GSH is not essential in *Escherichia coli* under normal growth conditions; moreover, transposon insertion mutants in which *gshA* was disrupted were unaffected in their resistance to compounds that cause oxidative stress (9).

Rhizobia, which are Gram-negative soil bacteria, can reduce N_2 (nitrogen fixation) in specific legume root organs called nodules, via stringent symbiotic interactions. Notably, the establishment of *Rhizobium*-legume symbiosis depends on the capacity for rhizobial GSH synthesis. A GSH-deficient mutant of *Rhizobium tropici* induced *Phaseolus vulgaris* to form root nodules with early senescence (10). For *Sinorhizobium meliloti*, GSH was reported to be essential for free-living growth and symbiosis with alfalfa (11). Mutation of *gshA* led to a severe growth defect under normal conditions, and the mutant did not induce nodule formation in alfalfa plants. A *gshB* mutant induced the formation of alfalfa nodules with low nitrogen fixation capacity. However, whether the GR-catalyzed recycling synthesis of GSH from GSSG is required for redox homeostasis and symbiosis has not been determined to date. In this study, a *gor* null mutant was constructed in a *S. meliloti* 1021 background, and its free-living and symbiotic phenotypes were investigated. Our results indicate that the recycling of glutathione is important for *S. meliloti* to grow, to adapt to oxidative stress, and to interact symbiotically with host plants.

RESULTS

Construction of an *S. meliloti* *gor* mutant. To investigate the roles of GR in free-living and symbiotic *Rhizobium* cells, a *gor* null mutant (gor19) was constructed in *S. meliloti* 1021 by using a plasmid insertion method (Fig. 1A). The mutant was identified by PCR, and the results showed that an ~4.5-kb DNA fragment containing the suicide
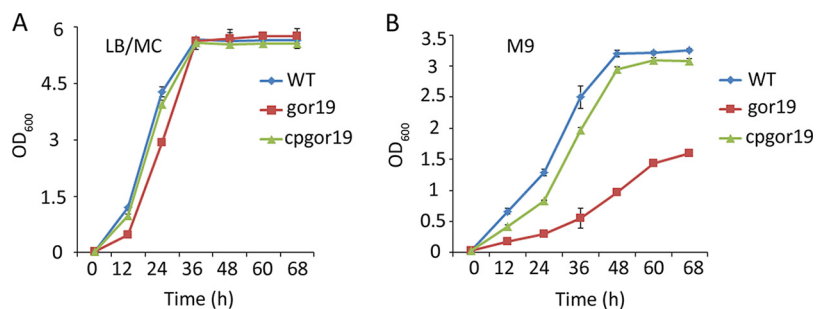
FIG 2 Growth curves of the *S. meliloti* strains. At time zero, rhizobial cells were diluted with LB/MC medium or M9 medium to an OD_{600} of 0.01. The growth of the WT, gor19, and cpgor19 strains in LB/MC and M9 media was monitored by measuring the optical density at 600 nm. (A) Growth curves of free-living *S. meliloti* strains grown in LB/MC medium. (B) Growth curves of free-living *S. meliloti* strains grown in M9 medium. The data points represent the averages of triplicate samples, with the error bars indicating the standard errors.

plasmid pK19mob2ΩHMB (3.5 kb) was amplified from the gor19 mutant, whereas an ~1-kb DNA fragment was amplified from the parent *S. meliloti* 1021 strain (Fig. 1B). DNA sequencing indicated that the suicide plasmid pK19mob2ΩHMB was inserted after nucleotide 994 of the *gor* open reading frame (ORF) (1,392 bp). To determine whether the plasmid insertion in gor19 disrupted the entire transcript, total RNA was extracted and reverse transcription (RT)-PCR was performed. The results showed the absence of an entire *gor* transcript in the insertion mutant, whereas such a fragment was detected in the wild-type (WT) strain and in the complementation strain (cpgor19) (Fig. 1C). Finally, cell extracts were made from the wild-type strain and the gor19 strain and were assayed directly for GR activity. The extracts of the gor19 strain showed activity of <0.11 ± 0.01 nmol/s/mg protein, but the wild-type strain and complementation strain (cpgor19) extracts reduced glutathione at rates of 28.4 ± 0.88 nmol/s/mg protein and 75.8 ± 0.79 nmol/s/mg protein, respectively (Fig. 1D). These results show that the gor19 strain lacks GR activity.

Growth defect of the *S. meliloti gor* mutant. The *gor* mutant was first evaluated for growth defects. In rich broth (Luria-Bertani [LB] medium supplemented with 2.5 mM MgSO$_4$ and 2.5 mM CaCl$_2$ [LB/MC medium]), the mutant grew slightly more slowly (the logarithmic phase was extended) than the wild-type strain or the complemented strain (Fig. 2A). Striking growth arrest was observed in minimal broth (M9 medium) for gor19, as its growth rate was 50% less than that of the wild-type strain; growth was restored by constitutively expressing *gor* from a plasmid in the complemented strain (Fig. 2B). These results indicate that *gor* is required for the free-living growth of *S. meliloti*.

Requirement for the *S. meliloti gor* gene for redox homeostasis. To evaluate the GSH and GSSG contents in the gor19 mutant, a liquid chromatography-mass spectrometry (LC-MS) method was used (see Materials and Methods). The data showed that the content of GSH was increased ~3-fold in the mutant, compared to the parent strain, while the level of GSSG was ~10-fold higher in gor19 than in *S. meliloti* 1021 (Fig. 3A and B). Thus, the GSH/GSSG ratio was 9:1 in gor19 and 49:1 in *S. meliloti* strain 1021, a decrease of ~5-fold in the mutant compared with the parent strain (Fig. 3C). This defect could be rescued by constitutively expressing *gor* from a plasmid in the mutant (Fig. 3). These results indicate that the *gor* gene is associated with the production of GSH and GSSG and is required for redox homeostasis in *S. meliloti* cells.

Activation of *gshA*, *gshB1*, *katA*, and *katB* expression in the *gor19* mutant. The level of GSH was strikingly increased in the gor19 mutant, which prompted us to analyze the expression of *gshA* and *gshB1* using quantitative RT-PCR (qRT-PCR). The data showed that the expression of both *gshA* and *gshB1* was increased >3-fold in the mutant compared with the parent strain but that the wild-type levels could be restored by expressing *gor* from a plasmid in the complementation strain (Fig. 4). The expression of the catalase gene was also investigated, to further evaluate the redox status of the
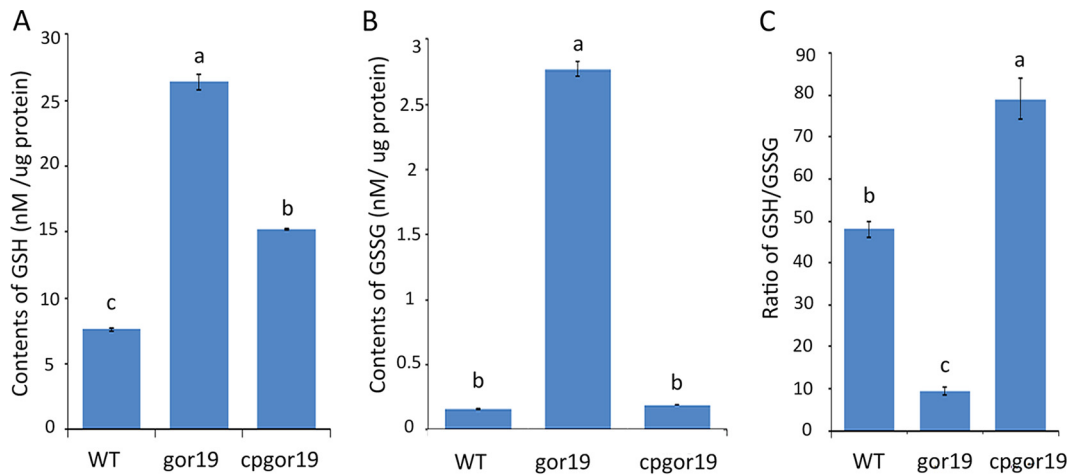
FIG 3 Accumulation of glutathione in *S. meliloti*. (A and B) High-performance liquid chromatography (HPLC) analysis of the GSH (A) and GSSG (B) contents in the WT, gor19, and cpgor19 strains. (C) GSH/GSSG ratios in the WT, gor19, and cpgor19 strains. The data represent the means ± standard errors for triplicate samples. Significant differences were identified by one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test, and are noted as different letters ($P < 0.05$).

cells. qRT-PCR data showed that the expression levels of *katA* and *katB* were elevated 2-fold in gor19, compared with *S. meliloti* 1021, whereas the expression of *katC* was almost not detected in any of the strains. Expression of *gor* from a plasmid restored catalase expression in the mutant. These results indicate that the lack of GR causes oxidative stress in *S. meliloti*.

Requirement for the *S. meliloti gor* gene for adaptation to oxidative stress. A *Rhizobium etli* strain with a Tn*5* insertion mutation in GR was reported to be sensitive to oxidative stress (12). To examine the possibility that *gor* is required for the adaptation of *S. meliloti* to oxidative stress, sensitivity to H_2O_2 and sodium nitroprusside (SNP) was assayed in LB/MC medium. The zone of inhibition induced by H_2O_2 increased from 2.31 ± 0.04 cm for the WT *S. meliloti* 1021 strain to 2.69 ± 0.06 cm for the gor19 mutant, which could be rescued in the cpgor19 strain (2.32 ± 0.1 cm). Furthermore, the results of the cell sensitivity assay confirmed that the gor19 mutant was sensitive to 100 mM H_2O_2, whereas the parent and complemented strains were sensitive to 150 mM H_2O_2 (Fig. 5A). The sensitivity of the gor19 strain to SNP stress was also estimated, and we observed that the gor19 mutant was hypersensitive to 3 mM SNP compared with
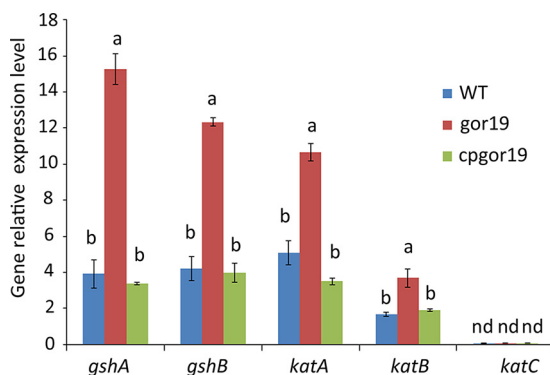

FIG 4 qRT-PCR analysis of the expression levels of various genes in the WT, gor19, and cpgor19 strains. The relative amount of mRNA for each gene was calculated using the threshold cycle (C_T) method and was normalized to that of the *S. meliloti rpsF* gene. Error bars indicate standard deviations. This experiment was performed in triplicate. Significant differences were identified by one-way ANOVA, followed by Tukey's *post hoc* test, and are noted as different letters ($P < 0.05$). Vertical bars indicate the standard errors for three biological replicates. nd, not detected.
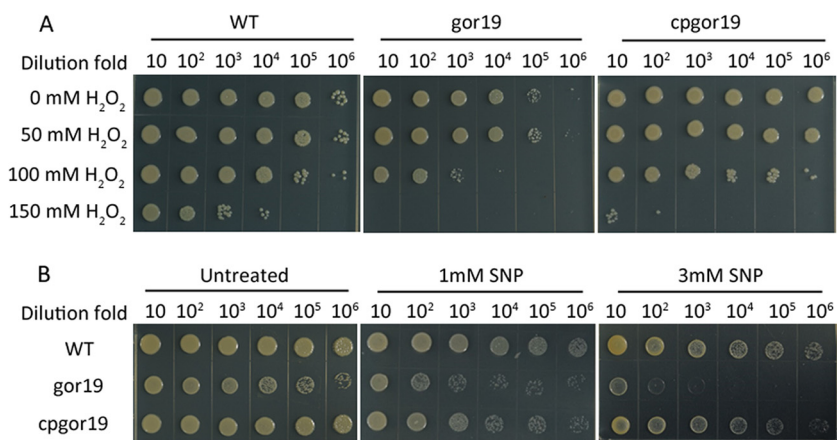
FIG 5 Sensitivity of the WT, gor19, and cpgor19 strains to oxidants. (A) H_2O_2 sensitivity of the WT, gor19, and cpgor19 strains grown in LB/MC medium. Cell cultures were adjusted to an OD_{600} of 0.5, treated for 30 min at 28°C with the indicated amount of H_2O_2, washed twice, resuspended in fresh LB/MC medium, and spotted onto LB/MC agar plates. The 10-fold serial dilutions are indicated above each column. The plates were incubated at 28°C for 4 days. (B) SNP sensitivity of the WT, gor19, and cpgor19 strains grown in LB/MC medium. Cell cultures were adjusted to an OD_{600} of 0.5 and spotted onto LB/MC agar in the absence or presence of 1 or 3 mM SNP. The 10-fold serial dilutions are indicated above each column. The plates were incubated at 28°C for 4 days.

the *S. meliloti* 1021 and complemented cpgor19 strains (Fig. 5B). These data indicate that *gor* is essential for the adaptation of *S. meliloti* to oxidative stress.

Symbiotic defects in the *S. meliloti gor* mutant. To determine the function of the *S. meliloti gor* gene during symbiotic interactions with an alfalfa host, the nodulation capacity of the gor19 mutant was first examined. The number of alfalfa nodules induced by gor19 was significantly decreased, compared with that induced by the parent strain, at 5 and 9 days postinoculation (dpi), but there was no significant difference in alfalfa nodulation with the mutant strain versus the parent strain at 14 dpi (Fig. 6D). To test the functionality of nodules, their nitrogen-fixing capacities and the dry weights of the alfalfa shoots were determined 4 weeks after inoculation with the WT, gor19, and cpgor19 strains. Using the acetylene reduction assay, it was found that the nitrogen-fixing capacity was severely affected in the gor19 mutant, with a reduction from 80.3 ± 4.8 nmol of C_2H_4/mg of nodules/min in WT *S. meliloti* 1021 or 74 ± 2.8 nmol of C_2H_4/mg of nodules/min in cpgor19 to 17.2 ± 2.8 nmol of C_2H_4/mg of nodules/min in the gor19 strain (Fig. 6C), which was consistent with the results of the dry weight of alfalfa shoots and the plant phenotype with gor19 (Fig. 6A and B). Finally, the competitive capacity of gor19 versus *S. meliloti* 1021 was evaluated. Coinoculation data from samples inoculated with WT/gor19 strain ratios of 1:1, 1:9, and 9:1 showed that nodule occupation proportions for the mutant were 6.5%, 13%, and 0%, respectively. These results indicate that infectivity and nitrogen fixation are affected by mutation of the *gor* gene.

DISCUSSION

In *S. meliloti*, glutathione plays key roles in the maintenance of redox homeostasis and the symbiotic root nodulation of alfalfa, according to a study of *gshA* and *gshB1* (11). In this study, we found that the pathway of GSH recycling from GSSG is important for the cellular redox status and alfalfa nodulation in *S. meliloti*. Our findings suggest a feedback regulatory mechanism for GSH biosynthesis in *S. meliloti*. The *gor* gene encodes an NADPH-dependent GR that belongs to a flavin adenine dinucleotide (FAD)-binding disulfide reductase superfamily. The crystal structure of the *S. meliloti* GR indicates that this enzyme consists of domain I (positions 1 to 141 and 295 to 335), domain II (positions 141 to 158 and 265 to 294), domain III (positions 159 to 264), and domain IV (positions 336 to 463) (see Fig. S1A in the supplemental material). According to the DNA sequencing data, the suicide plasmid disrupts the coding regions of
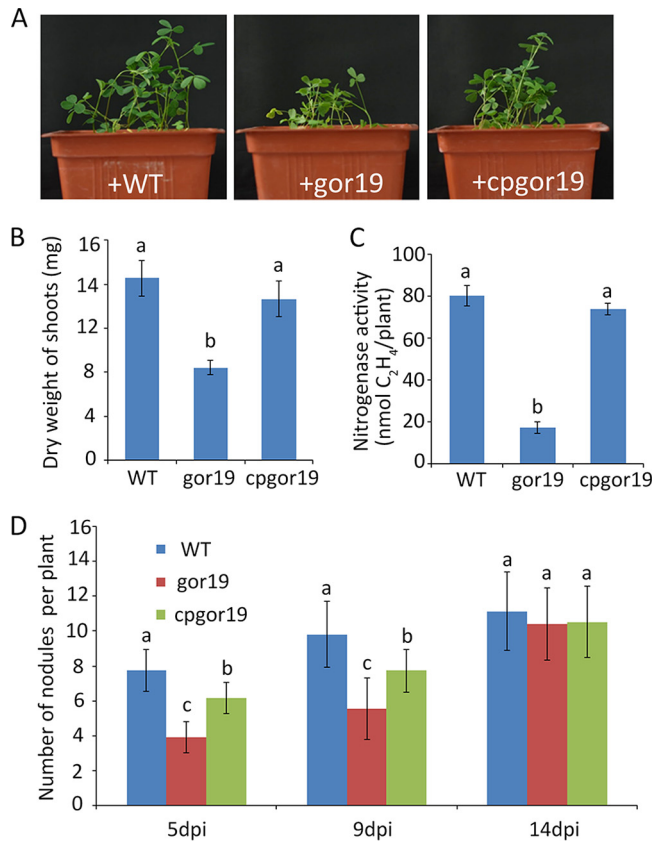
FIG 6 Deficiency of the gor19 strain in symbiosis with alfalfa. (A) Symbiotic phenotypes of the WT, gor19, and cpgor19 strains. (B) Dry weights of shoots inoculated with the WT, gor19, and cpgor19 strains. (C) Nitrogenase activity of alfalfa nodules per plant, as detected by C_2H_2 reduction, in plants inoculated with the WT, gor19, and cpgor19 strains. (D) Numbers of nodules per plant, assessed 5, 9, and 14 dpi. In panels B to D, the data represent means ± standard deviations ($n = 32$). The experiments were repeated four times, and a representative experiment is shown. Significant differences ($P < 0.05$) were identified by one-way ANOVA, followed by Tukey's *post hoc* test, and are noted as different letters ($P < 0.05$).

domains I and IV of GR in the gor19 mutant (Fig. S1B), possibly preventing the dimerization of GR and NADPH binding.

Transposon insertion mutants of *gor* were reported to be involved in defense against diamide but not H_2O_2 in *Rhizobium etli* E3 (13). However, we found that GR in *S. meliloti* 1021 is required for adaptation to oxidative stress, such as treatment with H_2O_2 or SNP (Fig. 5), which could be due to changes in the cellular redox status. The results indicate that GR is required for cellular redox homeostasis.

Catalase activity is often used as a physiological marker to assess whether *S. meliloti* cells are under oxidative stress. Three distinct hydroperoxidases have been reported to be involved with H_2O_2 in *S. meliloti*, i.e., two monofunctional catalases (KatA and KatC) and one bifunctional catalase-peroxidase (KatB) (14, 15). The *gor* mutant cells maintained their oxidative status because the expression of *katA* and *katB* increased (Fig. 4). At the same time, the oxidation status induced the expression of *gshA* and *gshB1* (Fig. 4). This finding is consistent with the observed GSSG/GSH ratio, although the production of GSH and GSSG was increased in the mutant (Fig. 3).

Our study demonstrated that the growth of an *S. meliloti* strain with a mutated *gor* gene was severely impaired in chemically defined minimal broth (M9 medium) supplemented with glucose as the sole source of carbon and NH_4Cl as the sole source of nitrogen, whereas only a slightly reduced growth phenotype was observed in rich broth (LB/MC medium). These results indicate that inactive GR may affect the uptake and/or utilization of sources of carbon and nitrogen in *S. meliloti* 1021. In fact, it has been reported that glutathione affects the transport activity of *Rhizobium leguminosarum*

TABLE 1 Strains and plasmids

Strain or plasmid	Relevant properties[a]	Reference or source
E. coli strains		
DH5α	F− *supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17*(r$_K$− m$_K$+) *recA1 endA1 gyrA96 thi-1 relA1*	TaKaRa Corp.
MT616	*pro-82 thi-1 hsdR17 supE44 recA56* (pRK600); Cm^r	18
S. meliloti strains		
1021	Wild type from SU47; Str^r	Laboratory store
gor19	Same as 1021 but with *gor*::pK19mob2ΩHMB; Str^r, Neo^r	This study
cpgor19	gor19 strain with pSRK19; Str^r, Neo^r, Gm^r	This study
Plasmids		
pK19	pK19mob2ΩHMB; Km^r	17
pK19gor	pK19 with 300-bp PCR-amplified fragment of *gor*; Km^r	This study
pSRK	Broad-host-range expression vector; Gm^r	19
pSRK19	pSRK with *gor* ORF; Gm^r	This study

[a]Cm^r, chloramphenicol resistant; Str^r, streptomycin resistant; Neo^r, neomycin resistant; Gm^r, gentamicin resistant; Km^r, kanamycin resistant.

3841 and is essential for efficient nodulation. A *gshB* mutant of *Rhizobium leguminosarum* 3841 failed to utilize glucose, succinate, glutamine, and histidine efficiently and caused a 50% reduction in the dry weight of plants and nitrogen-fixing ability (16). Therefore, we hypothesized that the slow growth of gor19 would alter bacterial infection and plant nodulation. This possibility was confirmed by inoculation and nodulation assays (Fig. 6), although the difference was minimal. In fact, another insertion mutant of *gor* (with an insertion 491 bp from the ATG of *gor*) may carry a second mutation since the expression of GR could not restore the phenotype (G. Tang, L. Yu, J. Yan, and L. Luo, unpublished data). Taken together, our findings reveal that the GSH recycling pathway is essential for cellular redox homeostasis and root-nodule symbiosis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Escherichia coli* DH5α and MT616 were grown in LB medium at 37°C. The *S. meliloti* 1021 (WT), gor19, and cpgor19 strains were grown at 28°C in LB medium supplemented with 2.5 mM MgSO$_4$ and 2.5 mM CaCl$_2$ (LB/MC medium). The following antibiotics were used at the indicated concentrations: streptomycin, 500 μg/ml; neomycin, 200 μg/ml; chloramphenicol, 10 μg/ml; kanamycin, 25 μg/ml; and gentamicin, 10 μg/ml. The plasmids and strains used in this study are listed in Table 1.

Construction of the gor19 and cpgor19 strains. First, a *gor* (SMc00154) DNA fragment (0.3 kb) from *S. meliloti* 1021 was PCR amplified using the primers P1 and P2. Next, the DNA fragments were digested with HindIII and BsrGI, ligated with a similarly treated pK19 plasmid (17), and transformed into DH5α cells. The resulting plasmid construct (pK19gor) was conjugated into *S. meliloti* 1021 using the helper strain MT616 in a triparental mating (18). Cells containing a plasmid insertion in the *gor* gene were selected for on solid LB/MC medium containing streptomycin and neomycin. A bacterial culture started from a single colony on the selective medium was used to prepare the *S. meliloti* phage φM12 lysate. The lysate was used to transduce the plasmid insertion of *gor* into the *S. meliloti* 1021 background, to ensure that the mutant (gor19) carried a single plasmid insertion in the *gor* gene. To construct a plasmid for complementation of the gor19 mutant, a 1,436-bp DNA fragment of the *gor* ORF was PCR amplified from the genomic DNA of *S. meliloti* 1021 by using primers P3 and P4. The PCR product was cloned into pSRK (19), and the resultant plasmid was named pSRK19. The recombinant plasmid was transferred into the gor19 recipient strain by triparental mating using the MT616 helper strain. The gor19(pSRK19) exconjugants, named cpgor19, were selected for on LB/MC medium containing neomycin, spectinomycin, and gentamicin. The primers used in this study are shown in Table 2.

Measurement of glutathione reductase activity. Measurement of GR activity was performed as described by Davis et al. (20), with minor modifications. Strains were grown in LB/MC broth to early stationary phase (optical density at 600 nm [OD$_{600}$] of 0.5); 1 ml was harvested and resuspended in 500 μl of 50 mM potassium phosphate buffer (pH 8). Cells were disrupted on ice with a Microson ultrasonic cell disruptor (Misonix, Farmingdale, NY) operating at 5 rpm for four periods of 15 s, and debris was removed by centrifugation for 5 min in an Eppendorf centrifuge. The protein concentration of the supernatant fraction was measured by the Lowry method. The reaction mixture contained 1.2 mM oxidized glutathione, 0.4 mM Na$_4$NADPH, 0.6 mM 5,5′-dithiobis-2-nitrobenzoic acid, and 50 mM potassium phosphate buffer (pH 8), in a final volume of 1 ml, at 28°C. The reaction was started with the

TABLE 2 Primers

Primer	Sequence (5′ to 3′)	Purpose
P1	GGGTGTACAAGGACATCATCCAGTCGGT	Construction of pK19gor
P2	CCCAAGCTTTGTTCTTGTATTCCGTCTCG	Construction of pK19gor
P3	GGAATTCCATATGATGAGCGCTTTCGACTATGAC	Construction of pSRK19
P4	CTAGCTAGCTCCTACCAGAAAACGTGG	Construction of pSRK19
P5	TTATGCCTCGCAGTTTGC	Identification of cpgor19
P6	TCGGCTGATACATCGTGA	Identification of cpgor19
P7	ATGTGCTTCATCGAGACG	qRT-PCR of *gor*
P8	GGCATTGACGACGAGCTT	qRT-PCR of *gor*
P9	AACTGGCGGAAGGTGACAT	qRT-PCR of *gshA*
P10	TTACCGAGGGCAAGCCTAA	qRT-PCR of *gshA*
P11	GGAATCGTCAATGTCGGGGTC	qRT-PCR of *gshB1*
P12	GCTTGTCGGGCGTGTAGTGG	qRT-PCR of *gshB1*
P13	CATCGCCGAAGAAGACAA	qRT-PCR of *katA*
P14	CGAAGACATCAGAGAAATGGTC	qRT-PCR of *katA*
P15	CTGACCGCACCGGAAATGAC	qRT-PCR of *katB*
P16	CATATCGAGCAGGTTGACGAAGAA	qRT-PCR of *katB*
P17	GTCGGGCGCGTGAACTACC	qRT-PCR of *katC*
P18	CCCCTGCTCCTCGGAAGG	qRT-PCR of *katC*

addition of supernatant and was monitored at 412 nm. Na_4NADPH and GSSG were always freshly prepared.

Determination of growth curves. The *S. meliloti* 1021 (WT), gor19, and cpgor19 strains were grown in LB/MC medium at 28°C, with shaking at 200 rpm, to an OD_{600} of 0.5. At time zero, 1 ml of rhizobial cells (OD_{600} of 0.5) was inoculated into LB/MC medium or M9 medium (50 ml). The growth of the *S. meliloti* 1021 (WT), gor19, and cpgor19 strains was monitored by measuring the optical density at 600 nm.

Assay of oxidative stress sensitivity. The sensitivity of *S. meliloti* to SNP was assessed with a spotting assay. The *S. meliloti* 1021 (WT), gor19, and cpgor19 strains were grown in LB/MC medium at 28°C, with shaking at 200 rpm, to an OD_{600} of 0.5 and then were harvested by centrifugation. After the cultures were serially diluted 10-fold in LB/MC broth, aliquots (5 μl) were spotted onto LB/MC agar supplemented with 0, 1, or 3 mM SNP and the plates were incubated at 28°C for 4 days to determine the oxidant resistance of the strains. The sensitivity of *S. meliloti* to H_2O_2 was assessed as follows. Cells were adjusted to an OD_{600} of 0.5, treated with 0, 50, 100, or 150 mM H_2O_2 at 28°C for 30 min, washed twice with distilled water, resuspended in fresh medium, and spotted onto LB/MC agar. The plates were incubated at 28°C for 4 days. The growth inhibition of strain gor19 with H_2O_2 exposure was tested with the halo assay method (21), which is a long-term test of resistance to H_2O_2 exposure. Aliquots (300 μl each) of *S. meliloti* cultures with OD_{600} values of 0.5 were plated on LB/MC agar plates. Paper disks (10 mm in diameter) were impregnated with 3 μl of H_2O_2 (1 M) and placed in the center of the *S. meliloti* plates. After 2 to 3 days of incubation at 28°C, the diameters of the halos were analyzed.

Assay of glutathione levels in *S. meliloti*. The extraction and quantification protocol for GSH was performed as described previously (22), with minor modifications. In brief, *S. meliloti* cells (OD_{600} of 2) from 50-ml cultures in LB/MC medium were prepared by centrifugation at 13,000 rpm for 1 min, washed twice with distilled water, and crushed in liquid nitrogen. The cell lysate was then suspended in 200 μl of distilled water. After centrifugation at 13,000 rpm for 10 min, one-half of each supernatant was used for analysis with a 6520 accurate-mass quadrupole time of flight LC-MS system (Agilent, San Jose, CA). The remaining supernatant was used to quantify total protein. The conditions for the purification and analysis of glutathione were as follows: column, Zorbax Extend-C_{18} (4.6 by 50 mm, 1.8 mm); injection volume, 5 μl; flow rate, 0.2 ml/min; solvent A, 0.1% trifluoroacetic acid (TFA) in water; solvent B, 0.1% TFA in methanol; detection wavelengths (diode array detector), 210, 254, 280, 320, 360, and 226 nm; mass range, m/z 50 to 400; nebulizer pressure, 40 lb/in^2; drying gas, N_2 (at 350°C and 9 liters/min); electrospray ionization voltages, 3,500 V (cap), 160 V (capillary and fragmentor), 65 V (skimmer), and 750 V (octupole radiofrequency voltage); and scanning mode, negative MS scan mode (2 GHz) with external dynamic range (to m/z 1,700). GSSG and GSH at 50 ng/μl were used as standards.

RNA extraction and real-time RT-PCR. *S. meliloti* subcultures were grown to exponential phase (OD_{600} of 0.5) in 50 ml of fresh LB/MC medium with 1% of the overnight cultures. RNA was extracted using the TRIzol reagent (Invitrogen, Shanghai, China) (23). The RNA quality was assayed using 1% agarose gel electrophoresis. Next, 1 μg of total RNA was used to prepare cDNA by using random hexamer oligonucleotide primers and the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China). Gene-specific primers were employed for real-time PCR using SYBR green supermix (TransGen, Shanghai, China). The expression of *rpsF* (a ribosomal protein gene) in each sample was used to normalize the expression of the gene(s) of interest. The real-time PCR primers used are listed in Table 2. The expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Assay of nodulation occupancy and competitiveness. Alfalfa (cv. aifeinite) was used as a host plant to test the nodulation of the *S. meliloti* strains. Seeds were surface sterilized and germinated on 1% agarose. Bacterial cells (1 ml, at an OD_{600} of 0.02) were plated on the surface of solid modified Fahraeus medium (24). Ten alfalfa seedlings were spread across each plate. The germinated alfalfa seeds were

coinoculated with the wild-type *S. meliloti* 1021 and gor19 mutant strains at different ratios (1:1, 1:9, and 9:1), ultimately reaching 10^4 CFU per seed. The strains were isolated from nodules using both resistance to streptomycin (Str) and neomycin (Neo) and PCR screening at 3 weeks postinoculation (wpi).

Plant nodulation assay. Sixteen germinated seedlings were transferred to a growth pot (10 by 10 cm) containing perlite and vermiculite at a ratio of 1:3 and supplied with modified Fahraeus medium. After 4 days of growth, plants were inoculated with 50 ml of *Sinorhizobium meliloti* culture (OD$_{600}$ of 0.02) per pot. Different strains were used, including the WT, gor19, and cpgor19 strains. Each strain harbored a *hemA-lacZ* construct that was used to observe the infection events, which were examined 5, 9, and 14 dpi. To examine β-galactosidase activity, whole roots were fixed for 1 h with fixative solution (1% [vol/vol] glutaraldehyde in 1× phosphate-buffered saline [PBS] [pH 7.5]). The fixed samples were subsequently washed twice with 1× PBS and incubated overnight at room temperature in a solution composed of 0.2× PBS, 2.5 mM K$_3$[Fe(CN)$_6$], 2.5 mM K$_4$[Fe(CN)$_6$], and 0.8 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside in *N,N*-dimethylformamide. Stained samples were rinsed three times with 1× PBS and cleared using different concentrations of ethanol. The dry weights of the shoots of the WT, gor19, and cpgor19 strain-inoculated plants were examined 3 wpi.

Acetylene reduction assay. Nitrogenase activity was assessed with the acetylene reduction assay as described previously (25). For this, 2 ml of acetylene was injected into sealed 20-ml tubes containing 4-week-old plants. Reduction of acetylene to ethylene was measured by flame ionization gas chromatography (Shanghai Analytic Instrument Factory, Shanghai, China) after 30 min of incubation at 28°C. For each sample, at least 30 plants, divided into three replicates, were analyzed.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM .01937-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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