Glutathione Is Required by *Rhizobium etli* for Glutamine Utilization and Symbiotic Effectiveness

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Here, we provide genetic and biochemical evidence indicating that the ability of *Rhizobium etli* bacteria to efficiently catabolize glutamine depends on its ability to produce reduced glutathione (L- γ -glutamyl-L-cysteinylglycine [GSH]). We find that GSH-deficient strains, namely a *gshB* (GSH synthetase) and a *gor* (GSH reductase) mutant, can use different amino acids, including histidine, alanine, and asparagine but not glutamine, as sole source of carbon, energy, and nitrogen. Moreover, L-buthionine(*S,R*)-sulfoximine, a GSH synthesis inhibitor, or diamide that oxidizes GSH, induced the same phenotype in the wild-type strain. Among the steps required for its utilization, glutamine uptake, occurring through the two well-characterized carriers (Aap and Bra systems) but not glutamine degradation or respiration, was largely reduced in GSH-deficient strains. Furthermore, GSH-deficient mutants of *R. etli* showed a reduced symbiotic efficiency. Exogenous GSH was sufficient to rescue glutamine uptake or degradation ability, as well as the symbiotic effectiveness of GSH mutants. Our results suggest a previously unknown GSH–glutamine metabolic relationship in bacteria.

The tripeptide glutathione (L- γ -glutamyl-L-cysteinylglycine [GSH]) is considered the most potent antioxidant produced by living cells. Usually, GSH is synthesized from the constituent amino acids by two ATP-dependent consecutive reactions catalyzed by two enzymes (i.e., γ -glutamylcysteine synthetase [EC 6.3.2.2] and GSH synthetase [EC 6.3.2.3], encoded by the *gshA* [*GSH1*] and *gshB* [*GSH2*] genes, respectively).

Based on the characterization of strains unable to synthesize GSH (*gshA* and *gshB* mutants) or depleted of intracellular GSH by using either L-buthionine(*S,R*)-sulfoximine (BSO), a specific inhibitor of GSH synthesis, or diamide, a thiol-oxidizing reagent, it was established that *Escherichia coli* cells i) require GSH when subjected to chemical or physical challenges such as radiation, osmotic shock, selenite, chlorine compounds, formaldehyde, acidic conditions, and electrophilic chemicals (i.e., methylglyoxal or *N*-ethylmaleimide) (Masip et al. 2006); ii) do not require GSH to grow efficiently in a chemically defined minimal medium and, therefore, for physiological cellular processes, including transport, transcription, and translation, such as in yeast and in mammalian cells (Greenberg and Demple 1986; Meister 1988).

Conversely, it has been shown that *Rhizobium* bacteria (namely, *Rhizobium tropici*, *Sinorhizobium meliloti*, and *Bradyrhizobium* spp.) require GSH not only to survive chemical or

physical challenges but also to grow in a chemically defined minimal medium (thus, in the absence of noticeable environmental stresses) (Harrison et al. 2005; Ricillo et al. 2000; Sobrevals et al. 2006). Rhizobia are gram-negative soil bacteria able to reduce N₂ (nitrogen fixation) in symbiotic association with legume plants, and it was established that their symbiotic proficiency depends on their capacity to synthesize GSH (Harrison et al. 2005; Pauly et al. 2006; Sobrevals et al. 2006). Notably, it has been recently reported that nodules induced by a GSH-deficient mutant of *R. tropici* on the root of *Phaseolus vulgaris* undergo early senescence (Muglia et al. 2008). Nevertheless, the cellular processes regulated by or dependent on GSH in either free-living rhizobia or N-fixing bacteroids (their intracellular symbiotic form) remain unknown.

In this study, we identify an intricate GSH–glutamine (Gln) metabolic relationship in *R. etli*. In searching for mutant strains unable to utilize Gln as sole source of carbon (C) and nitrogen (N), we substantiate our previous findings (Taté et al. 2004) supporting the idea of a strict GSH dependence of Gln catabolism in *R. etli*. Moreover, we show that Gln transport or uptake is the defective step of Gln utilization in GSH-deficient strains. Most remarkably, and unexpectedly, we provide evidence that Gln could delay or inhibit the growth of bacteria depending on the GSH status. All together, our data provide new insights on the role of GSH in free-living rhizobia and may be relevant for the efficiency or maintenance of its symbiotic form.

RESULTS

Isolation of a *gshB::Tn5* mutant by seeking for Gln-deficient mutants.

Tn5-induced mutants, derivative of the strain CE3 (wild type) of *R. etli* and unable to grow with Gln as sole source of C and N, were isolated and named CE3 Tn5 Gln utilization (CTGUT) mutants, as was previously done (Taté et al. 2004). Here, by means of a round of ampicillin-enrichment routines, four mutant strains affected in Gln but in neither glucose (Glc) nor in succinate (Suc) utilization were isolated. At first, exploiting the kanamycin-resistance cassette, the Tn5-flanking genomic DNA regions were cloned from all the mutants isolated. A DNA sequence analysis revealed that, among these mutants, only one strain, CTGUT43, was mutated in a gene that was not previously identified as required for Gln utilization (Taté et al. 2004). Indeed, strain CTGUT43 carried a single Tn5 insertion in an open reading frame (948 nucleotides, 316 codons) sharing up to 100% identity (over 315–amino acid overlap) with GSH synthetase (encoded by *gshB*) from various bacteria, including strain CFN42 of *R. etli* (RhizoBase: RHE_CH00322; National Center for Biotechnology Information: NC_007761.1), *R. tropici*, and *S. meliloti* (Gonzalez et al.

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2006; Harrison et al. 2005; Ricillo et al. 2000). GshB enzyme catalyses the second step of GSH biosynthesis (i.e., the conversion of γ -glutamylcysteine (γ -GluGly) and Gly into GSH. The growth defects of strain CTGUT43 were prevented either by adding exogenous GSH (100 μ M) or upon transformation with the plasmid pAR367 (strain CTGUT43-pAR367) (Fig. 1A). This plasmid harbors the *gshB* gene and its proximal 5'-flanking region (159 bp upstream from the ATG initiation codon) that was polymerase chain reaction (PCR) amplified using specific oligonucleotides (discussed below). As expected, strain CTGUT43 showed a high susceptibility to electrophilic chemicals (methylglyoxal and *N*-ethylmaleimide) and acidic conditions compared with strains CE3 and CTGUT43-pAR367 (data not shown). Altogether, these data indicated that the phenotype of strain CTGUT43 was a consequence of *gshB* gene disruption and, accordingly, strains CTGUT43 and CTGUT43-pAR367 were named CTGUT43(*gshB*⁻) and CTGUT43(*gshB*^{+/+}), respectively.

Growth behavior of the *gshB* mutant.

The strain CTGUT43(*gshB*⁻) grew more slowly than strains CE3 and CTGUT43(*gshB*^{+/+}) in all the growth media assayed. For instance, in liquid TYR medium, strains CE3, CTGUT43(*gshB*⁻), and CTGUT43(*gshB*^{+/+}) grew with a doubling time of 150, 246, and 162 min, respectively. In solid rhizobia minimal medium (RMM), the *gshB* mutant grew with usual sources of

C, such as Glc or Suc, as well as with different amino acids, including histidine (His), alanine (Ala), or asparagine (Asn), as sole source of C and N (Fig. 1A). Notably two different GSH-deficient mutants of *R. etli*, strain CTGUT43(*gshB*⁻) and a previously isolated mutant, strain CTGUT15(*gor*⁻), lacking GSH reductase enzyme (Tat et al. 2004), shared the same specific phenotype (Fig. 1A) (i.e., the incapacity to grow on RMM-agar supplemented with 5 mM Gln [or Glu] as sole source of N and C). The same growth phenotype was observed when GSH-deficient mutants were spotted at a density of 10³ to 10⁵ bacteria/ μ l (data not shown).

Which step of Gln utilization is impaired in GSH-deficient mutants?

In principle, a bacterium may utilize exogenous Gln as a source of C or energy by means of a four-step process: uptake, deamidation (Gln to Glu), oxidative deamination (Glu to α -ketoglutarate), and respiration through the citric acid cycle (CO₂ production) (Fig. 2). In *R. etli*, Gln uptake depends on the Aap and Bra transport systems pertaining to the ABC permease family (Hosie et al. 2002; Tat et al. 2004; Walshaw and Poole 1996), whereas Gln degradation requires the activity of two enzymes (i.e., glutaminase and glutamate dehydrogenase [Gdh] (Huerta-Saquerio et al. 2004; Tat et al. 2004). Likewise, Glu utilization occurs through the same pathway (Aap/Bra $\rightarrow$ Gdh), excluding the step catalyzed by Glis (Fig. 2). Hence, to

Fig. 1. Glutamine (Gln) utilization, transport, and respiration of glutathione (GSH)-deficient mutants of *Rhizobium etli*. **A**, Growth phenotype. Cells (10⁴ in 5 μ l) of strains CE3(WT), CTGUT43(*gshB*⁻), CTGUT43-pAR367(*gshB*^{+/+}), CTGUT15(*gor*⁻), CTGUT14(*aapM*⁻), CTGUT27(*braD*⁻), and CTGUT3(*gdhB*⁻), indicated to the left, were spotted onto either complete medium (TYR agar) or minimal medium (rhizobia minimal medium [RMM] agar) (indicated at top) containing 5 mM Gln, Glu, histidine (His), alanine (Ala), or asparagine (Asn) as sole C and N source; or onto RMM agar containing either 5 mM glucose (Glc) or 5 mM succinate (Suc) plus 5 mM NH₄Cl as C and N source, respectively. At a plate of RMM-Gln, 100 μ M GSH (bottom) was added. Bacterial colonies at 3 days postinoculation (dpi) (30°C) were individually photographed and displayed as a panel. **B**, Effect of Gln concentration (bottom) on the growth of *R. etli* mutants (left). Colonies at 3 and 9 dpi (top) are shown. **C**, Gln uptake activity of *R. etli* strains (left) was measured either before (TYR-grown cells, black bars) or after (white bars) the shift to RMM-Gln medium. **D**, Glutamate dehydrogenase (Gdh) activity measured in crude extracts prepared from cells incubated in RMM-Gln. **E**, Gln respiration capacity of *R. etli* strains (bottom) incubated in RMM-Gln with (dashed bars) or without (white bars) fluorocitrate addition (100 μ M). Values denote nmoles of Gln degraded to CO₂. In C, D, and E, data represent the means of three experiments and standard deviations are indicated by error bars.

get insight into the GSH-Gln relationship, the growth phenotype of a group of *R. etli* mutants (Table 1) was comparatively analyzed (Fig. 1A). Notably, both *gshB* and *gor* mutants could not even grow with Glu (Fig. 1A); thus, a putative falling of glutaminase expression or activity in a GSH-deficient background was promptly excluded. Likewise, Gdh activity seemed to be unrelated to GSH metabolism. The utilization of His as sole C source strictly depends on Gdh activity (His is converted into Glu) (Fig. 2) and, indeed, GSH-deficient mutants (*gshB* and *gor* mutants), unlike strain CTGUT3(*gdhB*⁻), an *R. etli* mutant lacking Gdh (Taté et al. 2004), were able to grow with His as sole C source (Fig. 1A). Finally, in order to grow on solid RMM-Gln (or Glu), the Aap transport system was required, whereas the Bra system was dispensable, as shown by the mutants CTGUT14(*aapM*⁻) and CTGUT27(*braD*⁻), respectively (Fig. 1A). All together, this phenotypic analysis led us to hypothesize a putative requirement of GSH in the Gln uptake process, likely occurring through the Aap permease. As expected, GSH-deficient strains, unlike *gdhB* and *aapM* mutants, grew on RMM-Gln when supplemented with 100 μ M GSH (Fig. 1A). Notably, the growth ability of the *gor* mutant but not that of the *gshB*, *aapM*, and *gdhB* mutants was recovered to some extent by increasing the Gln concentration up to 50 mM (Fig. 1B); thus, suggesting that Gln concentration was not the growth-limiting factor for the *gshB* mutant.

Effect of GSH metabolism on Gln transport.

Thus, we went on to assess and compare the Gln uptake capacity of *R. etli* strains. Because most of the mutants analyzed could not grow in RMM-Gln (Fig. 1A), *R. etli* strains were grown in TYR (up) to exponential growth phase and then shifted for 2 h to RMM-Gln. The Gln uptake activity of strain CE3 increased (approximately 3.5-fold) after incubation in RMM-Gln (Fig. 1C). Gln uptake in the *gshB* mutant was also induced (almost threefold), even though the activity values were lower (reduced by 70%) compared with those of strain

CE3 under both growth conditions (Fig. 1C). Determination of the CFU showed that the *gshB* mutant viability did not change (100% survival) after shifting to RMM-Gln. Furthermore, plasmid pAR367(*gshB*) reestablished (up to 80%) the Gln uptake capacity of the *gshB* mutant (Fig. 1C). Conversely, *R. etli* strains shifted to RMM-Suc/NH₄⁺ or RMM-Glc/NH₄⁺ displayed a Gln uptake activity similar to or slightly (up to 20%) lower than the activity of TYR-grown bacteria. Therefore, the induction of Gln uptake occurred when Gln became the sole source of C and N available. Furthermore, analyzing the *aapM* and *braD* mutants and assuming a functional autonomy when co-expressed, the relative contribution of Bra and Aap systems to the whole Gln uptake activity (110 nmol/mg/min displayed by strain CE3) was approximately 25% (27 nmol/mg/min) and 75% (85 nmol/mg/min), respectively (Fig. 1C). The activity values of the *gshB* and *gor* mutants were comparable (32 versus 42 nmol/mg/min) and similar to those displayed by the *aapM* mutant, thus suggesting that the process of Gln uptake is ineffective (i.e., not enough to support Gln catabolism) in GSH-deficient mutants. Finally, strain CTGUT3(*gdhB*⁻) lack-

Table 1. Mutant derivatives of *Rhizobium etli* CE3 (wild type)

Strain	Mutated gene	Reference
CTGUT43	<i>gshB</i> , glutathione synthetase	This work
CTGUT15	<i>gor</i> , glutathione reductase	Taté et al. 2004
CTGUT3	<i>gdhB</i> , NAD-dependent glutamate dehydrogenase	Taté et al. 2004
CTGUT14	<i>aapM</i> , amino acid permease (Aap system)	Taté et al. 2004
CTGUT27	<i>braD</i> , amino acid permease (Bra system)	Taté et al. 2004
CTNUX10	<i>gluD</i> , glutamate synthase (GOGAT)	Ferraioli et al. 2002
CFNX247	<i>nifA</i> , nitrogenase complex transcription	Girard et al. 1996
CFNX249	<i>nifH1/nifH2</i> double mutant, nitrogenase complex	Brito et al 1997

Fig. 2. Metabolic interplay between glutamine (Gln) and glutathione (GSH) in *Rhizobium etli* is schematically represented. His = histidine, γ GluCys = γ -glutamylcysteine, Suc = succinate, GDH = glutamate dehydrogenase, BSO = L-buthionine(S,R)-sulfoximine.

ing Gdh activity and, thus, intracellular Glu degradation showed a similarly reduced Gln uptake capacity (70% lower than strain CE3). The Gln uptake activity was significantly reduced also by the preincubation of bacteria with inhibitors of the citric acid cycle, namely fluorocitrate and malonate (data not shown). Hence, in *R. etli*, Gln uptake depends not only on the efficiency of Gln carriers (mainly the Aap system) but also on the efficiency of the Glu/Gln catabolic pathways; namely, the Gdh pathway and citric acid cycle (Fig. 2). Indeed, an efficient GSH metabolism appeared to be dispensable for the expression or activation of both the Gdh pathway and the citric acid cycle, when His became the sole source of C or energy (Fig. 1A).

Gln degradation or respiration in GSH-deficient mutants.

To rule out a role of GSH on Gdh activity, protein extracts were prepared from *R. etli* mutants and the Gdh activity was measured. Under conditions of induction (cells shifted to RMM-Gln) and considering the Gdh activity (mean value) of strain CE3 as 100%, the activities of strains CTGUT43(*gshB*⁻) and CTGUT43(*gshB*^{+/+}) were approximately 80 and 95%, respectively (Fig. 1D). Moreover, Gdh activity displayed by the *braD*, *aapM*, and *gor* mutants were either not significantly different or slightly increased (up to 15% higher than CE3). Strain CTGUT3(*gdhB*⁻), used as a control, produced NADH in the presence of Glu at a very low rate (less than 5% of the CE3 production). Thus, Gdh enzyme was not inhibited by defects in either GSH synthesis or reduction (*gshB* and *gor* mutants) or Gln transport (*aapM* and *braD* mutants).

To evaluate a putative influence of GSH on the last step of Gln utilization, the production of CO₂ from Gln (i.e., the flow of Gln through the citric acid cycle) (Fig. 2) was measured. Considering the rate (approximately 90 nmol/mg/min) at which strain CE3 produced CO₂ from Gln (Fig. 1E), and assuming that the complete degradation of each Gln molecule occurred, it is possible to conclude that approximately 80% of the Gln molecules transported were degraded to CO₂. Strain CTGUT43(*gshB*⁻) produced CO₂ from Gln at a lower rate (25 nmol/mg/min) compared with strain CE3, even if the fraction of Gln molecules degraded was comparable (approximately 80%). Finally, plasmid pAR367 improved Gln respiration capacity of the *gshB* mutant (Fig. 1E). As a control, we used fluorocitrate (100 μM) and malonate (2.5 mM), which inhibit two enzymes of the citric acid cycle; namely, aconitase and succinate dehydrogenase, respectively (Gardner and Fridovich 1991). These compounds reduced by more than 95% the capacity of *R. etli* strains, including the *gshB* mutant, to produce CO₂ from Gln (Fig. 1E). Finally, comparable results were obtained analyzing the *gor* mutant (data not shown), thus suggesting that the citric acid cycle is active in the GSH-deficient mutants. In correlation, we observed that GSH-deficient mutants grew with arabinose (5 mM) as C source, and it is known that rhizobia require an efficient citric acid cycle to grow catabolizing arabinose (Walshaw et al. 1997).

Effect of BSO on bacterial growth and Gln uptake.

It has been previously shown that i) *gshB* mutants accumulate γ-glutamylcysteine (γGluCys), the unique intermediate of GSH biosynthesis (Fig. 2); and ii) γGluCys may mask, even if partially, the lack of GSH (Harrison et al. 2005). Hence, to evaluate the occurrence of a putative link between γGluCys and the incapacity to utilize Gln or Glu, we used BSO, an inhibitor of the γGluCys synthetase enzyme (encoded by *gshA*) (Fig. 3A). Remarkably, at BSO concentrations of 500 μM to 2 mM, the growth of strain CE3 was i) inhibited on RMM-Gln or RMM-Glu; ii) slightly reduced on RMM-His; or iii) unaffected on either TYR or on RMM with Glc, Suc, or Asn as C source (Fig. 3A). Conversely, 5 mM BSO inhibited strain CE3

in RMM independently of the C source supplied. The BSO-induced phenotype was rescued by adding GSH (100 μM) (data not shown). Furthermore, the growth deficiencies of the *gshB* mutant were not reverted by BSO treatment (Fig. 3A); in contrast, the lack of *gshB* increased bacterial susceptibility to BSO. Actually, 1 mM BSO reduced the growth ability of strain CTGUT43(*gshB*⁻) in all growth media (Fig. 3A). Finally, strain CTGUT43(*gshB*^{+/+}) was more resistant to BSO than strain CTGUT43(*gshB*⁻) in all media assayed (Fig. 3A), thus indicating that, in the absence of *gshB* (i.e., in the absence of GSH), γGluCys is essential for bacterial growth. Finally, considering that BSO mimics the effect of a *gshA* mutation, it is not surprising that a *gshA* mutant was not isolated in the screening performed.

In order to assess the effect of BSO on the Gln uptake capacity of *R. etli*, bacteria were grown in liquid media with or without added BSO (Fig. 3B). Remarkably, 2 mM BSO significantly reduced the ability of the wild-type strain CE3 to use Gln, whereas its ability to use Suc or His, as well as to grow in TYR, was either slightly inhibited or unaffected (Fig. 3B). In correlation, the treatment with BSO reduced by approximately 70% the capacity of strain CE3 to transport Gln (Fig. 3C). The inhibitory effect of BSO was largely released (reaching approximately 80% of the maximal level of induction) (Fig. 1C) by shifting the cells (2 h) to RMM-Gln devoid of BSO. In contrast, BSO-treated cells of the *gshB* mutant showed almost undetectable Gln uptake activity even after incubation without BSO (Fig. 3C), thus supporting the idea that γGluCys alleviates the mutant deficiency. Furthermore, BSO-treated cells of strains CTGUT14(*aapM*⁻) and CTGUT27(*braD*⁻) transported Gln, although at lower rates (up to 70%) than BSO-untreated cells (Fig. 3C), thus indicating that BSO similarly inhibited Gln uptake mediated by both Aap and Bra systems.

Effect of diamide on Gln utilization.

Diamide treatment, by oxidizing thiol groups, depletes cells of reduced GSH (Kosower and Kosower 1995) and may cause the same specific growth phenotype induced by the lack of either *gshB* or *gor* gene (Fig. 3D). In fact, at 250 μM diamide, the growth of strain CE3 was i) halted on RMM-Gln or RMM-Glu; ii) slightly reduced on RMM-His; and iii) not appreciably affected on either TYR or RMM with Glc, Suc, or Asn as C source. Finally, 1 mM diamide completely blocked the growth capacity of strain CE3 in all the assayed media, including TYR (Fig. 3D). As expected, *gshB* and *gor* mutants were more susceptible to diamide treatment than its wild-type parent (Fig. 3D). For instance, the ability of the *gshB* mutant to grow in RMM was already abolished at 50 μM diamide (Fig. 3D). Surprisingly, the complemented mutant, strain CTGUT43(*gshB*^{+/+}), was more resistant to diamide than strain CE3. For instance, at 1 mM diamide, the growth ability of strain CTGUT43(*gshB*^{+/+}) on TYR, RMM-Suc, or RMM-Asn was reduced but not blocked, as observed with strain CE3.

Gln as N source.

Rhizobium spp. require Aap and Bra permeases to grow with Gln as C source but not to grow with Gln as N source (Hosie et al. 2002; Taté et al. 2004). We speculated that, if GSH is essential to achieve maximal levels of Gln uptake activity through Aap and Bra (Figs. 1 and 3), *gshB* and *gor* should be dispensable for Gln utilization as N source. To verify this assumption, *R. etli* strains were plated on RMM-Glc with different N sources (Fig. 4A). Notably, whereas *gshB* was essential, the *gor*, *aapM*, and *gdhB* genes were dispensable to use Gln (or Glu) efficiently as N source, even at a concentration of 50 μM to 5 mM. Conversely, all strains assayed grew with His or NH₄⁺ as N source. Certainly, the *gshB* mutant could utilize

Gln as N source when transformed with plasmid pAR367 (Fig. 4A) or when exogenous GSH (100 μ M) was supplied (data not shown). With Gln as N source, the growth of the *gshB* mutant was delayed but not blocked, because bacterial growth began to be perceptible 4 to 5 days postplating (data not shown). Furthermore, the *gshB* mutant, like strains CE3, CTGUT15(*gor*⁻), CTGUT14(*aapM*⁻), and CTGUT27(*braD*⁻), grew on RMM-Glc with 1 mM Glu-Glu dipeptide as sole N source. Thus, Aap

and Bra systems are dispensable for Glu-Glu or His uptake and, most remarkably, the *gshB* mutant is able to grow using alternative sources of intracellular Glu, such as His or Glu-Glu. Conversely, the wild-type strain of *R. etli* was unable to grow with Glu-Glu dipeptide as sole source of C and N.

The influence of GSH on the use of Gln or Glu as N source was also evaluated by analyzing a glutamate synthase (GOGAT) mutant of *R. etli*, strain CTNUX10(*gltD*⁻) (Ferraioli

Fig. 3. Effect of L-buthionine(S,R)-sulfoximine (BSO) and diamide on glutamine (Gln) utilization ability of *Rhizobium etli*. **A**, Effect of BSO on the bacterial ability to grow on solid medium. Cells (10^4 in 5 μ l) of strains CE3(WT), CTGUT43(*gshB*⁻), and CTGUT43-pAR367(*gshB*^{+/+}), indicated at the top, were spotted on TYR or rhizobia minimal medium (RMM) supplemented with 5 mM glucose (Glc), succinate (Suc), Gln, Glu, histidine (His), or asparagine (Asn), indicated to the left. BSO was added at the indicated concentration (bottom). Bacterial colonies (3 days postinoculation [dpi] at 30°C) are shown. **B**, Effect of BSO on the *R. etli* ability to grow in liquid medium. Strain CE3 was inoculated (initial optical density at 590 nm [OD₅₉₀] = 0.05) on liquid media TYR (black bars) or RMM supplemented with Gln (white bars), Suc (dashed bars), or His (gray bars), with or without BSO addition (2 mM) (left). Values of culture turbidity measured after incubation (24 h at 30°C) are shown. Data represent the means of three different experiments. Standard deviations (SDs) are indicated by error bars. **C**, Effect of BSO on Gln uptake activity. *R. etli* strains (left) were grown in TYR plus 2 mM BSO (inoculated at OD₅₉₀ = 0.01, harvested at OD₅₉₀ = 0.6) and shifted (OD₅₉₀ = 0.3) to either RMM-Gln alone (white bars) or supplemented with BSO (2 mM; black bars). After incubation (2 h at 30°C), the cells were harvested and Gln uptake capacity was measured. Data represent the means of three experiments. SDs are indicated by error bars. **D**, Effect of diamide on the bacterial ability to grow on solid medium. Cells (10^4 in 5 μ l) of strains CE3(WT), CTGUT15(*gor*⁻), CTGUT43(*gshB*⁻), and CTGUT43-pAR367(*gshB*^{+/+}), indicated at the top, were spotted on TYR or RMM supplemented with 5 mM Glc, Suc, Gln, Glu, His, or Asn, indicated to the left. Diamide was added at the concentration indicated (bottom). Bacterial colonies (3 dpi at 30°C) are shown.

et al. 2002). In *Rhizobium* spp., GOGAT is essential for NH_4^+ assimilation (Patriarca et al. 2002). As expected, strain CTNUX10(*gltD*⁻) grew on RMM-Glc/ NH_4^+ (or RMM-Suc/ NH_4^+) only when Glu or a source of Glu (namely Gln, Asn, or His) was available (Fig. 4B). The ability of Glu to overcome the Glu auxotrophy of strain CTNUX10(*gltD*⁻) was prevented by BSO (500 μM). Conversely, BSO could not prevent the growth rescue induced by the other intracellular sources of Glu (i.e., Asn and His) (Fig. 4B).

Gln interfere with the growth of the *gshB* mutant.

The growth of the *gshB* mutant was prevented when 5 mM Gln (or Glu) was added to RMM containing either Glc, Suc, His, or Asn as an alternative C source (Fig. 4C). The Gln-induced inhibitory effect could be avoided either by adding GSH (100 μM) (Fig. 4C) or by transforming the mutant with plasmid pAR367 (Fig. 4D). Remarkably, Gln strongly delayed (colony formation began to be evident 5 to 6 days postinoculation [dpi]) but did not block the growth of strain CTGUT43 (*gshB*⁻). Unexpectedly, the lowest concentration of Gln (50 μM) capable of inhibiting the growth of the *gshB* mutant corresponds to the minimal concentration required to rescue the auxotrophy of the *gltD* mutant (lacking GOGAT) (Fig. 4D). Furthermore, Gln was unable to influence markedly the growth

of *gor*, *aapM*, and *gdhB* mutants, independently from the concentration used. Finally, as a control, the *gshB* mutant grew on RMM-Glc/ NH_4^+ supplemented with 5 mM His (Fig. 4D) or Glu-Glu dipeptide (not shown). These data support the occurrence of a specific mechanism of growth interference induced by Gln or Glu in a *gshB* background.

The symbiotic behavior of the *gshB* mutant.

In order to assess the nodulation capacity of the *gshB* mutant, *P. vulgaris* seedlings were inoculated with *Rhizobium* bacteria and, 3 weeks later, the nodules developed on the primary root of the plants were counted and examined. Strain CTGUT43(*gshB*⁻) induced up to 3 nodules per plant, whereas strain CE3 induced, on average, approximately 15 nodules per plant (Fig. 5A). Moreover, 21-day-old nodules induced by the *gshB* mutant were highly heterogeneous in size, frequently reaching a notable dimension (fresh weight up to twofold higher), and displayed a substantially lower (approximately fivefold) acetylene reduction activity (ARA) compared with normal (CE3-induced) nodules (Fig. 5A). It is worth noting that strain CTGUT43(*gshB*⁻) was reisolated from 100% of the few nodules induced. In contrast, on the secondary and tertiary roots, the *gshB* mutant induced a higher (up to threefold) number of nodules compared with the wild-type strain (data not shown). This delayed nodulation was

Fig. 4. Effect of glutamine (Gln) on the growth of *Rhizobium etli* mutants. **A**, Gln as N source. Cells (10^4 in 5 μl) of strains CE3(WT), CTGUT43(*gshB*⁻), CTGUT43-pAR367(*gshB*^{-/+}), CTGUT15(*gor*⁻), CTGUT14(*aapM*⁻), and CTGUT3(*gdhB*⁻), indicated to the left, were spotted on rhizobia minimal medium (RMM)-glucose (Glc) containing, as N source Gln, Glu, histidine (His), or ammonium (NH_4^+), as indicated (bottom). Concentration used is indicated at the bottom. **B**, Effect of L-buthionine(S,R)-sulfoximine (BSO) on the growth of strain CTNUX10(*gltD*⁻). Cells were spotted (10^4 in 5 μl) onto RMM agar containing NH_4Cl as N source and either Glc or succinate (Suc) as C source; 5 mM Gln, His, or asparagine (Asn) (left) was added to specific plates. A subgroup of plates was also supplemented with BSO. **C**, Effect of Gln on the *gshB* mutant growth. Strain CTGUT43(*gshB*⁻) was spotted onto RMM agar supplemented with either His or Asn (5 mM) as C and N source or onto RMM agar containing either 5 mM Glc or 5 mM Suc plus NH_4Cl as C and N source, respectively (left). To a group of plates, 100 μM glutathione (GSH) was added (bottom). **D**, Gln concentration and bacterial growth. Strains (left) were spotted on RMM-Glc/ NH_4^+ supplemented with either Gln, Glu, or His as indicated (bottom). Colonies are shown at 3 days postinoculation and 30°C.

characterized by the formation of undeveloped and ineffective nodules, resembling the nodules induced by the *gshB* mutant of *R. tropici* on the roots of common bean (Muglia et al. 2008). As expected, the nodulation ability of strain CTGUT43(*gshB*⁻) was improved by watering the inoculated plants with Jensen medium supplemented with 100 μ M GSH (Fig. 5A). Furthermore, strain CTGUT43(*gshB*^{+/+}) induced an almost normal number (approximately 12 per plant) of efficient (ARA⁺) nodules. The competitiveness of the *gshB* mutant was evaluated as previously described (Tat et al. 1997), inoculating the roots of 24 seedlings of common bean with a mixture (1:1 ratio) of strain CTGUT43 (*gshB*⁻) and CE3. The *gshB* mutant was able to invade approximately 0.5% of the nodules (21 dpi) located on the primary roots (192 nodules tested).

The 21-day-old nodules induced by strains CE3 and CTGUT43(*gshB*⁻) show a similar tissue organization (Fig. 5B). However, the invaded cells (ICs) located at the periphery of the central tissue were less regular in CTGUT43(*gshB*⁻)-induced nodules than in normal nodules, with some ICs showing a heterogeneous staining and abnormal symbiosomes (Fig. 5B). Moreover, the uninvaded cells of CTGUT43(*gshB*⁻)-induced nodules contained prominent starch grains. As previously shown (Cermola et al. 2000; Ferraioli et al. 2002), these features are indicators of premature senescence. Finally, the plants nodulated by the *gshB* mutant developed sclerotic leaves, a symptom of nitrogen deficiency (Fig. 5C). This phenotype was as severe as that observed in plants inoculated with two different Fix⁻ (unable to fix nitrogen) mutant strains of *R. etli*; namely, CFNX247(*nifA*⁻) and CFNX249(*nifH1*⁻/*nifH2*⁻). Hence, as in the case of other rhizobia, a normal GSH metabolism is essential for the symbiotic efficiency of *R. etli*.

DISCUSSION

Here, we have described the identification of a previously unforeseen role of GSH in bacteria. Briefly, an efficient process of GSH synthesis or reduction is essential to allow the

growth of *R. etli* with or in the presence of Gln. In a series of experiments, we have demonstrated that, among the few steps required for Gln utilization or catabolism, only the process of Gln transport (uptake) depends strictly on GSH homeostasis (Fig. 1). More than 70% of the total Gln uptake capacity of *R. etli* depends on Aap permease and, accordingly, only a functioning Aap system guaranteed the use of Gln as sole C and N source. Remarkably, the Gln transport capacity of *gshB*, *gor*, and *aapM* mutants was similarly reduced, allowing us to conclude that the Gln uptake capacity of the GSH-deficient mutants (*gshB* and *gor*) is inadequate to permit their growth with Gln (or Glu) as sole source of C and N.

The requirement of GSH for Gln utilization was supported by the results obtained assaying the effect of BSO and diamide (Fig. 3). In fact, when supplied at appropriate sublethal concentrations, these chemicals alone were sufficient to mimic the effect of either a *gshB* or *gor* mutation. Moreover, exogenously added GSH was sufficient to overcome the growth inhibition induced by BSO or diamide, thus indicating that they reduced the bacterial capacity to utilize Gln (or Glu) only by affecting the GSH homeostasis. Remarkably, BSO induced a substantial reduction of Gln uptake activity in all strains assayed, including the *gshB* mutant, thus supporting genetic evidence indicating that GSH influences, in a positive manner, the process of Gln uptake, and excluding a negative role of γ Glu-Cys in this process. Notably, BSO at a high concentration prevents the growth of the wild-type strain of *R. etli* in all media assayed, including minimal medium, thus suggesting that *R. etli* strictly depends on GSH or γ GluCys to grow. Moreover, considering that i) BSO mimics the effect of a *gshA* mutation and ii) Gln-deficient mutants were identified by replica plating of bacteria in minimal medium, it is not surprising that a *gshA* mutant (i.e., a strain completely defective in both γ GluCys and GSH and, hence, unable to grow in minimal medium) was not isolated from the screening performed.

Even though the role of GSH in Gln catabolism remains to be defined, its positive influence on the Gln uptake activity

Fig. 5. Symbiotic behavior of *Rhizobium etli* mutants. **A**, Germinated seedlings of *Phaseolus vulgaris* were inoculated with the indicated strains (left) and, 21 days postinoculation (dpi), the number, fresh weight, and acetylene reduction activity (ARA) of the nodules developed on the main roots were determined. Numbers are the means and standard deviations of data obtained from three experiments. In a single experiment, the main root of 60 plants was inoculated with each bacterial strain. **B**, Upper: photographs of nodules elicited by strain CTGUT43(*gshB*⁻) and CE3(WT). Central tissue (CT) and peripheral tissue (PT) are indicated. Lower: photographs of representative invaded cells (ICs). Starch granules (SG) located in the uninvaded cells are indicated (blue arrows). **C**, Leaves of *P. vulgaris* plants nodulated by *R. etli* mutants. Strains inoculated were CE3(WT), CFNX247(*nifA*⁻), CFNX249(*nifH1*⁻/*nifH2*⁻), or CTGUT43(*gshB*⁻), as indicated. The central one of the first triplet of leaves developed in each symbiotic pair was harvested (21 dpi) and photographed.

may be considered as a metabolic paradox. In fact, because the Gln degradation product Glu is a precursor of GSH synthesis, a negative feedback regulation (i.e., low GSH levels → high Gln uptake activity) could be expected. However, GSH is required for Glu transport in some eukaryotic cell lines (Agostinho et al. 1997). Moreover, in both yeast and some mammalian cell lines, a direct role of GSH in amino acid transport, through a complex cycling process named the γ -glutamyl cycle, has been demonstrated (Griffith et al. 1978). The presence in *R. etli* of a similar GSH-dependent cycle, essential for Gln uptake, remains to be established, even though it recently has been shown that, in *Helicobacter pylori*, the γ -glutamyl transpeptidase, a key enzyme in the γ -glutamyl cycle, is expressed and exhibited Gln hydrolysis activity (Shibayama et al. 2007).

Surprisingly, we observed that Gln significantly delayed the growth of *GshB*-knockout bacteria even when plated in the presence of alternative sources of C and N. Overall, our data suggest that i) Gln should be metabolized (transported and degraded) to interfere with bacterial growth and ii) GOGAT activity is required to avoid growth inhibition induced by Gln of GSH-deficient (*BSO*-treated) bacteria (Fig. 4). Certainly, exogenously adding Gln (or Glu) by inducing the *Aap/Gdh* pathway (for uptake or catabolism) may reduce the amount of Glu available to be funneled into the GSH synthesis pathway; this, in turn, should be compensated for by inducing GOGAT enzyme (Glu synthesis) (Fig. 2). In correlation, after shifting the cells into RMM-Gln, GOGAT activity was twofold higher in the *gshB* mutant than in its wild-type parent (*our unpublished results*). Therefore, in the presence of exogenous Gln and in the absence of GSH synthesis, a futile cycle (Glu synthesis and degradation operating simultaneously) may be generated which slows down the bacterial growth. It is worth noting that evidence for a fine regulated Gln cycle has been previously obtained in *Neurospora crassa* (Mora 1990), *S. meliloti*, and *R. etli* (Encarnacion et al. 1998), even if a putative role of GSH in its regulation was never hypothesized.

The symbiotic performance of the *gshB* mutant was altered at different levels of nodule formation, function, and maintenance (Fig. 5). A low number of nodules may be due to a reduced invasion capacity and using green fluorescent protein-labeled bacteria (Sartorius et al. 2009). We have observed that most of the initial invasion events (i.e., the development of infection threads) induced by the *gshB* mutant on the main root of inoculated plants undergo early abortion (*our unpublished observations*). Moreover, similar to the *R. tropici gshB* mutant (Ricciolo et al. 2000), the *R. etli gshB* mutant was unable to compete efficiently for nodulation with its wild-type parent. During plant invasion, *Rhizobium* bacteria should adapt their metabolism to the micro-environment generated by the host partner and, hence, GSH-deficient bacteria should be susceptible to these chemical and physical challenges. However, because *aapM* and *braD* mutants of *R. etli*, which are defective in Gln transport or utilization, are able to induce a normal number of nodules on the main root of *P. vulgaris*, the nodulation inefficiency of the *gshB* mutant should not be due to its inability to uptake or utilize Gln. On the other hand, the reduced nitrogenase activity displayed by the few nodules induced by the mutant may be dependent on a reduced functionality of the Gln transporters in the GSH-deficient bacteroids. In fact, *Aap* and *Bra* systems are key players of a mechanism of amino-acid cycling involving Gln or Glu and essential for an efficient symbiotic interaction (Lodwig et al. 2003). A putative key role of GSH in the regulation of this amino-acid cycling in bacteroids should be further investigated. Finally, as in *R. tropici* and *S. meliloti* (Harrison et al. 2005; Muglia et al. 2008), nodules elicited by the *gshB* mutant of *R. etli* on the main root of *P. vulgaris* exhibit clear-cut signs of early senescence. However, a similar phenotype was also ob-

served in ineffective nodules elicited by *Fix⁻* mutant strains of *R. etli* (Cermola et al. 2000). Thus, the lack of bacterial GSH production could adversely influence the nodule maintenance, directly or indirectly reducing the expression or the activity of the nitrogenase complex.

MATERIALS AND METHODS

Bacterial strains and growth media.

R. etli strains (Table 1) CE3 (wild-type), CTGUT3(*gdhB⁻*), CTGUT14(*aapM⁻*), CTGUT15(*gor⁻*), CTGUT27(*braD⁻*), CTNUX10(*gltD⁻*), CFNX247(*nifA⁻*), and CFNX249(*nifH1⁻/nifH2⁻*) described elsewhere (Brito et al. 1997; Ferraioli et al. 2002; Girard et al. 1996; Taté et al. 2004) were grown at 30°C with shaking in complete medium TYR or in a chemically defined RMM as described elsewhere (Taté et al. 1997). *E. coli* strain DH5 α was grown at 37°C with shaking in TY medium. All media were solidified with 1.5% agar (wt/vol) (Difco Laboratories, Detroit). Antibiotics were added to the media in the following final concentrations: tetracycline at 15 μ g/ml for *E. coli* and 5 μ g/ml for *R. etli*, ampicillin at 20 μ g/ml, nalidixic acid at 20 μ g/ml, and kanamycin at 50 μ g/ml for *R. etli*. Antibiotics and Glu-Glu dipeptide (G3640) were purchased from Sigma-Aldrich (St. Louis). To test the bacterial growth phenotype on RMM solid media, *R. etli* strains were grown in liquid TYR to exponential growth phase and harvested by centrifugation (2,500 $\times$ g for 10 min at 4°C). The bacterial pellet was washed twice with sterile RMM and resuspended at the required density in RMM without added C and N sources.

Tn5 mutagenesis.

Transposon mutagenesis of *R. etli* was carried out as previously described (Taté et al. 1997). For the ampicillin-mediated enrichment procedure, a suspension of approximately 10⁵ kanamycin-resistant transconjugants was used to inoculate RMM supplemented with Gln (5 mM) plus ampicillin (20 μ g/ml). Bacteria were incubated for 2 days at 30°C, with two changes of the medium every day, and then poured on TYR supplemented with kanamycin (50 μ g/ml). Auxotrophs were excluded by replica plating of the resulting kanamycin-resistant colonies (approximately 200) on minimal medium (RMM) containing Glc or Suc as C source and NH₄Cl as N source. Four mutant strains unable to grow with Gln but able to grow with Glc or Suc as sole source of C were isolated and characterized.

DNA manipulation.

To clone the Tn5-flanking DNA regions, digestion of total DNA (500 ng) was performed with *EcoRI* followed by ligation to *EcoRI*-digested pGEM7Zf+ (Promega Corp., Madison, WI, U.S.A.). After transformation of *E. coli* DH5 α , kanamycin-resistant colonies were isolated. In the case of strain CTGUT43, a DNA fragment (12 kb) carrying the Tn5- and genomic DNA-flanking regions was cloned (plasmid pAR363); plasmid DNA was purified and the nucleotide sequence was established using a standard Tn5 outward-reading primer 5'-GAAAGGTTCCG TTCAGGACGCTAC-3' and appropriate oligonucleotides. The *gshB* gene was PCR amplified using the following oligonucleotides: *gshBf* 5'-CGGAATTCGAAGCCTTTGGCAATCG CCTA-3' and *gshBr* 5'-GCTCTAGACCTTCTATGCCCTGGA ATG-3' as primers (restriction sites for *EcoRI* and *XbaI* are underlined) and total DNA, isolated from strain CE3, as a template. The amplified DNA fragment (1,323 bp) was digested with *EcoRI* and *XbaI*, and ligated with plasmid pMP220 (Spaink et al. 1987) digested with the same restriction enzymes. The resulting plasmid, pAR367, was used to transform strain CTGUT43 by electroporation as previously described (Taté et al. 1997).

Gln transport assays and CO₂ production from Gln.

An uptake assay was performed by a rapid-filtration method as described elsewhere (Taté et al. 1998). Briefly, bacteria were grown in TYR at 30°C under aeration up to an optical density at 590 nm (OD₅₉₀) of 0.6 and harvested by centrifugation (2,500 × g for 10 min at 4°C). The bacterial pellet was washed twice with sterile water and resuspended (OD₅₉₀ of 0.3) in either RMM-Gln (5 mM), RMM-Suc/NH₄Cl (5 mM each), or RMM-Glc/NH₄Cl (5 mM each); incubated (2 h at 30°C) under aeration; and harvested by centrifugation (2,500 × g for 10 min at 4°C). The bacterial pellets were washed twice with sterile water and suspended in RMM. Before filtration, Gln (30 μM final concentration) and L-[U-¹⁴C]Gln (50 μCi/ml, 244 μCi/mmol) (10 μM final concentration) was added.

Gln respiration was measured as previously described (Taté et al. 2004). Bacteria were grown as for Gln transport and re-suspended in 0.2 ml (OD₅₉₀ value of 3.0) of RMM-Gln (40 μM final concentration). After addition of 10 μl (2 nmoles) of a labeled L-[U-¹⁴C]Gln solution (50 μCi/ml, 244 μCi/mmol), the mixture was incubated (15 min, 30°C) and the reaction was stopped by addition of cold Gln (50 mM final concentration) plus 1 ml of 0.9 M HClO₄. The ¹⁴CO₂ liberated from this acid mixture was captured in a CO₂ trap (appended from the cap) containing 1 M benzethonium hydroxide (Sigma-Aldrich) in CH₃OH. After 3 h of incubation, the trap was removed and its contents were diluted in 50% scintillation mix (Ultima Gold; PerkinElmer, Foster, CA, U.S.A.) and counted (β-Counter, Beckman Coulter, Fullerton, CA, U.S.A.). The protein content of the bacterial suspension was determined with the Bio-Rad kit.

Glutamate dehydrogenase activity.

R. etli strains grown in TYR were harvested by centrifugation ($2,500 \times g$), washed, and resuspended in RMM-Gln (OD_{590} of 0.3). Cells were incubated (2 h at 30°C), harvested, resuspended in extraction buffer (20 mM HEPES pH 8.5, 100 mM NaCl, and 1 mM EDTA), and disrupted by sonic oscillation at 0°C . Cellular debris were removed by centrifugation ($27,000 \times g$ for 20 min at 4°C) and, with the resulting crude extracts used as a source of Gdh, the initial rate of glutamate-dependent NADH appearance was measured by monitoring the absorbance at 340 nm (initial rates of NADH formation) in 2 ml of a reaction mixture containing 100 mM HEPES buffer (pH 8.5), 50 mM L-glutamate, and 2.5 mM NAD. The rates were corrected for the production of NADH in the absence of added glutamate.

Nodulation test.

Seed of *P. vulgaris* 'Negro Jamapa' were surface sterilized with 5% H₂O₂ for 30 min, washed with sterile water, and germinated at 23°C for 72 h on a petri dish (1% water agar). The main root of germinated seed was inoculated with 100 µl of a bacterial suspension (10⁶ cells/ml) in 50 mM phosphate buffer (pH 7.0). Plants were cultured in a growth chamber maintained at 70 to 75% relative humidity at 23°C in light and 19°C in darkness, with a 16-h-daylight period, using an N-free medium (Taté et al. 1998). To test the nodule occupancy, nodules located in the primary root were surface sterilized (5% sodium hypochlorite for 1 min, 70% ethanol for 1 min, and two rinses with sterile water) and crushed in 96-multiwell plates (100 µl of RMM per well). Cell suspensions were then streaked (using toothpicks) on plates of TYR agar supplemented with nalidixic acid (20 µg/ml) or with nalidixic acid (20 µg/ml) plus kanamycin (50 µg/ml). Aliquots were also streaked on RMM-Gln and RMM-His. A nodule was considered invaded by strain CTGUT43(*gshB*⁻) when only Gln⁻, His⁺ kanamycin-resistant bacteria were isolated from that nodule. For the measurement

of ARA of nodulated roots, plants were harvested and the root system was immediately detached and incubated at 25°C in 15-ml rubber-cap tubes containing 1/10 (vol/vol) acetylene. The ethylene produced at different time points was quantified with a Sigma 3B gas chromatograph (Perkin-Elmer, Foster, CA, U.S.A.) equipped with a Porapak T column.

Optical microscopy.

For histological analysis, 21-day-old nodules were excised from the primary root with a razor blade and fixed in a 50 mM potassium phosphate buffer (pH 7.4) containing 4% paraformaldehyde, 3% glutaraldehyde, and 4% sucrose. To observe nodule tissues, samples were embedded in agarose (4% [wt/vol]) sections (70 μ m) were obtained using a Leica vibrating-blade microtome (model VT1000S; Leica, Nussloch, Germany) and observed with a Leica microscope (model MZ16FA; Leica Microsystems, Wetzlar, Germany). Digital images were captured with a video camera (Leica DFC320). To observe the invaded cells, nodule samples were rinsed, dehydrated, and embedded in Poly/Bed 812 resin (Polyscience, Warrington, PA, U.S.A.) as previously described (Taté et al. 2004). Semithin sections (2 μ m thick), obtained with a Leica microtome equipped with a diamond knife (Diatome, Biel, Switzerland), were stained with toluidine blue (0.04% in distilled water) and observed.

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