A cysG Mutant Strain of Rhizobium etli Pleiotropically Defective in Sulfate and Nitrate Assimilation

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The soil bacterium Rhizobium etli has the ability to induce the formation of nitrogen-fixing nodules on the root of the common bean (Phaseolus vulgaris). From the point of view of the bacterial partner, the development of these new plant organs is a multistage process involving bacterial multiplication in the rhizosphere, the recognition and infection of the root hairs, bacterial growth inside a network of infection threads, and the release of bacteria from the threads into the cytoplasm of host cells. Within the infected plant cells, the bacteria differentiate into nondividing bacteroids which are equivalent to plant cell organelles able to reduce atmospheric dinitrogen (28, 30).

In poor soils, where Rhizobium-legume symbiosis takes place, and prior to the onset of N₂ fixation, the host plant should provide nutrients (e.g., carbon, nitrogen, and sulfur sources) to support the growth of the developing nodules. It has recently been proposed (6) that bacterial nutrients are delivered by the plant only at the tip (and not at the base) of the infection threads, where bacterial growth was observed. It has also been speculated (19) that provision of nutrients from seed storage reserves may serve as a mechanism for regulating both the bacterial growth inside the infection threads and the bacteroid differentiation inside the invaded plant cells. However, the kind of nutrients used by Rhizobium bacteria (as carbon, nitrogen, or sulfur sources) during the early steps of the symbiotic interaction remain undefined. Furthermore, since both root metabolism and nodule metabolism are different for different legumes (19), the bacterial nutrient(s) might be specific for each symbiotic relationship.

Considering the compounds commonly used by bacteria as a sulfur source, inorganic sulfate is particularly interesting, since its assimilation represents the pathway by which inorganic sulfur is fixed into an organic linkage. Moreover, the process by which sulfate is reduced to sulfide is analogous to nitrate reduction and N₂ fixation (see reference 10 for a review). Finally, while many genetic and biochemical details of sulfate assimilation are well characterized in enteric bacteria (10), little is known about members of the family Rhizobiaceae.

It has previously been demonstrated that the nodP and nodQ gene products of Rhizobium meliloti catalyze the conversion of ATP and sulfate into 3′-phosphoadenosine 5′-phosphosulfate (PAPS), the first intermediate in cysteine biosynthesis (20). The nod gene products of Rhizobium were identified as being involved in the synthesis of Nod factors, which are morphogenetic signals able to elicit host-specific plant responses (30). The Nod factors (N-acylated oligosaccharides) may be sulfated by using PAPS as a precursor (11, 20), and this could explain the presence of genes involved in sulfate activation located in the nod gene cluster (20). However, an R. meliloti strain with a mutation or deletion of both copies of nodQ was found to be severely defective in symbiotic phenotypes but could grow with sulfate as the sole sulfur source (remain prototrophic). Thus, the existence in R. meliloti of structural genes for enzymes that generate PAPS (sulfate activation) other than nodP and nodQ was proposed (21).

As a starting point in the study of rhizobial sulfur metabolism, we report here on the isolation of a mutant strain of R. etli, CTNUX8, which failed to grow on chemically defined minimal medium containing sulfate as the sole sulfur source. Strain CTNUX8 contains a single Tn5 insertion in a gene showing a high degree of identity only with the cysG gene (siroheme synthetase) of other bacteria. Siroheme is a uroporphyrinogen III derivative used as a prosthetic group by sulfite and nitrite reductase (12). In Escherichia coli, siroheme synthetase is a multifunctional protein able to catalyze all of the reactions (two methylations, ring oxidation, and iron insertion) necessary to transform uroporphyrinogen III into siroheme (7, 25, 33). The cysG-homologous gene from R. etli was cloned and sequenced, and its sulfur-dependent transcriptional regulation was demonstrated. Despite its pleiotropic phenotype, strain CTNUX8 was able to induce efficient nitrogen-fixing nodules on roots of P. vulgaris. In contrast, the lack of an efficient sulfate assimilation pathway led to a decreased competitiveness of strain CTNUX8 in nodulation of P. vulgaris in the presence of its wild-type parent (strain CE3). Based on the evidence obtained, the nature of the sulfur sources used by...
Rhizobium to grow both in the rhizosphere and inside the invaded plant cells will be discussed.

MATERIALS AND METHODS

Growth conditions and bacterial strains. R. etli (formerly Rhizobium leguminosarum biovar phaseoli) (22) strains were grown at 30°C in TYR medium, which contained 5 g of tryptone, 3 g of yeast extract, and 0.88 g of CaCl_2 \cdot 2H_2O per liter. RMM was used as a chemically defined medium, and it contained 0.25 g of MgSO_4 \cdot 7H_2O, 0.1 g of CaCl_2 \cdot 2H_2O, 1 g of KH_2PO_4, and 1 g of KH_2PO_4 per liter and 10 mg of each of FeCl_3 \cdot 6H_2O and 20 mg of each of MnSO_4 \cdot H_2O, ZnSO_4 \cdot 7H_2O, CuSO_4 \cdot 5H_2O, CoCl_2 \cdot Na_2MoO_4, biotin, calcium pantothenate, and thiamine per liter. Escherichia coli strains were grown at 37°C in TYM medium containing 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter.

Antibiotics, unless otherwise indicated, were added to the medium as needed at the following concentrations: tetracycline, 5 \( \mu \)g/ml; nalidixic acid, 20 \( \mu \)g/ml; kanamycin, 50 \( \mu \)g/ml; rifampicin, 100 \( \mu \)g/ml; and ampicillin 100 \( \mu \)g/ml. All media were solidified with 1.5% agar (Difco).

To clone the Tn5 DNA flanking region from strain CTNUX8 of R. etli, a partial restriction map of the Tn5 DNA (boxed) is shown. The black bar indicates the DNA fragment used as a probe to clone the corresponding DNA region from the wild-type strain, CE3. The Tn5-flanking reading map is indicated by the arrowhead. (B) Physical map of R. etli strain cloned into pAR179 with a partial restriction map and sequencing strategy. The hatched area in pAR179 represents the translated sequence of cystG. The position of the Tn5 insertion in the R. etli mutant strain, CTNUX8, is indicated. B, BamHI; E, EcoRI; H, HindIII; P, PstI; S, SacI; X, XhoI.

**FIG. 1.** (A) Strategy to clone the Tn5 DNA flanking region from strain CTNUX8 of R. etli. A partial restriction map of the Tn5 DNA (hatched box) is shown. The black bar indicates the DNA fragment used as a probe to clone the corresponding DNA region from the wild-type strain, CE3. The Tn5-flanking reading map is indicated by the arrowhead. (B) Physical map of R. etli strain cloned into pAR179 with a partial restriction map and sequencing strategy. The hatched area in pAR179 represents the translated sequence of cystG. The position of the Tn5 insertion in the R. etli mutant strain, CTNUX8, is indicated. B, BamHI; E, EcoRI; H, HindIII; P, PstI; S, SacI; X, XhoI.
and 10^6 bacteria per plant) ratio and applied (spotted) to the root of 5-day-old P. vulgaris seedlings. Before inoculation bacteria were grown in TYR medium, centrifuged at low speed, and resuspended in sterilized water. Three weeks postinoculation, nodules located on the primary roots (5 nodules per plant, 40 plants) were surface sterilized (5% sodium hypochlorite treatment for 1 min, 70% ethanol for 1 min, and two rinses with sterile water) and crushed in 100 ml of RMM medium with multiple-well (a single nodule for each well) plates (Corning, Corning, N.Y.). Cell suspensions were then streaked (with a toothpick) on plates of TYR agar supplemented either with nalidixic acid (20 \( \mu \)g ml\(^{-1} \)) or with nalidixic acid plus kanamycin (50 \( \mu \)g ml\(^{-1} \)). Aliquots of cells were also streaked on defined sulfate medium with or without supplemental cysteine. A nodule was considered to be invaded by strain CTNUX8 when only cysteine-auxotrophic kanamycin-resistant bacteria were isolated from that nodule. Based on these criteria, the percent of nodules invaded by each strain was calculated.

Nucleotide sequence accession number. The sequence data in Fig. 2 will appear in the EMBL, GenBank, and DDBJ sequence data libraries under accession no. AJ001731.

RESULTS

Isolation of Cys\(^{-}\) strains of R. etli. Cysteine auxotrophy (Cys\(^{-}\) strains) was defined operationally as the inability to utilize sulfate as the sole sulfur source (10). We observed that the wild-type strain of R. etli, CE3, could grow on a chemically defined medium containing only inorganic sulfur sources (e.g., sulfate, sulfite, or thiosulfate), thus indicating that it is capable of synthesizing cysteine. To isolate Cys\(^{-}\) strains, the suicide plasmid pSUP1011 (24) was used to deliver Tn\(^{5}\) to the R. etli wild-type strain, CE3. A control experiment was performed with pSUP1 carrying the genetic markers of pSUP1011, except for Tn\(^{5}\) (24). Plasmid DNA was introduced into R. etli by electroporation, and kanamycin-resistant bacteria were observed.
tained at high frequency only with pSUP1011. To our knowledge, this is the first time that electroporation has been used to transform \textit{R. etli}. The efficiency of transformation was increased (at least fivefold) when a modified medium in which the NaCl (10 mM) had been replaced with CaCl\textsubscript{2} (18 mM) was used after electroporation (see Materials and Methods).

Mutants showing cysteine-dependent growth were identified by being screened simultaneously on plates of sulfate-containing minimal medium (RMM) with or without supplemental cysteine (1 mM). Two strains, CTNUX6 and CTNUX8, able to grow only in the presence of cysteine, were recovered from 1,500 Tn5 recipients. Both putative Cys\textsuperscript{−} strains were isolated and confirmed by their failure to grow on sulfate-containing RMM medium.

Cloning of the Tn5 flanking region from the CTNUX8 mutant. Probing of the total genomic DNA of strains CTNUX6 and CTNUX8 with a labelled Tn5 DNA fragment established that in both cases, the insertion was located on a single 7.5-kb \textit{EcoRI} fragment. Moreover, when the genomic DNAs were digested with \textit{BamHI}, two fragments with sizes of 7 and 9 kb hybridized (data not shown). Therefore, each strain carried only one Tn5, located in a very closely linked genomic site. Since both strains showed identical growth phenotypes, only one of them (strain CTNUX8) was chosen for further characterization.

By selecting transformants of \textit{E. coli} DH5\textalpha{} which were kanamycin resistant, the plasmid pAR176, carrying a portion of Tn5 (including the kanamycin resistance cassette) plus the adjacent genomic DNA, was isolated from strain CTNUX8 (Fig. 1A). The DNA sequence of the flanking DNA region, including that of the insertion point, was established with the Tn5out oligonucleotide. Sequence analysis revealed an open reading frame (amino acids [aa] 378 to 478 of the deduced peptide sequence shown in Fig. 2A) highly similar to those of numerous prokaryotic and eukaryotic \textit{S}-adenosyl-\textit{L}-methionine (SAM)-dependent methyltransferases. These proteins belong to a family that includes SUMT (SAM-dependent uroporphyrin III C-methyltransferase), CysG (siroheme synthetase), and CbiI/CobiI (SAM-dependent precorrin-2 methyltransferase), which are all involved in the biosynthesis of corrinoids, such as vitamin B\textsubscript{12}, siroheme, and coenzyme F430 (1, 16). Among the proteins with methyltransferase activity, only the CysG protein (encoded by \textit{cysG}) is involved in sulfate assimilation. The \textit{cysG} mutant (Cys\textsuperscript{−} auxotrophs) strains of enteric bacteria are unable to grow with sulfate as the sulfur source because of their failure to produce siroheme, the co-factor of the sulfite reductase (the \textit{cysH} gene products) (4, 12). It is thus likely that the mutant strain of \textit{R. etli}, CTNUX8, contains a \textit{cysG}:Tn5 insertion.

Cloning, sequence, and sequence analysis of the \textit{R. etli} \textit{cysG}-like gene. Southern hybridization of various restriction digests of DNA obtained from the wild-type strain of \textit{R. etli}, CE3, probed against a DNA fragment from pAR176 (Fig. 1A), yielded at least one strongly hybridizing band for each digest (data not shown). Of the hybridizing bands, one (an about 10-kb \textit{BamHI} fragment) was chosen for construction of an enrichment library (see Materials and Methods). The vector used was pTR101, a plasmid that specifies tetracycline resistance and that was found to be very stable during vegetative and in planta growth of \textit{R. melloti} (31). Before its utilization, experiments were performed to demonstrate that pTR101 is stable in \textit{R. etli} as well as in \textit{R. melloti} (data not shown). This characteristic was essential in our choice of this plasmid, since bacteria used for nodulation tests should be grown under non-selective conditions.

After screening of the library with the same probe (350-bp XhoI-\textit{EcoRI} fragment obtained from pAR176), a plasmid called pAR179 carrying a 10.5-kb \textit{BamHI} DNA fragment was isolated, and a restriction map was obtained (Fig. 1B). By Southern hybridization analysis of various restriction digests of pAR179, two overlapping DNA fragments were identified, cloned (pAR180 and pAR183 [Fig. 1B]), and sequenced (Fig. 2A). A sequence analysis revealed one major open reading frame of 1,434 bp showing an ATG start codon at position 39 of the nucleotide sequence and a TGA stop codon at position 1473 (Fig. 2A). The predicted protein, with a size of 478 aa, shares 38.1% identical residues (70.7% similar over a 448-aa overlap) with the product of the \textit{E. coli} \textit{cysG} gene (accession no. P11098) (Fig. 2B), 37.9% identical residues (452-aa overlap) with the product of the \textit{Salmonella typhimurium} \textit{cysG} gene (accession no. P25924), and 38.9% identical residues (452-aa overlap) with the product of the \textit{Neisseria meningitidis} \textit{cysG} gene (accession no. Y10177). Thus, it was assumed that a \textit{cysG}-homologous gene is present in \textit{R. etli}. The length of the \textit{R. etli} \textit{cysG} gene (1,434 bp, 478 aa) is similar to those of \textit{E. coli} (1,371 bp, 457 aa) and \textit{N. meningitidis} (1,425 bp, 475 aa), and the coding region was preceded by a putative ribosomal binding site (Fig. 2A). The most conserved regions with respect to other reported CysG proteins were those corresponding to the consensus patterns (SUMT\textsubscript{1} and SUMT\textsubscript{2}), which are conserved in all known SUMT (uroporphyrin III C-methyltransferase)-related proteins (Fig. 2A). In contrast, the N-terminal region (the first 200 aa), shows similarity to the corresponding regions of CysG proteins and to the product of the \textit{MET8} gene of \textit{Saccharomyces cerevisiae}, but not to other methyltransferases. Unlike the situation observed in enteric bacteria, in which the three enzymatic steps (methylation, oxidation, and metal insertion) that transform uroporphyrinogen III into siroheme are catalyzed by a single multifunctional protein, CysG, siroheme biosynthesis in \textit{S. cerevisiae} requires the products of both the \textit{MET1} and \textit{MET8} genes. It has recently been suggested that while \textit{MET1} encodes uroporphyrinogen III methyltransferase, \textit{MET8} encodes a protein with both ring oxidation and chelation activities necessary for siroheme formation (8). Therefore, in strain CTNUX8, the gene interrupted by Tn5 probably encodes a multifunctional siroheme synthetase, the \textit{cysG} gene product, rather than a protein with only SUMT activity.

The sulfur sources used by strain CTNUX8. To test the ability of \textit{R. etli} strains to grow on various sulfur sources, a modified chemically defined RMM medium was used (see Materials and Methods). It was observed that strain CTNUX8 could grow as well as the parent (CE3) strain when tested on defined medium containing an organic sulfur source, such as cysteine, glutathione, or methionine. The mutant strain could also grow in thiosulfate-containing medium, albeit only at higher concentrations and at lower rates than the parent strain, CE3 (Fig. 3). Moreover, unlike the parent strain, the CTNUX8 mutant could not grow on defined medium containing either sulfate or sulfite as the sole sulfur source (Fig. 3), thus suggesting that it is unable to reduce sulfite. To test this possibility, bacteria were grown in TYR rich medium, harvested by low-speed centrifugation, washed, and resuspended in sulfate-containing minimal medium. Cells were maintained for 1 h with shaking at 30°C, harvested by centrifugation, and disrupted by sonic oscillation. With the resulting crude extracts used as a source of sulfite reductase, the initial rate of sulfite-dependent NADPH oxidation was measured spectrophotometrically at 340 nm as previously described (23). The rates were corrected for oxidation of NADPH in the absence of sulfite. Considering the NADPH-sulfite reductase activity measured with the wild-type strain (0.12 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\)) as 100%, the activity of...
the CTNUX8 mutant was less than 2% (0.002 μmol min\(^{-1}\) mg\(^{-1}\)).

To test the ability of the wild-type locus to complement the growth phenotype, either pTR101 or pAR179 was transformed into R. etli CTNUX8 by electroporation. Transformants bearing pAR179 (CTNUX8-pAR179) could grow in the absence of cysteine, whereas those bearing pTR101 (CTNUX8-pTR101) could not. Strain CTNUX8-pAR179, but not CTNUX8-pTR101, could grow with sulfite as the sole sulfur source. Therefore, the DNA fragment carried by pAR179 contains the information necessary to complement the Tn5 mutation.

The level of \(cysG\) mRNA depends on the sulfur source. RNase protection analysis (Fig. 4) was carried out to determine the level of \(cysG\) mRNA when \(R. etli\) was grown under various conditions. RNA purified from the wild-type CE3 strain grown on sulfate, thiosulfate, cysteine, or methionine as the sole sulfur source was hybridized to an excess of \(^{32}\)P-labelled antisense RNA (riboprobe) generated from clone pAR182 (Fig. 1B) and digested with RNase A and RNase T1. Quantitative analysis of the protected bands indicated that the intracellular level of \(cysG\) mRNA was higher (threefold) when the wild-type strain was grown on sulfate than when it was grown on thiosulfate or cysteine. The level of \(cysG\) mRNA was even higher (10-fold) in bacteria grown with methionine. Hybridization of the same preparations of RNA against total genomic DNA of \(R. etli\) yielded comparable intensities for all RNA samples (Fig. 4, bottom), indicating that the observed differences in the \(cysG\) transcript levels were not due to variations in the amount of RNA used. These data suggest that methionine and sulfate may be, directly or indirectly, inducers of \(cysG\) transcription.

The CTNUX8 mutant has a Nir\(^{−}\) phenotype. It was previously established that in enteric bacteria, the siroheme cofactor (the product of the CysG activity) is also required for nitrite reductase activity. As a consequence, the \(cysG\) mutants could not grow with nitrate as the sole nitrogen source (Nir\(^{−}\)) (2). To test the ability of the CTNUX8 strain of \(R. etli\) to grow in minimal medium with various nitrogen sources, a modified RMM medium with thiosulfate substituted for sulfate was prepared. Thiosulfate was used as the sulfur source, since glutathione, methionine, or cysteine could also be used as nitrogen sources by \(R. etli\). The mutant strain CTNUX8 was able to grow on ammonia-containing minimal medium (Fig. 5A). In contrast, it was observed that unlike strains CE3 and CTNUX8-pAR179, strain CTNUX8 could not grow on nitrate-containing minimal medium, probably because it is unable to reduce nitrite (Fig. 5B). To test this possibility, the nitrite reductase activities of various strains of \(R. etli\) were measured (Fig. 5C). Bacteria were grown in TYR rich medium, harvested by low-speed centrifugation, washed, and resuspended in nitrate-containing minimal medium. Cells were maintained for 3 h with shaking at 30°C. The reaction was colorimetrically monitored by nitrite disappearance with sodium dithionite as a reductant and methyl viologen as an artificial electron carrier (29). Considering the activity measured with the wild-type strain (33 nmol min\(^{-1}\) mg\(^{-1}\)) as 100%, the activity of the mutant CTNUX8 was less than 5% (1 nmol min\(^{-1}\) mg\(^{-1}\)), and it was about 70% (23 nmol min\(^{-1}\) mg\(^{-1}\)) when the transformed strain CTNUX8-pAR179 was assayed. Essentially the same values

\[\text{RNA samples (Fig. 4, bottom), indicating that the observed differences in the cysG transcript levels were not due to variations in the amount of RNA used. These data suggest that methionine and sulfate may be, directly or indirectly, inducers of cysG transcription.}\]

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were obtained when the nitrite reductase activity was measured 8 h after the induction (data not shown). These experiments showed that the mutant strain could not induce nitrite reductase activity and that the DNA fragment carried by pAR179 could rescue the mutant’s ability to reduce nitrite and its capacity to grow with nitrate as the sole nitrogen source.

**The CTNUX8 mutant has a Nod⁺ Fix⁺ phenotype.** To test the capacity of the cysG::Tn5 (Cys⁻ Nit⁻) mutant of *R. etli* to nodulate *P. vulgaris*, roots of germinated seeds were inoculated either with strain CTNUX8 or with the wild-type strain, CE3, used as a control. In both cases, the localization, shape, and histological organization of nodules were comparable. In contrast, the total number of nodules induced by the mutant strain, CTNUX8, was lower (about 50%) relative to the number induced by the reference strain, but only when a low number of bacteria (10⁵ to 10⁸ bacteria per root) were inoculated.

In the case of the mutant strain, the original inoculum of 10⁵ bacteria/plant was unlikely to have been contaminated with prototrophic revertants, because prototrophic derivatives arose spontaneously at a frequency of about 10⁻³ and were kanamycin sensitive (data not shown). To test this possibility, bacteria were reisolated from nodules (five nodules per plant, five plants) induced by strain CTNUX8 and tested for the auxotrophic marker. The nodules were harvested, surface sterilized by sodium hypochlorite treatment, and crushed once at a time (see Materials and Methods). The bacteria were then diluted and plated on TYR medium with addition of either nalidixic acid or nalidixic acid plus kanamycin to determine the number of CFU present. The same numbers of colonies were observed in both types of plates. Moreover, aliquots of cells were also streaked on plates of defined sulfate medium (RMM) with or without supplemental cysteine. After 3 days of incubation, colonies could be observed only on cysteine-containing plates. The absence of nodulation in un inoculated controls and the presence of Cys⁺ kanamycin-resistant bacteria in all nodules tested indicated that nodules were formed by auxotrophic bacteria and not by prototrophic revertants. In conclusion, strain CTNUX8 has a nodulating phenotype (Nod⁺).

To study the nitrogen fixation capacity of the nodulated roots, the acetylene reduction activity was measured. The acetylene reduction activities of 21-day-old nodules induced by strains CE3 and CTNUX8 were almost the same (between 15 and 16 μmol of ethylene h⁻¹ g of fresh nodule⁻¹). Furthermore, the acetylene reduction activity was not different even when 4- and 5-week-old nodules were assayed. These results demonstrated that the cysG::Tn5 mutant strain has a fixing phenotype (Fix⁺). In conclusion, the mutant strain, CTNUX8, of *R. etli* was able to establish an efficient symbiotic interaction (Nod⁺ Fix⁺) with common beans.

**Competitiveness of the CTNUX8 mutant.** The relative ability of a given bacterial strain to infect its host plant and cause development of nodules in a multistrain environment has been termed “competitiveness.” Many studies have demonstrated that inactivation of a single bacterial gene can result in a detectable phenotypic change in competitiveness (27). To determine the effect of the cysG inactivation on the nodulation competitiveness of *R. etli*, a plant food minimal medium (9) containing sulfate as the sole sulfur source was used to test mixed inoculation. The competitiveness of strain CTNUX8 was studied by analysis of nodule occupancy following coinoculation with the parent strain (see Materials and Methods). In competition experiments performed with mixtures of strain CTNUX8 (cysG mutant) and CE3 (wild type) in a 1:1 ratio applied to the roots of 40 seedlings of common beans, the mutant strain infected about 20% of the nodules (200 nodules were tested). This experiment was repeated three times with essentially the same result: the mutant was reisolated from only 16 to 27% of total nodules. Moreover, the percentage of nodules invaded by the mutant strain was higher (52 to 65% of the total) when a mixture in a 10:1 CTNUX8/CE3 ratio was tested. Therefore, inactivation of cysG gene led to a decreased (at least fivefold) competition ability of mutant CTNUX8 to nodulate roots of *P. vulgaris* in the presence of its wild-type parent. Finally, the number of nodules invaded and the competition ability of strain CTNUX8 transformed with pAR179, but not with the vector pTR101 alone, were nearly the same as those of the wild-type strain. These experiments indicate that the DNA fragment carried by pAR179 was able to rescue the loss of competitiveness of the mutant strain, probably allowing its growth in the rhizosphere. In fact, in competition experiments
performed with mixtures of CTNUX8 and CE3 in a 1:1 ratio, but after addition of either methionine or glutathione (the nutritional requirement of the auxotrophic mutant) to the inoculation buffer, the mutant strain was able to generate 39 to 51% of the total nodules.

**DISCUSSION**

A Tn5 insertion mutant, strain CTNUX8 of *R. etli*, was isolated and characterized. The mutation in this strain was concluded to be due to a single Tn5:cysG insertion in its chromosome, based on the following evidence. (i) When DNA prepared from this strain was digested with *Eco*RI (which does not cut Tn5), electrophoresed, and hybridized with a nick-translated probe containing Tn5, it was found that only one 7.5-kb DNA fragment hybridized. (ii) Sequence analysis of the Tn5 flanking region (and of the corresponding DNA region cloned from the wild-type strain) showed a significant degree of identity only with the sequence of the cysG (siroheme synthetase) gene of bacteria. (iii) A plasmid (pAR179) carrying the corresponding DNA region from the wild-type strain was shown to rescue prototrophic growth. (iv) Southern analysis of a mutant lysate showed no Tn5 insertions in plasmid DNA. (v) The resulting pleiotropic phenotype (Cys−Nir−) of this strain is the same as that observed in a cysG mutant strain of enteric bacteria.

The mutant strain CTNUX8 was unable to use sulfate or sulfite as an inorganic sulfur source, thus indicating that under the growth conditions tested, the sulfite reductase involved in the sulfur assimilation pathway of *R. etli* depends on cysG and most probably requires siroheme as a cofactor. It is important to note that sulfite added to the medium at a concentration higher than 50 μM inhibited the growth of *R. etli*, likely due to a toxic effect (Fig. 3). Therefore, on a sulfate-containing medium, the growth of a mutant strain lacking sulfite reductase, such as CTNUX8, should not be possible as a consequence of sulfite accumulation. In contrast, the CTNUX8 mutant strain could grow in a medium containing both cysteine and sulfate, thus suggesting the presence of *R. etli* of a regulatory system that reduces or abolishes the expression of genes involved in the reduction of sulfate to sulfite when cysteine is present. In fact, RNase protection assays showed that the intracellular level of cysG transcript depended on the sulfur source used by *R. etli* to grow (Fig. 4). High levels of cysG mRNA were observed in cells grown with methionine or sulfate, while low levels were observed when cysteine was used. The methionine-mediated induction of cysG expression is probably not simply the consequence of sulfur limitation, since when tested, methionine did not appear to be a growth-limiting sulfur source (data not shown). Presumably, cysteine or a metabolite thereof represses the cysG transcription, whereas methionine or SAM acts as an inducer of cysG transcription in *R. etli*.

Whether cysG expression is regulated in enteric bacteria during aerobic growth has not been well established, but in *S. typhimurium*, cysG is not part of the cysteine regulon and has a basal level of expression (7, 33). The cys regulon consists of those genes (at least 16) required for cysteine biosynthesis (including sulfite reductase) that are coordinately repressed in cysteine-grown cells, but derepressed in cysteine-grown cells (3, 26). Differences between enteric bacteria and *R. etli* in the genomic organization and the transcriptional regulatory circuits have also been observed in genes involved in nitrogen metabolism (15).

To obtain more information about sulfur metabolism in *R. etli*, strains CTNUX8 (cysG mutant) and CE3 (wild type) were tested for their ability to grow on various sources (organic or inorganic) of sulfur. It was previously established that in *S. typhimurium*, the O-acetylserine sulfhydrylase B can use thiolsulfate in place of sulfide to give cysteine thiosulfonate, which is then converted to cysteine (14). Moreover, this pathway of cysteine biosynthesis, acting in thiosulfate-grown cells, did not require siroheme cofactor. Since strain CTNUX8 of *R. etli* was able to grow by using thiolsulfate as the sole sulfur source (although less well than the wild-type strain [Fig. 3]), it is likely that *R. etli* contains a pathway of thiolsulfate assimilation independent of the product of the cysG gene (likely siroheme independent). On the other hand, enteric bacteria cannot use methionine as the sole sulfur source, because there is no efficient pathway from methionine to cysteine (10). Because the cysG mutant (CTNUX8) and its parent strain (CE3) were able to use methionine as the sole sulfur source, we inferred the existence in *R. etli*, as in fungi and plants (10), of an efficient pathway from methionine to cysteine. The same pathway seems to exist in other members of the family *Rhizobiales*, since a mutant strain of *R. meliloti* unable to grow with sulfate as the sulfur source could grow as well as the wild-type strain on either methionine or cysteine (21).

In *E. coli*, the cysG gene product is also required for the activity of the nitrate reductase complex (2, 10). Strain CTNUX8 was able to grow on ammonia-containing medium, but was unable to grow on nitrate-containing minimal medium and was unable to induce a high level of nitrite reductase activity (Fig. 5). Actually, the mutant strain showed less than 5% of the nitrite reductase activity measured in the wild-type strain. Therefore, the principal nitrate assimilatory pathway of *R. etli*, when growing under aerobic conditions, includes a cysG-dependent (siroheme-dependent) nitrite reductase complex.

The symbiotic behavior of the cysG mutant strain was studied. The CTNUX8 strain of *R. etli* was able to induce (Nod+) and maintain efficient fixing nodules (Fix+) on the root of *P. vulgaris*, and only Cys- mutants (cysteine-requiring auxotrophs) were recovered from the nodules. Since under freeliving conditions, a cysG-dependent (siroheme-dependent) sulfite reductase is essential to allow the growth of *R. etli* with sulfate (or sulfite) as the sulfur source, it is reasonable to speculate that the root cells are able to provide the symbiotic bacteria with a sufficient amount of a sulfur source other than sulfate or sulfite (such as cysteine, methionine, or glutathione), allowing bacterial growth inside the infection thread. It has previously been found that the tripeptide thiol glutathione (γ-Glu–Cys–Gly) is the major low-molecular-weight thiol compound of the plant cell (18). Since glutathione can be exported and degraded, we speculate that during the first steps of *Rhizobium* invasion, the synthesis of glutathione in the cytoplasm of the invaded plant cells may be induced as part of their initial stress response. Thus, bacteria growing inside the plant may use glutathione or a product of its degradation as a sulfur source. The isolation of a mutant strain of *R. etli* unable to use glutathione as a sulfur source will be useful to clarify this hypothesis.

Finally, the CTNUX8 strain of *R. etli* is poorly competitive in nodulation with respect to its parent strain, CE3. It has recently been shown (5) that the CysG protein of *S. typhimurium* also catalyzes the first step of the biosynthesis of cobalamin (vitamin B12). If this is also the case in *R. etli*, a vitamin B12 defect in strain CTNUX8 could explain its lower competitiveness. However, the competitiveness of strain CTNUX8 could be restored almost completely, not only by transformation of the mutant with the complementing pAR179, but also by ad-
dation of an organic sulfur source. This result indicates that for a successful root colonization, R. etli requires cysteine or methionine, which apparently are not present in root exudates, but not the activity of the CysG protein per se. In other words, the cysG gene product is essential to allow the growth of R. etli in a sulfate-containing root environment.

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