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Isolation, Characterization, and Complementation of *Rhizobium meliloti* 104A14 Mutants That Lack Glutamine Synthetase II Activity

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The glutamine synthetase (GS)-glutamate synthase pathway is the primary route used by members of the family *Rhizobiaceae* to assimilate ammonia. Two forms of glutamine synthetase, GSI and GSII, are found in *Rhizobium* and *Bradyrhizobium* species. These are encoded by the *glnA* and *glnII* genes, respectively. Starting with a *Rhizobium meliloti glnA* mutant as the parent strain, we isolated mutants unable to grow on minimal medium with ammonia as the sole nitrogen source. For two auxotrophs that lacked any detectable GS activity, *R. meliloti* DNA of the mutated region was cloned and partially characterized. Lack of cross-hybridization indicated that the cloned regions were not closely linked to each other or to *glnA*; they therefore contain two independent genes needed for GSII synthesis or activity. One of the cloned regions was identified as *glnII*. An *R. meliloti glnII* mutant and an *R. meliloti glnA glnII* double mutant were constructed. Both formed effective nodules on alfalfa. This is unlike the *B. japonicum*-soybean symbiosis, in which at least one of these GS enzymes must be present for nitrogen-fixing nodules to develop. However, the *R. meliloti* double mutant was not a strict glutamine auxotroph, since it could grow on media that contained glutamate and ammonia, an observation that suggests that a third GS may be active in this species.

The infection of *Medicago sativa* (alfalfa) by its endosymbiont, *Rhizobium meliloti*, initiates a complex developmental sequence that leads to the formation of root nodules in which the bacteria can reduce atmospheric dinitrogen to ammonia. During this development, bacterial nitrogen metabolism switches from nitrogen acquisition behavior appropriate for a free-living bacterium to a symbiotic behavior in which fixed nitrogen is released to the plant (2, 5, 10). A critical enzyme in bacterial nitrogen metabolism is glutamine synthetase (GS) (1, 4, 10, 28, 30, 41). GS makes the glutamine used in biosynthesis of other nitrogen-containing molecules and is the first enzyme in the GS-GOGAT (glutamate synthase) pathway used for assimilating ammonia. Most rhizobia possess two distinct GS enzymes (10, 11), as do *Agrobacterium tumefaciens* (17, 38) and *Frankia* sp. strain CpI1 (13), both of which are intimately associated with plants. Bacteria with these two types of GS enzymes appear to be uncommon, and it has been suggested that the second enzyme is important in the metabolic changes needed for a successful association with plants (10, 11).

Rhizobial GSI is similar to the GS of enteric bacteria: it is a dodecameric protein with a subunit molecular mass of ca. 58 kilodaltons and is regulated posttranslationally by an adenylylation-deadenylylation cascade (10, 18, 30, 31). GSII is a heat-sensitive, octameric protein with a subunit size of ca. 36 kilodaltons (10) and is related to the GS enzymes found in eucaryotes (6; R. G. Shatters and M. L. Kahn, J. Mol. Evol., in press). The kinetics and substrate affinities of GSI and GSII are similar (10, 16). The gene that encodes GSI, *glnA*, has been isolated from several members of the family *Rhizobiaceae* (7, 14, 15, 39, 41), but isolation of the GSII structural gene, *glnII*, has been confirmed only for *Bradyrhizobium japonicum* (6) and *A. tumefaciens* (38). *B. japonicum* strains with mutations in either *glnA* or *glnII* are effective in symbiosis (Fix^+) (8). A strain with mutations in both genes cannot form nodules unless glutamine is added to the plant medium; these nodules are Fix^- (8). Other mutants lacking one or both GS activities have been isolated (25, 30), but the mutations leading to the GS^- phenotype have not been precisely defined.

To establish the role of the GS enzymes in *Rhizobium* nitrogen metabolism, we have isolated mutants with defects in genes necessary for *glnII* expression and/or GSII function. We have been able to distinguish three genetic loci that are involved in the production of GSII, and we show here that one of these regions contains *glnII*. Another contains *ntrA* (40a), and the function of the third has not been determined. Using the cloned *R. meliloti glnII* gene, we have constructed mutants that lack GSI or GSII or both enzymes. We have used the mutants to investigate the role of these enzymes in nitrogen metabolism by free-living bacteria and to determine whether they are essential for symbiotic nitrogen fixation. A preliminary account of this work has been presented (23).

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains, bacteriophages, and plasmids not constructed for this work are listed in Table 1. Plasmid pBJ196A (8) was provided by Todd Carlson and Barry Chelm, and pJK11 was provided by Jennifer Kraus. Media for the growth of *Escherichia coli* and *R. meliloti* have been described previously (41), with the exception of the rich medium 3×YMB+gln, which is yeast mannitol broth (YMB) medium (41) supplemented with 3 g of yeast extract per liter and 2 g of glutamine per liter. The methods used to evaluate nodulation, nitrogen fixation, and

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TABLE 1. Genetic materials

Strain or plasmid	Relevant properties	Reference or source
E. coli		
ET8051	ET8000 Δ(*glnA-rha*)	33
MV12*thy*	*thyA deo* derivative of MV12	19
R. meliloti		
104A14	Wild type	41
GLN210	104A14 *glnA210*	41
WSU100	Nitrous acid Gln$^-$ mutant of GLN210	This work
WSU300	Nitrous acid Gln$^-$ mutant of GLN210	This work
WSU414	Tn*5* Gln$^-$ mutant of GLN210	This work
WSU515	Tn*5* Gln$^-$ mutant of GLN210	This work
WSU650	*glnII* mutant of 104A14	This work
WSU660	*glnA glnII* mutant of 104A14	This work
Plasmids		
pBJ196A	*B. japonicum glnII* in pBR322	8
pBR322	Tet^r Amp^r	3
pGS9	Tn*5* Tra$^+$ (N type)	40
pJK11	*lacZYA* Tet^r Amp^r	J. Kraus
pMC1513	*lacZYA* Amp^r	9
pMK318	Kan^r Tet^r P4 *cos* IncP1	41
pPH1JI	Gen^r Str^r IncP1	41
pRZ102	ColE1::Tn*5*	20

reversion have been described previously (26, 41). *Rhizobium* media were supplemented with kanamycin sulfate at 200 μg/ml, gentamicin sulfate at 25 μg/ml, streptomycin sulfate at 150 μg/ml, and tetracycline hydrochloride at 10 μg/ml, except when otherwise indicated.

General and recombinant genetic methods. The construction of a bacteriophage P4 cosmid library by inserting *R. meliloti* 104A14 DNA partially digested with *Hin*dIII into the *Hin*dIII site of pMK318 has been described previously (41), as has the use of this bank in rescuing auxotrophic mutants (26). DNA manipulations have also been described (21, 27, 41). Transposon Tn*5* mutagenesis of a recombinant plasmid was performed by the first method of Kerppola and Kahn (27).

Mutagenesis of *R. meliloti*. Nitrous acid mutagenesis of *R. meliloti* was performed as described earlier (26), except that the medium used for mutant isolation was supplemented with 2 g of sodium L-glutamine per liter and 2 g of NH_4Cl per liter instead of $(NH_4)_2SO_4$ and penicillin was not used to enrich for auxotrophs. Colonies from cultures with survival rates between 0.1 and 0.01% were screened for glutamine auxotrophy by replica picking to minimal mannitol medium (MM). The suicide vector pGS9 (40) was used for transposon Tn*5* mutagenesis of *R. meliloti*. Mid-log-phase cultures of *E. coli* MV12*thy*(pGS9) and GLN210 were mixed, allowed to mate on nitrocellulose filters that had been placed on YMB medium, and then spread on MM supplemented with 2 g of sodium L-glutamine per liter, 1 g of sodium L-glutamate per liter, and 200 μg of L-arginine, kanamycin sulfate, and streptomycin sulfate per ml. After 4 to 5 days, presumptive Tn*5* mutants were replica picked to screen for glutamine auxotrophs. Some isolates that grew well on glutamine-supplemented YMB plate medium did not grow well in YMB broth culture, even when glutamine was added at 2 g/liter. We therefore used spread plates to grow many of the auxotrophs for DNA isolation and transduction and for GS assays.

Isolation of marker exchange mutants. *R. meliloti* 104A14 *glnII* deletion-insertion mutants were constructed by marker exchange mutagenesis as described previously (41). *R. meliloti* 104A14(pPH1JI)(pRS18) was constructed by infecting *R. meliloti* 104A14(pPH1JI) with a P2 *vir*1 lysate that contained pRS18. This strain was restreaked twice on YMB containing gentamicin sulfate, streptomycin sulfate, and kanamycin sulfate and then tested for the Tet^r trait carried on pRS18. Tet^s colonies were presumed to have lost pRS18 but to have transferred Kan^r to the chromosome by recombination. Recombination was confirmed by Southern blotting the chromosomal DNA; the strains were also tested for heat-labile GS activity. The same procedure was used to construct a *glnII* mutation in *R. meliloti* GLN210, except that 3×YMB+gln medium was used instead of YMB. The genomic Tet^r gene in GLN210 confers resistance to 2 μg of tetracycline hydrochloride per ml and did not interfere with the use of tetracycline hydrochloride at 10 μg/ml to screen for the loss of pRS18.

Determination of GS activity. GS activity was determined by measuring the production of γ-glutamylhydroxamate (41). To test the glutamine auxotrophs for GS activity, cells were first grown in 2 g of sodium L-glutamine per liter and 2 g of NH_4Cl per liter and then grown for 4 h in medium containing 1 g of sodium L-glutamate per liter as the sole nitrogen source to induce GS expression. The cells were washed with 1% KCl and then ruptured by three cycles of freezing and thawing in the presence of 0.5 μg of DNase I per ml. When appropriate, cell debris were removed by centrifugation and samples of the crude extract were layered onto 5 to 20% sucrose gradients. GSI and GSII activities were separated by centrifugation (10). We confirmed the slower-migrating peak to be GSII by heating the fractions at 50°C for 45 min prior to the assay.

RESULTS

Isolation of *R. meliloti* glutamine auxotrophs. *R. meliloti* GSI and GSII are encoded by the *glnA* and *glnII* genes, respectively. Our objective was to isolate mutants with defects in genes necessary for *glnII* expression or GSII function. Since it seemed unlikely that a mutation in *glnII* or in regulatory genes needed for *glnII* expression would lead to glutamine auxotrophy if GSI was active, we searched for glutamine auxotrophs by using the previously described *R. meliloti* 104A14 *glnA* mutant, GLN210 (41), as the parental strain.

Cultures of GLN210 were treated with nitrous acid. Glutamine auxotrophs were identified by their ability to grow on minimal media supplemented with 2 g of NH_4Cl per liter and 2 g of L-glutamine per liter and their inability to grow on the same media without glutamine. Two relatively stable glutamine auxotrophs, WSU100 and WSU300, were found in 4,050 colonies screened. Glutamine auxotrophs created by the insertion of transposon Tn*5* were isolated by conjugating the suicide vector pGS9 (40) into GLN210. Kan^r exconjugants were screened as above; 17 glutamine auxotrophs were isolated from among 5,800 screened colonies. Two mutants, WSU414 and WSU515, were chosen for further study. WSU414 and WSU515 reverted to prototrophy at frequencies of 10^{-8} and 10^{-4}, respectively. Reversion to prototrophy occurred primarily by pseudoreversion, as indicated by the conservation of the original Tn*5* insertion in most of the WSU414 and all of the WSU515 revertants tested (data not shown). No GS transferase activity could be detected in WSU100, WSU300, WSU414, or WSU515.

Complementation of glutamine auxotrophs. WSU100, WSU300, and WSU414 were infected with a pMK318-derived cosmid library of *R. meliloti* 104A14 (41). Cosmids

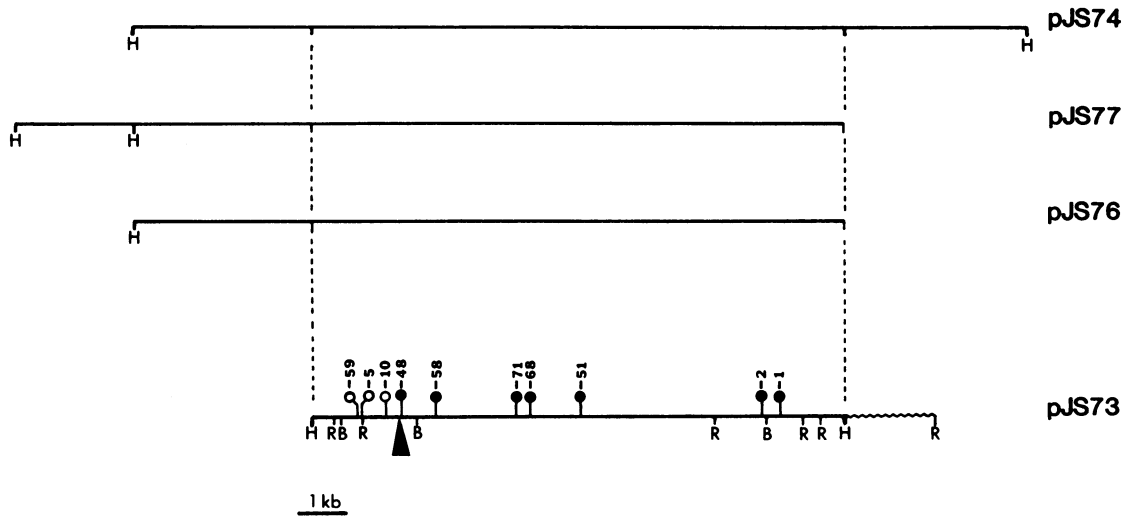

FIG. 1. Restriction map of plasmids that complement WSU100 and derivatives of these plasmids. Symbols: ~, DNA not from *R. meliloti*; ○, transposons that abolish complementation; ●, transposons that do not affect complementation; ▲, site of the ca. 300-bp deletion of pJS73::Tn*5*-48 that gives pRS8. Restriction enzyme abbreviations: B, *Bgl*II; H, *Hin*dIII; R, *Eco*RI.

that complemented the mutations in these strains were isolated by selecting Tet^r colonies able to grow on MM with 2 g of NH_4Cl per liter. All of the plasmids from these Tet^r colonies conferred Kan^s when they were transferred to *E. coli*, as would be expected if they contained DNA insertions in the *Hin*dIII site of pMK318. Each set of plasmids was characterized by using agarose gel electrophoresis. Recombinant plasmids that complemented WSU100 could be divided into six related groups. Four of these, pJS73, pJS74, pJS76, and pJS77, contained a common 10.2-kilobase-pair (kb) *Hin*dIII fragment which had not undergone any gross rearrangement during isolation (Fig. 1). pJS73 contained only this 10.2-kb *Hin*dIII fragment.

Plasmids that complemented WSU300 and WSU414, such as pJS86, pJS87, pJS88, and pJS96, contained a common 6.6-kb *Hin*dIII fragment (Fig. 2). pJS90, a deletion derivative of pJS87, contained only this 6.6-kb fragment and was able to complement both WSU300 and WSU414.

To further characterize the region mutated in WSU414, we cloned the Tn*5* insertion and flanking DNA directly by digesting DNA from WSU414 with *Eco*RI and ligating this to similarly digested pBR322 vector DNA. Since there are no

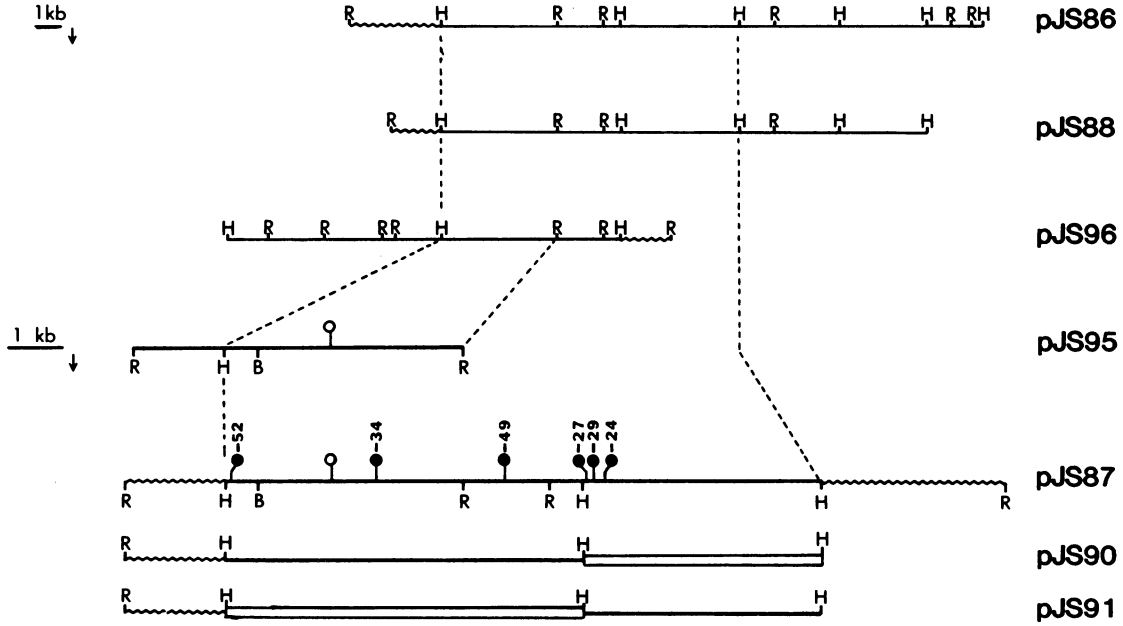

FIG. 2. Restriction map of plasmids that complement WSU300 and WSU414 and derivatives of these plasmids. Boxed regions indicate deleted DNA. Dashed lines align the lower set of plasmids with the upper set, as indicated by the scales on the left. Other symbols and abbreviations are the same as in Fig. 1.

*Eco*RI sites in Tn*5*, Kan^r colonies produced by transforming this ligation mixture into *E. coli* contained pBR322, Tn*5*, and the *R. meliloti* DNA adjacent to the Tn*5* insert. The plasmid containing this DNA was designated pJS95 (Fig. 2). Plasmids that complemented WSU100 and those that complemented WSU300 and WSU414 were distinct: they did not have common restriction fragments and did not hybridize to each other. Plasmids isolated by complementation of WSU100 did not complement WSU300 or WSU414.

Owing to the high pseudoreversion frequency of WSU515, we were unable to isolate the wild-type allele of the gene by complementation. Instead, the Tn*5* insertion and adjacent DNA were cloned as an *Eco*RI fragment to give pJS97. Restriction site and hybridization analysis indicated that the mutation in WSU515 was in a region unlinked to the regions that rescued WSU100, WSU300, or WSU414 or to *glnA* (data not shown). This gene has not been studied further.

Defining cloned regions responsible for complementation. To define the regions of pJS73 and pJS87 responsible for complementation, we mutagenized the plasmids with Tn*5*. Kan^r plasmids were screened by electrophoresis to determine which had Tn*5* insertions in the *R. meliloti* DNA. Plasmids with such inserts were transduced into the original glutamine auxotrophs, and the ability of the plasmid-containing strains to grow on minimal medium with ammonia as the sole nitrogen source was determined. pJS73::Tn*5*-5, pJS73::Tn*5*-10, and pJS73::Tn*5*-59 (Fig. 1) were unable to complement WSU100, but pJS73::Tn*5*-48 retained this ability. These insertions delimit a 1.8-kb region of pJS73 that contains the information needed to complement WSU100. In a similar fashion, the approximate region of pJS87 responsible for complementation of WSU414 was mapped (Fig. 2). We have shown that this region contains the *ntrA* gene (40a).

pJS73 contains *glnII*, the structural gene for GSII. None of the recombinant plasmids described above were able to complement ET8051, an *E. coli* glutamine auxotroph. However, when ET8051(pJS73::Tn*5*-48) (Fig. 1), a strain that carries Tn*5* inserted adjacent to the complementing region of pJS73, was placed on minimal ammonia medium, prototrophs were isolated at low frequency. pRS8, isolated from one of these colonies, was shorter than pJS73::Tn*5*-48 because of a deletion that removed approximately 250 base pairs (bp) from the left end of Tn*5* and 250 to 300 bp of *R. meliloti* DNA (Fig. 1).

ET8051(pRS8) contained a high level of heat-labile GS transferase activity (data not shown). The sedimentation behavior of this GS was compared with that of GS activities in crude extracts of *R. meliloti* GLN210 and *R. meliloti* 104A14 (Fig. 3). The *R. meliloti* 104A14 crude extract showed two GS peaks. As determined by heat sensitivity experiments, the faster-sedimenting peak corresponds to heat-stable GSI, whereas the slower-sedimenting peak corresponds to heat-labile GSII. The activity in ET8051 migrates at the same rate as the single GSII peak from GLN210 extracts and the heat-labile GSII peak from *R. meliloti* 104A14. No activity was present in any of the ET8051(pRS8) fractions after they were heated to 50°C. These observations show that the activity that permits ET8051(pRS8) to grow in the absence of glutamine is GSII. As further evidence, antibody prepared against this GS reacts with a *Rhizobium* polypeptide of 36 kilodaltons, as expected (data not shown).

Plasmid pBJ196A is a derivative of pBR322 that contains the *B. japonicum glnII* gene (8). pBJ196A hybridized to the 10.2-kb *Hin*dIII fragment, which was common to all of the plasmids that complemented WSU100 (Fig. 1). The 10.2-kb fragment therefore contains the *R. meliloti* homolog of *B. japonicum glnII*. DNA sequence data confirm the relationship between the two genes, but our analysis of these two sequences (Shatters and Khan, in press) does not support the speculation that the *glnII* gene is of plant origin (6).

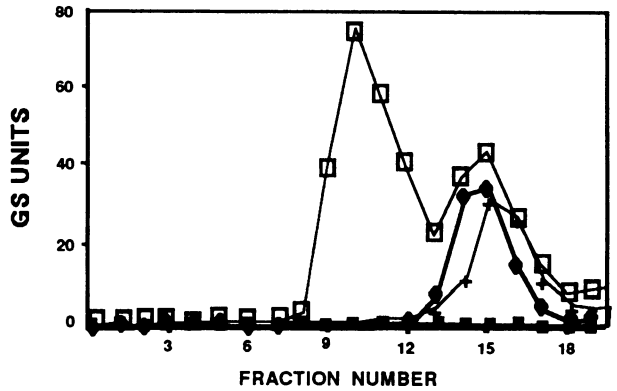

FIG. 3. Sedimentation behavior of GS activity from various strains. Crude extracts were prepared and analyzed by using sucrose gradients, as described in Materials and Methods. Fractions from one gradient of *E. coli* ET8051(pRS8) were heated at 50°C for 45 min. GS units are expressed as nanomoles of γ-glutamylhydroxamate produced per minute. Symbols: □, *R. meliloti* 104A14; ◆, *R. meliloti* GLN210; +, *E. coli* ET8051(pRS8); ■, *E. coli* ET8051(pRS8), heated.

Construction of *R. meliloti glnII* and *glnA glnII* mutants. The nitrous-acid-induced mutant, WSU100, was not a strict glutamine auxotroph, since it was able to grow in media containing 1.1 g of sodium glutamate per liter and 0.5 g of NH_4Cl per liter (23). It seemed possible that WSU100 had a point mutation in *glnII* that was leaky, even though we could detect no GS activity. The reversion frequency of WSU100 was high enough that although it produced Fix$^+$ nodules, these nodules always contained prototrophs. We therefore constructed a stable mutation in *glnII* (Fig. 4). Plasmid pRS18 contains a deletion-insertion mutation in the *glnII* gene in which a 555-bp *Eco*RI restriction fragment within *glnII* was replaced with a 9.8-kb *Eco*RI restriction fragment containing the *lacZYA* genes from pMC1513 and the Kan^r gene from Tn*5*. This *lac*-Kan^r fragment is flanked on both side by *R. meliloti* 104A14 DNA. As described in Materials and Methods, this arrangement allowed us to select for recombinants that had transferred the mutation to the chromosome. The *glnII* mutant, WSU660, was isolated by using *R. meliloti* 104A14 as the parent strain, and the *glnA glnII* double mutant, WSU650, was isolated by using *R. meliloti* GLN210 as the parent strain.

GS activity in *R. meliloti* 104A14, GLN210, and WSU660. The contribution of GSI and GSII to the total GS transferase activity present in free-living cultures of *R. meliloti* 104A14, GLN210, and WSU660 is shown in Fig. 5. The transferase assay conditions used did not distinguish between adenylation levels of GSI. As expected, GLN210 lacks heat-stable GS activity and WSU660 lacks heat-labile activity. All three strains have a higher GS transferase activity when grown on glutamate as the sole nitrogen source than when grown on NHY_4Cl as the sole nitrogen source. GSII activity was the more sensitive to the available nitrogen source. GSII activity in *R. meliloti* 104A14 grown on glutamate was double that in cells grown on ammonia. GSII activity in GLN210 was 5.6-fold higher when cells were grown with glutamate as the sole nitrogen source instead of ammonia. GSI activity increased less dramatically and was only 1.4-fold higher in *R.*

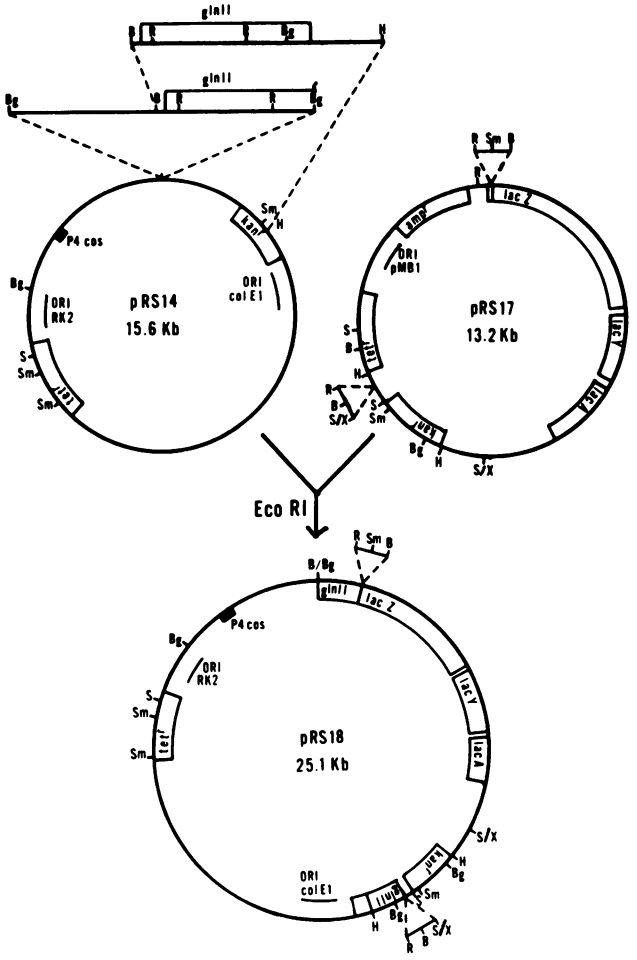

FIG. 4. Construction of the *glnII* deletion-insertion mutation in pRS18. The cosmid vector pRS14 has no *Eco*RI sites and was constructed by digesting pMK318 with *Eco*RI, treating the digest briefly with S1 nuclease to remove the cohesive ends, and then ligating the two *Eco*RI fragments. The *glnII* gene and flanking DNA were cloned as a 2.4-kb *Hin*dIII-*Bgl*II fragment into pRS14 in a two-step procedure. First, the 1.8-kb *Bgl*II fragment from pJS73 was inserted into the *Bam*HI site of pRS14. The resulting 3.1-kb *Bgl*II-*Hin*dIII insertion-vector junction fragment was then replaced with the 1.5-kb *Hin*dIII-*Bam*HI fragment containing all of *glnII* to give pRS16. To inactivate *glnII*, the 555-bp *Eco*RI fragment in *glnII* was replaced with a 9.8-kb *Eco*RI *lac*-Kan^r cartridge from pRS17. pRS17 was constructed by inserting the 2.4-kb *Xho*I fragment containing the Tn*5* Kan^r gene from pRZ102 (20) into one of the *Sal*I sites in pJK11. pJK11 is a derivative of pMC1513 (9) that has the entire Tet^r gene restored by insertion of a 650-bp *Sal*I fragment (J. Kraus, unpublished work). Restriction enzyme abbreviations: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; R, *Eco*RI; S, *Sal*I; Sm, *Sma*I; X, *Xho*I.

meliloti 104A14 and 1.8-fold higher in WSU660 than in the same strains grown on ammonia. In both GLN210 and WSU660, the activity of the remaining GS was higher than its corresponding activity in *R. meliloti* 104A14, suggesting that the activity or synthesis of each enzyme increased to compensate for the loss of the other.

Phenotypes of mutants that lack GSII activity. Table 2 summarizes various phenotypes of *R. meliloti* 104A14, GLN210, WSU414, WSU660, and WSU650. The *glnII* mutant, WSU660, was similar to the wild type in its ability to grow in free-living culture with various nitrogen sources and in forming Fix$^+$ nodules on alfalfa. WSU650 was unable to

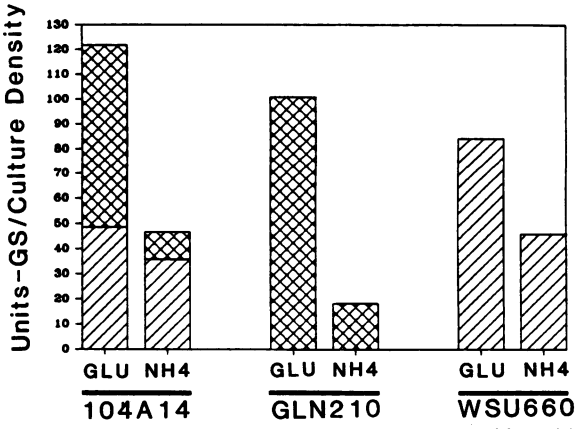

FIG. 5. GSI and GSII transferase activity in *R. meliloti* 104A14, GLN210, and WSU660. Cells were grown to mid-log phase in MM with either glutamate or ammonium as the nitrogen source and assayed for transferase activity. GSI (▨) and GSII (▩) were distinguished by the heat lability of GSII. Units are expressed as nanomoles per minute per A_{600}.

grow on either 0.5 g of NH_4Cl per liter or 1.1 g of sodium glutamate per liter as the sole nitrogen source. However, like the nitrous-acid-induced mutant WSU100, WSU650 could grow at about half the rate of wild-type *R. meliloti* 104A14 when both glutamate and ammonia were added to the medium (data not shown). Lowering either the glutamate or the ammonia concentration resulted in a proportional decrease in the growth rate. In media that contained both glutamate and glutamine, the growth rates of WSU650 and *R. meliloti* 104A14 were similar. The mutations that inactivated the GS genes should not be leaky, and we conclude that under these conditions there is another pathway that can allow glutamine synthesis. We were unable to detect GS transferase activity in WSU650 grown in MM containing glutamate and ammonia, although we have found glutamate-dependent biosynthetic activity (data not shown). No glutamine prototrophs appeared when WSU650 was placed on MM with either glutamate or ammonia as the sole nitrogen source.

WSU650 forms Fix$^+$ nodules on alfalfa. Since the nodule environment can select for reversion of auxotrophic mutations (36), all of the nodules on a number of plants were excised and crushed. Nodule bacteria were serially diluted and placed onto 3×YMB+gln and MM-ammonia agar. None

TABLE 2. Phenotypes of various *R. meliloti* 104A14 GS mutants

Strain	Transferase activity[a]		Growth on[b]:			Symbiotic phenotype	
	GSI	GSII	MM + glutamate	MM + NH_4^-	MM + glutamate + NH_4^-	Nod	Fix
104A14	+	+	+	+	+	+	+
GLN210	−	+	+	+	+	+	+
WSU660	+	−	+	+	+	+	+
WSU650	−	−	−	−	±	+	+
WSU414	−	−	−	−	−	+	−
WSU515	−	−	−	−	−	+[c]	+[c]

[a] Differentiation between GSI and GSII activity is based on heat sensitivity.
[b] Symbols: +, growth rate similar to the wild type on the indicated media; −, no growth on media; ±, growth rate of WSU650 approximately one-half that of the wild type.
[c] All nodules contained prototrophic pseudorevertants.

of the nodule preparations contained bacteria that were able to grow on minimal plates. This shows that the Fix$^+$ phenotype was not produced by prototrophic revertants of WSU650.

WSU414 was unable to grow on any medium we have tested that does not contain glutamine. This mutant was Nod$^+$ Fix$^-$. The Fix$^-$ phenotype is not the result of glutamine auxotrophy, since introducing the *glnA* wild-type allele into WSU414 to produce strain WSU415 (40a) leads to a Gln$^+$ Nod$^+$ Fix$^-$ phenotype. The mutation in WSU414 is in the *ntrA* gene (40a), so the symbiotic phenotype is consistent with that found for *ntrA* mutants in *R. meliloti* 1021 (37).

DISCUSSION

Using *R. meliloti* 104A14 GLN210 as the parent strain, we have isolated glutamine auxotrophs and identified and cloned three loci that affect the expression or function, or both, of the GSII protein. Strains that carry mutations in these three loci (WSU100, WSU300, WSU414, and WSU515) do not produce any detectable GS transferase activity. One of these mutants, WSU100, probably has a defect in *glnII*, the GSII structural gene. Although the *glnII* promoter is not active in *E. coli*, we were able to obtain expression of GSII and functional complementation of an *E. coli* glutamine auxotroph by selecting for a spontaneous deletion that removed sequences at the 5′ end of the gene.

R. meliloti GSII is not necessary for the establishment or maintenance of the symbiosis with alfalfa, since both WSU650 and WSU660 are Fix$^+$. Carlson et al. (8) have found that a *glnII* mutant of *B. japonicum* is Fix$^+$, a result consistent with ours. Donald and Ludwig (12) have also found that *Azorhizobium caulinodans* ORS751 apparently lacks a second GS enzyme and forms Fix$^+$ nodules.

However, the Fix$^+$ phenotype of our *glnA glnII* double mutant, WSU650, was different from that of the corresponding *B. japonicum* mutant. Carlson et al. (8) have found that a *B. japonicum glnA glnII* mutant is Nod$^-$ on soybeans when standard nitrogen-free plant medium is used and Nod$^+$ Fix$^-$ when the plant growth medium is supplemented with glutamine. A glutamine auxotroph of the cowpea strain 32H1 was Fix$^-$, but this mutant may have a defect in a regulatory gene (32). Glutamine auxotrophs of *Azorhizobium caulinodans* ORS751 are also Fix$^-$ (12).

This difference between the double mutants of *R. meliloti* and *B. japonicum* is similar to the difference seen with glutamate auxotrophs of these bacteria. Several groups have reported *R. meliloti* glutamate auxotrophs that are missing GOGAT and are Fix$^+$ (1, 28, 35), whereas the only reported GOGAT mutant of *B. japonicum* is Fix$^-$ (34). The difference in effectiveness of the glutamine auxotrophs may therefore reflect some basic difference between these two symbioses in how glutamate and glutamine are metabolized.

Many auxotrophic rhizobia are able to form Fix$^+$ nodules and presumably get the needed nutrients from the host plant (literature reviewed in reference 26). It might be argued that WSU650 requires only a small amount of glutamine, which could be provided by the plant. However, glutamate is thought to be made primarily by the GS-GOGAT pathway in *Rhizobium* species (1, 28, 35), and both glutamine and glutamate are needed for the synthesis of other small metabolites. Of 13 classes of *R. meliloti* 104A14 auxotrophs with alterations in amino acid or nucleic acid precursor biosynthesis, 10 were Fix$^-$ on alfalfa (26). Some of these Fix$^-$ auxotrophs had defects in biosynthetic pathways that require glutamine as a substrate, such as those for asparagine, pyrimidines, and purines. If the plant provides glutamine to the bacteria, the quantity must be substantial to satisfy these other requirements in addition to that for glutamine itself.

Since WSU650 grew at about half the rate of the wild-type strain when the medium was supplemented with glutamate and ammonium, another possible explanation for the Fix$^+$ phenotype of WSU650 is that the mutation in *glnA* or *glnII* is leaky. However, both of the GS structural genes in WSU650 carry well-defined mutations that appear to completely inactivate their products. The insertion in *glnA* splits the gene (41), and the mutation blocks the growth of WSU414; the deletion in *glnII* removes about half of the gene. We therefore suggest that there is a third enzyme that has sufficient GS activity to support growth when both ammonia and glutamate are present.

It has recently been reported that some rhizobia contain a third glutamine synthetase. Kumar and Rao (29) have chromatographically separated three forms of GS from *R. leguminosarum*, *R. phaseoli*, and *R. meliloti*. Kaush et al. (24) have also obtained evidence that a third GS exists in *R. lupini*. The enzyme that allows *R. meliloti* WSU650 to grow without a glutamine supplement is unlikely to be exactly what is described in these reports, since we detected no GS transferase activity in extracts of strain WSU650.

Alternative routes for glutamine synthesis may exist that use unique enzymes. Rossbach et al. (39) have reported that they can rescue an *E. coli* glutamine auxotroph with a region of *R. meliloti* DNA, *glnT*, which is neither *glnA* nor *glnII*. *E. coli* strains that contain the *glnT* gene derived from *R. phaseoli* are reported to have GS biosynthetic activity (14). We have detected GS biosynthetic activity in strain WSU650 but not in WSU414 (data not shown). These observations suggest that *glnT* could be important in the growth of WSU650 on glutamate and ammonia.

Because WSU650 is Fix$^+$, it is clear that the nodule environment permits this mutant to grow, but it is not clear whether this growth is related to the observation that WSU650 can grow without glutamine if glutamate and ammonia are provided. Streeter (42) has recently measured concentrations of glutamate in bacteroids and found that these concentrations may be high enough to support growth similar to what we see in glutamate and ammonia. However, to maintain a high ammonia concentration might require nitrogen fixation by the bacteria, which in turn would require protein synthesis that would be dependent on the ability to obtain glutamine. Therefore, although nitrogen fixation may be stable once it has begun, initiating the process may depend critically on details of the interaction between the symbionts. Whether the growth of WSU650 is supported by glutamine or glutamate, the ability of this strain to establish Fix$^+$ nodules suggests that the plant feeds the bacteria at least one of these amino acids. This implies that there is a flow of nitrogen from the plant to the bacteria, a view consistent with our hypothesis that amino acids may serve as important carbon sources for the bacteroids (22).

To understand the symbiotic differences between *R. meliloti* and *B. japonicum glnA glnII* double mutants, we are trying to characterize the activity that allows *R. meliloti* WSU650 to grow on media with glutamate and ammonia. It is important to determine the symbiotic phenotype of a true glutamine auxotroph and to understand why there is a difference in symbiotic phenotype between *R. meliloti* and *B. japonicum* mutants with defects in ammonia metabolism.

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ADDENDUM IN PROOF

An expanded version of the work by Rossbach et al. (39) has been recently published by de Bruijn et al. (F. J. de Bruijn, S. Rossbach, M. Schneider, P. Ratet, S. Messmer, W. W. Szeto, F. M. Ausubel, and J. Schell, J. Bacteriol. **171**:1673–1682, 1989).

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