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Rhizobium meliloti 1021 Has Three Differentially Regulated Loci Involved in Glutamine Biosynthesis, None of Which Is Essential for Symbiotic Nitrogen Fixation†

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We have cloned and characterized three distinct *Rhizobium meliloti* loci involved in glutamine biosynthesis (*glnA*, *glnII*, and *glnT*). The *glnA* locus shares DNA homology with the *glnA* gene of *Klebsiella pneumoniae*, encodes a 55,000-dalton monomer subunit of the heat-stable glutamine synthetase (GS) protein (GSI), and complemented an *Escherichia coli glnA* mutation. The *glnII* locus shares DNA homology with the *glnII* gene of *Bradyrhizobium japonicum* and encodes a 36,000-dalton monomer subunit of the heat-labile GS protein (GSII). The *glnT* locus shares no DNA homology with either the *glnA* or *glnII* gene and complemented a *glnA E. coli* strain. The *glnT* locus codes for an operon encoding polypeptides of 57,000, 48,000, 35,000, 29,000, and 28,000 daltons. *glnA* and *glnII* insertion mutants were glutamine prototrophs, lacked the respective GS form (GSI or GSII), grew normally on different nitrogen sources (Asm^+), and induced normal, nitrogen-fixing nodules on *Medicago sativa* plants (Nod^+ Fix^+). A *glnA glnII* double mutant was a glutamine auxotroph (Gln^-), lacked both GSI and GSII forms, but nevertheless induced normal Fix^+ nodules. *glnT* insertion mutants were prototrophs, contained both GSI and GSII forms, grew normally on different N sources, and induced normal Fix^+ nodules. *glnII* and *glnT*, but not *glnA*, expression in *R. meliloti* was regulated by the nitrogen-regulatory genes *ntrA* and *ntrC* and was repressed by rich N sources such as ammonium and glutamine.

Glutamine synthetase (GS; L-glutamate-ammonia ligase [ADP-forming]; EC 6.3.1.2.), coupled in a two-step pathway to glutamate synthase (GOGAT; glutamine-oxoglutarate aminotransferase; EC 2.6.1.53), plays an essential role in bacterial nitrogen assimilation. In enteric bacteria, the GS-GOGAT pathway is primarily responsible for nitrogen assimilation under low concentrations of available ammonia (44). The gene encoding GS (*glnA*) in enteric bacteria is controlled via two promoters by a complex nitrogen-regulatory system (*ntr*) that coordinately regulates a number of loci involved in nitrogen metabolism. One of the two *glnA* promoters resembles general bacterial promoters and is repressed by the product of the *ntrC* gene (NR1). The second *glnA* promoter consists of a DNA sequence motif typical of *ntr*-regulated promoters and requires the *ntrC* product (NR1) as well as an alternative sigma factor (*ntrA* or *rpoN* product) for activation (29, 30, 32, 54). In addition to transcriptional regulation of *glnA* by the *ntr* system, GS activity is regulated posttranslationally via adenylylation (44).

In contrast to enteric bacteria, members of the family *Rhizobiaceae*, including *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium* species, contain at least two distinct forms of GS (13, 27, 38, 62). GSI is a homolog of the *glnA* gene product of enteric bacteria in terms of its heat stability, molecular mass (monomer ~55,000 daltons), subunit structure (12 subunits), and adenylylation properties. GSII, on the other hand, is composed of eight identical subunits of ~36,000 daltons each, heat labile, and not subject to adenylylation control (14, 28, 40, 47). In fact, GSII is structurally similar to GS forms found in eucaryotic organisms, such as plants (9). Recently, we described the structure and regulation of the *Agrobacterium tumefaciens gln* loci (56, 57). Here we extend this analysis to *Rhizobium meliloti* and show that *R. meliloti* contains three distinct loci involved in glutamine synthesis (*glnA*, *glnII*, and *glnT*), which are differentially regulated in response to fluctuating ammonium concentrations and which appear to be nonessential for symbiotic nitrogen fixation.

(Preliminary aspects of this work have been described by de Bruijn et al. [20, 21].)

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. The bacterial strains and recombinant plasmids used are listed in Table 1. Strain FDB213 was isolated as a Tc^s derivative of FDB128 after cycloserine enrichment. For *Escherichia coli* the rich medium was LB and the minimal medium was M9 (48). Rich medium for *R. meliloti* was TY (3) and minimal medium was GTS (35). The media were supplemented with 0.2% glutamine in the case of Gln^- strains. Antibiotics were added to the following final concentrations: ampicillin, 40 μg/ml; chloramphenicol, 60 μg/ml; gentamicin, 50 μg/ml; kanamycin, 25 μg/ml for *E. coli* or 200 μg/ml for *R. meliloti*; rifampin, 100 μg/ml; streptomycin, 250 μg/ml (*R. meliloti*); tetracycline, 10 μg/ml for *E. coli* and *R. meliloti*.

Plasmid DNA isolation. Large-scale and small-scale plasmid DNA preparations were carried out according to Ish-Horowicz and Burke (33) and de Bruijn and Lupski (18).

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TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant characteristics or genotype	Source or reference
Escherichia coli		
HB101	*recA hsdR hsdM pro leu lacZ supE*	6
YMC11	*thi-1 endA hsdR* Δ(*glnA-ntrC*)	2
FDB128	K-12 *pro* B1 *r endA* Δ(*glnA-ntrC*) *recA56 srl*::Tn*10*	16
FDB213	K-12 *pro* B1 *r endA* Δ(*glnA-ntrC*) *recA56 srl*	16
CSR603	*recA1 uvrA6 phr-1*	58
GJ23	JC2926(pGJ28) (R64 *drd*11); ColE1 (Mob$^+$ Tra$^+$)	63
MC4100 (Mu *cts*) (MudIIPR46)	*araD139* Δ(*argF-lac*)*U169*, *rpsL150* (Sm^r) *relA1 flbB5301 pstF25 deoC1* (Mu *cts*) (MudIIPR46)	53
MC4100 (Mu *cts*) (MudIIPR48)	*araD139* Δ(*argF-lac*)*U169 rpsL150* (Sm^r) *relA flbB5301 pstF25 deoC1* (Mu *cts*) (MudIIPR48)	53
Rhizobium meliloti		
1021	Wild type, Sm^r	46
5001	*ntrC*::Tn*5*, Sm^r Km^r	61
1680	*ntrA*::Tn*5*, Sm^r Km^r	55
1-20	*glnA*::Tn*5*, Sm^r Km^r	This work
1-21	*glnA*::Tn*5*, Sm^r Km^r	This work
2-37	*glnII*::Tn*5*, Sm^r Km^r	This work
3-5	*glnT*::Tn*5*, Sm^r Km^r	This work
3-53	*glnT*::Tn*5*, Sm^r Km^r	This work
3-62	*glnT*::Tn*5*, Sm^r Km^r	This work
3-63	*glnT*::Tn*5*, Sm^r Km^r	This work
4-15	*glnA* (MudIIPR46) *glnII*::Tn*5*, Sm^r Km^r Gm^r	This work
4-17	*glnA* (MudIIPR46) *glnII*::Tn*5*, Sm^r Km^r Gm^r	This work
Agrobacterium tumefaciens GV3101(pTiC58)	Rif^r derivative of C58, nopaline type	31
Plasmids		
pBR322	Ap^r Tc^r	5
pACYC184	Tc^r Cm^r	11
pRK290	Tc^r Tra$^-$ Mob$^+$ IncP	22
pWB5	Km^r Tc^r pRK290 derivative, polylinker	W. Buikema and F. M. Ausubel
pLAFR1	Tc^r Cos Tra$^-$ Mob$^+$ IncP	26
pPHIJI	Cm^r Sm^r Sp^r Gm^r Tra$^+$ IncP Mob$^+$	4
pRK2013	Km^r Tra$^+$ Mob$^+$ IncP	22
pFB68	Tc^r, *R. meliloti glnA* region in pLAFR1	This work
pFB682	Ap^r, *Bam*HI-*Eco*RI fragment of pFB68 in pBR322	This work
pFB69	Tc^r, *R. meliloti glnII* region in pLAFR1	This work
pFB691	Tc^r, *Pst*I fragment of pFB69 in pBR322	This work
pFB61	Tc^r, *R. meliloti glnT* region in pLAFR1	This work
pFB616	Tc^r, *R. meliloti glnT* region in pLAFR1*	This work
pFB6162	Cm^r, *Bam*HI fragment of pFB616 in pACYC184	This work
pSR1301	Cm^r, *A. tumefaciens glnA* region in pACYC184	57
pJS44	Ap^r, *R. meliloti* 104A14 *glnA* region in pBR322	59
pJS196A	Ap^r *B. japonicum glnII* region in pBR322	9
pVSP9A	Tc^r Km^r, *R. meliloti nifH-lacZ* gene fusion in pWB5	60; T. Nixon and F. M. Ausubel
pCE116	Tc^r, *R. meliloti fixABCX* region in pBR325	61
pFB54	Cm^r, *K. pneumoniae glnA* region in pACYC184	16

Total chromosomal DNA isolation. *Rhizobium* DNA was isolated by using a protocol modified from Meade et al. (46), as described by Rossbach et al. (56).

Restriction endonuclease analysis, ligations, and transformations. Restriction endonucleases were purchased from Bethesda Research Laboratories (BRL; Gaithersburg, Md.), New England BioLabs (Waltham, Mass.), or Boehringer (Mannheim, Federal Republic of Germany) and used according to the specifications prescribed by the manufacturers. Agarose gel electrophoresis, ligations, and transformation were carried out as described before (45).

Nick translations, Southern blotting, and hybridizations. Nick translations were carried out with a kit purchased from Amersham, as specified by the manufacturer. Southern blotting and DNA-DNA hybridizations were carried out as described before (45). Colony hybridizations were performed according to Maas (43).

Transposon Tn*5* mutagenesis. Transposon Tn*5* mutagenesis was carried out as described before (18).

Maxicell analysis. Maxicell analysis was carried out by a modification of the protocol of Sancar et al. (58), as described elsewhere (17).

Genomic library construction. The genomic library of *R. meliloti* was constructed as described before (56).

Conjugations and gene replacement experiments. Conjugations and gene replacement experiments were performed as described before (15). Strains 1-20 and 1-21 were obtained by mobilizing pFB682:Tn*5* 20 and 21, respectively (Fig. 1A), into *R. meliloti* 1021 by using GJ28 (63) and screening for loss of vector sequences by colony hybridization (43). Strain

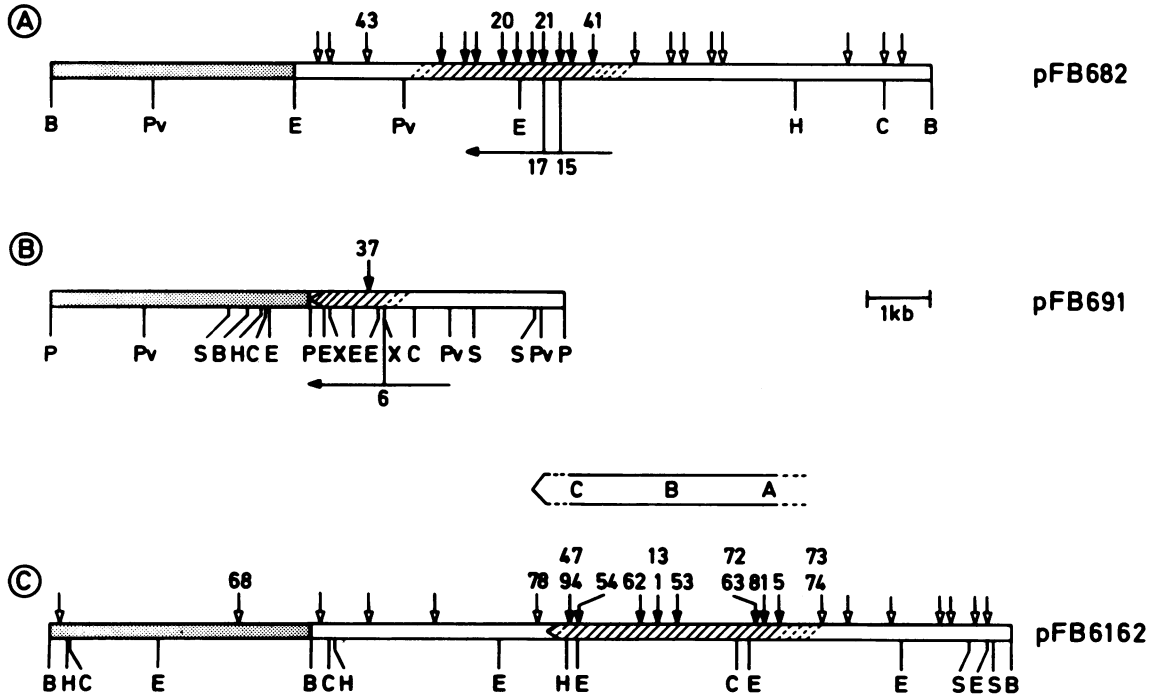

FIG. 1. Physical and genetic maps of the *Rhizobium meliloti* 1021 *gln* loci. (A) *glnA* locus. Solid arrows indicate the positions of Tn*5* insertions leading to a Gln^- phenotype, and open arrows indicate those leading to a Gln^+ phenotype. The solid arrows numbered 20 and 21 indicate the Gln^- Tn*5* insertions used for homogenotization. The arrows numbered 15 and 17 indicate the positions and orientations of MudIIPR46 insertions used for homogenotization (see Table 1 and text). The stippled region denotes pBR322 vector sequences. The hatched region denotes the approximate limits and direction of transcription of the *glnA* gene. (B) *glnII* locus. The arrow indicates the position of Tn*5* insertion 37, used for homogenotization. The arrow numbered 6 indicates the position and orientation of the MudIIPR48 insertion. The stippled region denotes the vector pBR322, and the hatched region denotes the approximate limits and direction of transcription of the *glnII* gene. (C) *glnT* locus. Solid arrows indicate the positions of Tn*5* insertions leading to a Gln^- phenotype, and open arrows indicate those leading to a Gln^+ phenotype. Numbered arrows represent the Tn*5* insertions used for homogenotization. The stippled region denotes the vector pACYC184. The hatched region denotes the approximate limits and direction of transcription of the *glnT* locus. Abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; X, *Xho*I.

2-37 was constructed by cloning pFB691::Tn*5* 37 (Fig. 1B) into pWB5 (Table 1), replacing its Km^r gene, and mobilizing the resulting plasmid into *R. meliloti* by using the triparental mating protocol (15). A homogenote was isolated by using pPHIJI (Table 1), as described before (15). Strains 3-5, 3-63, 3-53, and 3-62 were constructed by cloning pFB6162::Tn*5* 5, 63, 53, and 62, respectively (Fig. 1C), into pRK290 (Table 1) and creating homogenotes as described above. To create strains 3-15 and 3-17, plasmid pFB682 was mutagenized with MudIIPR46 (Table 1) as described before (53), and Gln^- Ap^r Cm^r pFB682 *glnA*::MudIIPR46 plasmids were mobilized into *R. meliloti* 1021 2-37 by using the *oriT* carried by MudIIPR46. *glnA*::MudIIPR46 *glnII*::Tn*5* homogenotes were identified by screening for loss of vector sequences (colony hybridization). The structure of all insertion mutations was verified by Southern blotting.

RNA biochemistry. Isolation of RNA, dot blots, and hybridizations were performed as described before (61).

GS assays. *E. coli* and *Rhizobium* cells were inoculated into 5 ml of LB or TY medium and grown overnight to saturation. A 0.5-ml sample was reinoculated in 50 ml of minimal medium supplemented with 0.2% $(NH_4)_2SO_4$ and 0.2% glutamine, and the culture was incubated for 24 h at 37°C (*E. coli*) or 28°C (*R. meliloti*). The cells were harvested and resuspended in 50 ml of minimal medium without an N source (derepressing conditions) and grown for 10 h. Crude extract preparations, GS enzyme assays, and sucrose gradient analyses were performed as described before (56).

Plant tests. Plant tests were carried out in 30-cm glass tubes. *Medicago sativa* seeds were surface sterilized by immersion for 3 min in 75% ethanol followed by 2 min in 0.1% $HgCl_2$ and washed five times with sterile distilled water. The seeds were then germinated in the test tubes on a slant of B+D medium (7) under artificial light at 21°C (16-h day) and after 1 week were inoculated with an H_2O-washed culture of bacteria which had been grown in TY medium with the appropriate antibiotics. Acetylene reduction assays were carried out as described before (61). Bacteria were reisolated from nodules as described before (61).

Mini-Mu mutagenesis. Plasmid mutagenesis and *lacZ* gene fusion construction with mini-Mu *lac* transposons were carried out as described by Ratet et al. (53) and in the text.

Construction of a *glnII-lac* fusion. Plasmid pFB691 was mutagenized with MudIIPR48 as described before (53), and the mutagenized culture was conjugated en masse with *Agrobacterium tumefaciens* C58 (Table 1) cells. Blue transconjugants were selected on MinA plates (56) supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactose (X-gal), 0.1% nitrate, and 0.01% arginine (as nonrepressing nitrogen sources), rifampin (C58 marker), tetracycline (pFB691), and gentamicin (MudIIPR48). Fifty blue clones were streaked onto MinA medium with 0.2% glutamine as a repressing N source, and five clones showed repression of β-galactosidase activity. The plasmids were mobilized back to *E. coli*, the position and orientation of MudIIPR48 in pFB691 were mapped with restriction enzymes, and one pFB691 *glnII*::

MudIIPR48 plasmid was selected for further studies (no. 6; Fig. 1B).

β-Galactosidase assays. β-Galactosidase activity was determined as described before (48) except that 50 μl of chloroform was used per assay. β-Galactosidase activity of *M. sativa* nodules was determined as described before (52).

RESULTS

Cloning the *R. meliloti glnT* locus. Initially, we attempted to clone the *glnA* and *glnII* genes from *R. meliloti* 1021 by genetic complementation of an *E. coli glnA* deletion. This strategy has been used successfully to clone the *glnA* gene from *R. meliloti* 104A14 (59), *Azorhizobium caulinodans* (20), and *A. tumefaciens* (57). Therefore, *E. coli* FDB128 (*recA* Δ*glnA*) was transformed with an *R. meliloti* 1021 cosmid library constructed in pLAFR1 (26), and Gln^+ transformants were selected. This resulted in the isolation of four slow-growing Gln^+ colonies which carried identical cosmids with ~25-kilobase (kb) inserts of *R. meliloti* DNA that were colinear with the *R. meliloti* genome (data not shown). One cosmid (pFB61) was transferred to a Tc^s derivative of *E. coli* FDB128 (FDB213) and chosen for further studies.

Although *E. coli* FDB213(pFB61) grew very slowly on minimal medium, taking 5 days to form colonies at 37°C, occasionally fast-growing derivatives arose. Cosmid DNA was isolated from eight of these fast-growing colonies, and in one case (pFDB616), we demonstrated that the fast-growing phenotype was due to a 0.7-kb deletion in the *oriV* region of the pLAFR1 vector. The deleted pLAFR1 vector (pLAFR1*) was cloned from pFB616. Both pFB616 and pLAFR1* were found to have a significantly higher copy number in *E. coli* than pFB61 and pLAFR1, as determined by both small- and large-scale plasmid isolation procedures (data not shown). Interestingly, pLAFR1* could not be transferred to (or to replicate in) *R. meliloti*, *A. tumefaciens*, and *Azorhizobium caulinodans* ORS571. Both pFB61 and pFB616 were used in subsequent experiments.

Unexpectedly, no GS enzymatic activity (either transferase activity or biosynthetic activity; see Materials and Methods) could be detected in two *E. coli glnA* deletion mutants, FDB213 and YMC11, carrying either pFB61 or pFB616, despite the fact that both cosmids complemented the *E. coli glnA* deletions. The GS assays suggested that the *gln* locus cloned in pFB61 was neither *glnA* or *glnII*, and this conclusion was supported by Southern blotting experiments, which showed that pFB61 DNA failed to hybridize to cloned *glnA* genes from *Klebsiella pneumoniae*(pFB54), *R. meliloti* (pJS44), and *A. tumefaciens*(pSR1301) or the cloned *glnII* locus from *B. japonicum*(pJB196A) (data not shown). The *gln* locus cloned in pFB61 was called *glnT*.

In a second attempt to clone the *R. meliloti* 1021 *glnA* and *glnII* genes, *E. coli* YMC11 (Δ*glnA*) was transformed with a newly constructed *R. meliloti* 1021 cosmid library in pLAFR1 (see Materials and Methods), and 1,400 Tc^r transformants were individually screened for clones with a Gln^+ phenotype. Six slow-growing Gln^+ colonies were obtained, each of which carried a cosmid with an insert that overlapped the region of the *R. meliloti* 1021 genome already cloned in pFB61. Since these six cosmids apparently did not carry the *glnA* or *glnII* locus (see below), they were not examined further.

Cloning of *glnA* and *glnII* by using DNA probes. Because two attempts to clone the *R. meliloti* 1021 *glnA* and *glnII* genes by genetic complementation had failed, we used previously cloned *glnA* and *glnII* genes as hybridization probes to clone the cognate *R. meliloti* 1021 genes. Southern blotting experiments showed that two *R. meliloti* 1021 *Eco*RI fragments (4 and 20 kb) hybridized to an *R. meliloti* 104A14 *glnA* probe (*Hin*dIII fragment of pJS44) and that three *R. meliloti* 1021 *Eco*RI fragments (two 0.5-kb fragments and a 10-kb fragment) hybridized to a *B. japonicum glnII* probe (*Sal*I fragment of pJB196A) (data not shown). To clone these hybridizing fragments, 1,800 clones from the newly constructed *R. meliloti* 1021 pLAFR1 library (see Materials and Methods) were screened by colony hybridization as described before (43). With the *R. meliloti* 104A14 *glnA* probe, two cosmids that carried both the 4-kb and 20-kb hybridizing *Eco*RI fragments were identified (data not shown), one of which (pFB68) was selected for further analysis. With the *B. japonicum glnII* probe, one cosmid (pFB69) was identified that carried the two hybridizing 0.5-kb *Eco*RI fragments and the 10-kb hybridizing *Eco*RI fragment.

Despite the failure to clone a *glnA* gene by using the genetic complementation procedure, pFB68 complemented the Gln^- phenotype of YMC11, and a high level of heat-stable GS activity was observed in YMC11(pFB68) cells (data not shown). Both the 4- and 20-kb *Eco*RI fragments of pFB68 were required for complementation (data not shown), corroborating the hybridization data. YMC11(pFB69), on the other hand, failed to grow on minimal medium.

Characterization of the presumptive *glnA* gene on pFB68. Two adjacent restriction fragments of pFB68 (a 6-kb *Eco*RI-*Bam*HI fragment and a 3.6-kb *Eco*RI fragment) that hybridized to the *R. meliloti* 104A14 *glnA* probe were subcloned into pBR322 to form plasmid pFB682 (Fig. 1A). *E. coli* YMC11(pFB682) was Gln^+ and expressed a high level of GS transferase activity (data not shown). To delimit the presumptive *glnA* locus, pFB682 was mutagenized with Tn*5* as described before (18), and pFB682::Tn*5* plasmids were mapped by restriction enzyme analysis, delimiting the presumptive *glnA* gene to a ~2.5-kb region surrounding the internal *Eco*RI site (Fig. 1A). Maxicell analysis showed that pFB682 encoded a ~55,000-dalton polypeptide that was not synthesized by pBR322 or a Gln^- pFB682::Tn*5* plasmid (Fig. 2, lanes 2 and 3) and that comigrated with the *A. tumefaciens glnA* product (Fig. 2, lane 1).

Characterization of the presumptive *glnII* gene of pFB69. A 4.3-kb *Pst*I fragment from pFB69 that hybridized to the *B. japonicum glnII* probe was subcloned into pBR322, forming pFB691 (Fig. 1B). Further hybridization experiments narrowed the region of *glnII* homology to a ~1.2-kb region of pFB691 between the *Pst*I and *Cla*I sites (Fig. 1B, striped area). As was the case with pFB69, pFB691 failed to complement the Gln^- phenotype of YMC11, and no GS transferase or biosynthetic activity was observed in YMC11(pFB691) cells. On the other hand, subculturing YMC11(pFB691) in minimal medium resulted in the isolation of a few Gln^+ colonies, which were found to contain rearranged and multimerized pFB691 plasmids (data not shown). Similar results were obtained by Rossbach et al. (57) in the case of the *A. tumefaciens glnII* gene.

Forty-five pFB691::Tn*5* insertions were mapped, and one insertion (37) that was located in the middle of the *glnII*-homologous region was selected for further study. Maxicell analysis showed that pFB691 encoded a 36,000-dalton polypeptide that was not synthesized by pBR322 or pFB691::Tn*5* (37) (data not shown).

Characterization of the *glnT* locus on pFB616. Sixty pFB616::Tn*5* plasmids were screened for their Gln phenotype in *E. coli* FDB213, and seven insertions were identified which resulted in a Gln^- phenotype, all of which mapped

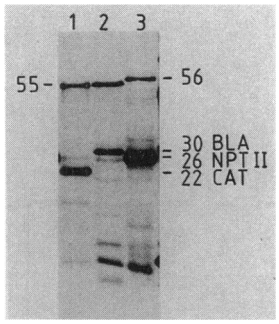

FIG. 2. Identification of the *glnA* (GSI) product. Lanes 1–3 show an autoradiogram of a 10% SDS-polyacrylamide gel, containing [^{35}S]methionine-labeled polypeptides synthesized by maxicells harboring plasmid pSR1301 (*A. tumefaciens glnA*; lane 1), pFB682 (*R. meliloti* 1021 *glnA*; lane 2), and pFB682::Tn*5* 20 (*R. meliloti glnA*::Tn*5*; lane 3). The sizes of the polypeptides encoded by these plasmids were determined relative to protein size markers (not shown). *glnA* gene product encoded by pSR1301 and pFB682, ~55,000 daltons; Tn*5*-encoded transposase, ~56,000 daltons (lane 3); β-lactamase (BLA) encoded by the pBR322 vector of pFB682, ~30,000 daltons (lane 2); neomycin phosphotransferase II (NPTII) encoded by Tn*5*, ~26,000 daltons; chloramphenicol acetyltransferase (CAT) encoded by the pACYC184 vector of pSR1301, ~22,000 daltons (lane 1).

within an 11.2-kb *Bam*HI fragment. The 11.2-kb *Bam*HI fragment was subcloned in pACYC184 to form pFB6162 (Fig. 1C), which complemented the Gln$^-$ phenotypes of *E. coli* YMC11 and FDB213 as efficiently as did pFB54, which carries the *K. pneumoniae glnA* gene.

To delimit the *glnT* locus further, 200 pFB6162::Tn*5* insertions were screened for their Gln phenotype, and 30 Gln$^-$ and 70 Gln$^+$ insertions were mapped, delimiting the *glnT* locus to a ~5-kb region bounded by Tn*5* insertions 78 and 73/74 (Fig. 1C).

Maxicell analysis showed that pFB6162 encoded at least five polypeptides (57,000, 48,000, 38,000, 29,000, and 28,000 daltons; Fig. 3, lane 1). When selected pFB6162::Tn*5* plasmids were tested in maxicells, insertions further to the right (Fig. 1) had a more severe effect on the synthesis of these polypeptides than insertions further to the left. Insertions 5,

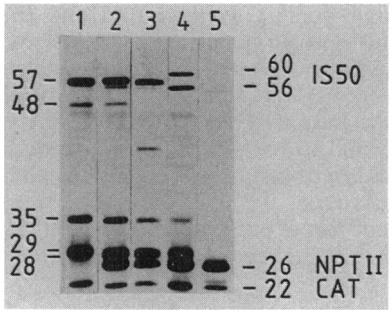

FIG. 3. Identification of the *glnT* gene products. Lanes 1–5 show the polypeptides synthesized by maxicells harboring plasmids pFB6162 (lane 1), pFB6162::Tn*5* 68 (lane 2), pFB6162::Tn*5* 94 (lane 3), pFB6162::Tn*5* 53 (lane 4), and pFB6162::Tn*5* 81 (lane 5). The sizes of the polypeptides encoded by these plasmids were determined relative to protein size markers (not shown). pFB6162-encoded polypeptides, 57,000, 48,000, 35,000, 29,000, and 28,000 daltons; Tn*5* (IS*50*)-encoded transposases, 60,000 and 56,000 daltons; neomycin phosphotransferase II (NPTII) encoded by Tn*5*, 26,000 daltons; chloramphenicol acetyltransferase (CAT) encoded by pACYC184 vector of pFB616, 22,000 daltons.

TABLE 2. Phenotypes of *R. meliloti* 1021 *gln* mutants[a]

Strain (genotype)	GS activity		Phenotype[b]			
	GSI	GSII	Gln	Asm	Nod	Fix
1021 (wild type)	+	+	+	+	+	+
1-20 (*glnA*)	−	+	+	+	+	+
2-37 (*glnII*)	+	−	+	+	+	+
3-53 (*glnT*)	+	+	+	+	+	+
4-17 (*glnA glnII*)	−	−	−	−	+	+
5001 (*ntrC*)	+	−	+	+	+	+
1680 (*ntrA*)	+	−	+	+	+	+

[a] GS (transferase activity) was determined on sucrose gradient fractions before and after heat treatment (see Materials and Methods).

[b] Gln phenotype was determined by growth on GTS medium lacking glutamine with 0.2% $(NH_4)_2SO_4$ as the sole nitrogen source. Asm phenotype, growth in GTS with nitrate, arginine, histidine, or asparagine (0.2%) as the sole nitrogen sources. Nod phenotype, ability to nodulate *M. sativa* plants. Fix phenotype, ability to reduce C_2H_2 as measured by the whole-plant assay.

63, and 81 blocked the synthesis of all five polypeptides (Fig. 3, lane 5); insertion 53 blocked the synthesis of the 57,000- and 48,000-dalton polypeptides (Fig. 3, lane 4); insertions 1, 13, and 62 blocked the synthesis of the 48,000-dalton polypeptide but caused the appearance of a new polypeptide (~42,000 daltons; Fig. 3, lane 3), which presumably represents a truncated version of the 48,000-dalton polypeptides (18). These data suggest that the *glnT* locus is an operon consisting of several genes involved in the biosynthesis of glutamine.

Construction and characterization of *R. meliloti* 1021 *gln* mutants. Selected *glnA*::Tn*5* mutations carried on plasmids pFB682, pFB691, and pFB6162 were marker exchanged into the *R. meliloti* genome as described in Materials and Methods. To construct a *glnA glnII* double mutant, the *glnA* gene carried on pFB682 was first mutagenized with the mini-Mu *lac* transposon MudIIPR46, which confers chloramphenicol resistance (see Materials and Methods). Two *glnA*::MudIIPR46 mutations (15 and 17; Fig. 1A) were then marker exchanged into *R. meliloti* 2-37 (*gln*II::Tn*5*) (see Materials and Methods).

The single *R. meliloti* 1021 *glnA*::Tn*5*, *glnII*::Tn*5*, and *glnT*::Tn*5* mutants were all Gln$^+$, suggesting that none of the three *gln* loci is absolutely required for glutamine biosynthesis. These strains also grew like the wild type on a variety of nitrogen sources, including nitrate, arginine, ammonium, and glutamine (Asm$^+$). In contrast, the double *glnA*::MudIIPR46 *glnII*::Tn*5* mutant was a glutamine auxotroph (Table 2).

French press extracts were prepared from wild-type and *gln* mutant strains and analyzed by sucrose gradient centrifugation as described in Materials and Methods. *R. meliloti* 1021 (wild type) yielded two peaks of GS activity (GSI and GSII), the second of which (GSII) was heat sensitive (Fig. 4A). The *glnA*::Tn*5* mutant lacked the GSI peak (Fig. 4B), and the *glnII*::Tn*5* mutant lacked the GSII peak (Fig. 4D). The *glnT*::Tn*5* mutant yielded both GSI and GSII peaks of activity, although the relative levels of GSI and GSII activity were different than in the wild type (Fig. 5).

Interestingly, all of the *R. meliloti gln*::Tn*5* mutants, including the *glnA glnII* double mutant, elicited normal nitrogen-fixing nodules on *M. sativa* (Table 2), indicating that none of the three GS activities are required for effective symbiosis. Reisolation of bacteria from the nodules revealed that no significant reversion of the mutations had occurred (data not shown).

Regulation of the *R. meliloti gln* genes. We first measured GSI and GSII activity following sucrose gradient centrifuga-

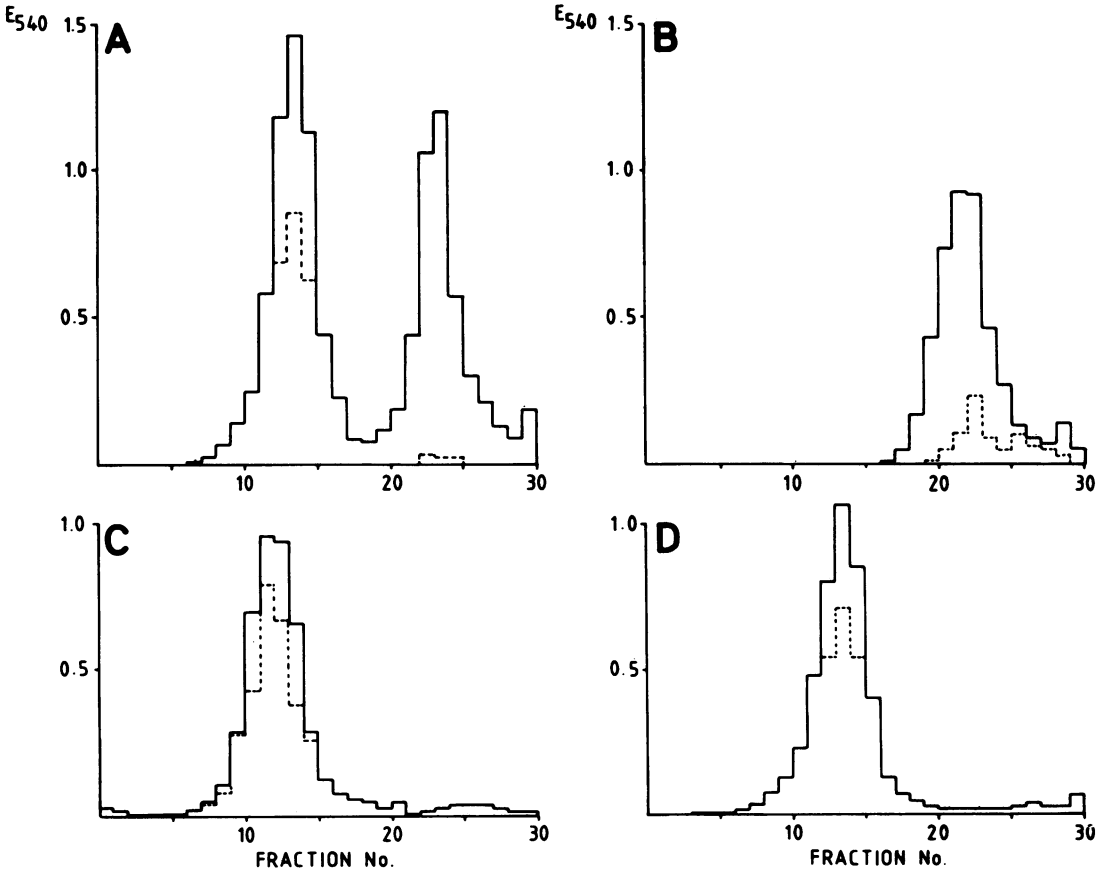

FIG. 4. GS activity profiles of crude extracts. The optical density of the transferase reaction mix at 540 nm (E_{540}) is indicated on the *y* axis; the fraction number is indicated on the *x* axis. The left-hand peaks represent GSI and the right-hand peaks represent GSII activity. The broken lines indicate the E_{540} of the peak fractions after heat treatment of the samples at 50°C for 1 h. (A) *R. meliloti* 1021 (wild type); (B) *R. meliloti* 1-20 (*glnA*::Tn*5*); (C) *R. meliloti* 5001 (*ntrC*::Tn*5*); (D) *R. meliloti* 2-37 (*glnII*::Tn*5*).

tion of extracts from wild-type *R. meliloti* cells grown in the presence (0.2%) or absence of ammonia. GSII but not GSI activity was found to be significantly repressed by the high ammonia level (data not shown). Moreover, GSII activity could not be detected in *R. meliloti ntrC* (strain 5001) or *ntrA* (strain 1680) mutants, whereas GSI activity was not affected in these mutants (Fig. 4C).

To determine whether regulation of GSII activity was occurring at the level of expression of the *glnII* gene, a *glnII-lacZ* fusion was constructed in pFB691 by using MudIIPR48 as described in Materials and Methods. Because MudIIPR48 carries an *oriT* site for efficient conjugal transfer and the origin of replication of the Ri plasmid of *A. rhizogenes* for stable low-copy-number replicon maintenance in *Rhizobiaceae* (53), the *glnII-lacZ* fusion could be stably transferred to wild-type *R. meliloti* 1021 and to *R. meliloti ntrC* and *ntrA* mutants. In wild-type cells, high levels of β-galactosidase activity (4,200 Miller units; 48) were found under derepressing conditions (GTS medium) plus 0.2% nitrate or 0.2% arginine) with succinate and lactate as the carbon source, whereas a low level of activity (180 Miller units) was found under repressing conditions (GTS medium plus 0.2% ammonium sulfate or 0.2% glutamine). No β-galactosidase activity was found in the *ntrA* or *ntrC* mutant strains under repressing or derepressing conditions.

R. meliloti 1021 carrying the *glnII-lacZ* fusion was used to elicit *M. sativa* root nodules, but no β-galactosidase activity could be detected in 6-week-old nodules. pFB691::MudIIPR48 was still present in more than 80% of the bacteria that were reisolated from the nodules. In contrast, high levels of β-galactosidase were found in nodules elicited by *R. meliloti* 1021 carrying a *nifH-lacZ* fusion (pVSP9A).

To study regulation of the *glnT* locus, an RNA dot blot analysis was carried out as described by Szeto et al. (61). RNA isolated from *R. meliloti* 1021 and *R. meliloti* 5001 (*ntrC*) cultures grown under repressing (N) or derepressing (no N) conditions was spotted onto nitrocellulose filters and hybridized to ^{32}P-labeled *R. meliloti* total DNA, pFB6162 (*glnT*) DNA, and pCE116 (*fixABCX*) DNA. Expression of *glnT* was found to parallel *fixABCX* expression, being repressed under nitrogen excess conditions and controlled by *ntrC* (Fig. 6) (61).

The phenotypes of each of the *R. meliloti gln* mutants and of other *R. meliloti* regulatory mutants are summarized in Table 2.

DISCUSSION

We have shown that in addition to *glnA* and *glnII*, *R. meliloti* has a novel complex locus, *glnT*, involved in the biosynthesis of glutamine when present in multiple copies in *E. coli*. Surprisingly, we were able to clone the *glnT* locus but not the *glnA* or *glnII* gene by direct complementation of an *E. coli glnA* deletion mutant. The failure to clone *glnA* and *glnII* by direct complementation was not due to the absence of these genes in our cosmid libraries, since we were

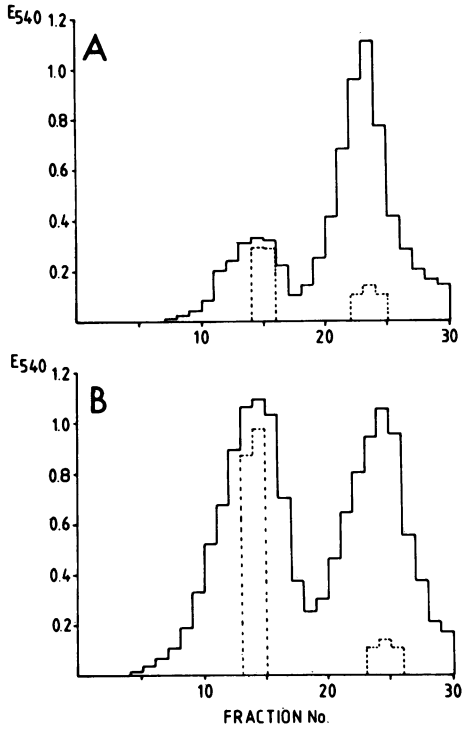

FIG. 5. GS activity profiles of crude extracts. For details, see the legend to Fig. 4. (A) *R. meliloti* 1021; (B) *R. meliloti* 3-53 (*glnT*::Tn*5*).

able to identify cosmid clones containing the *glnA* and *glnII* genes by using heterologous hybridization probes. Our failure to clone *glnII* by complementation appears to be due to the fact that the *R. meliloti glnII* gene is not expressed in *E. coli*. Our failure to clone *glnA* by complementation may be due to the fact that the *R. meliloti glnA* gene is located at the junction of a 4-kb *Eco*RI fragment and a 20-kb *Eco*RI fragment. Because the insert size limit in pLAFR1 is approximately 25 kb, it is possible that cosmids that carry both the 4- and 20-kb *Eco*RI fragments are generally underrepresented in pLAFR1 libraries containing *Eco*RI partial digest inserts. Indeed, this was true in the second pLAFR1 library which we constructed for this project, in which cosmids that carried both *Eco*RI fragments were present at a frequency of 1 in 900, about fourfold less frequent than expected.

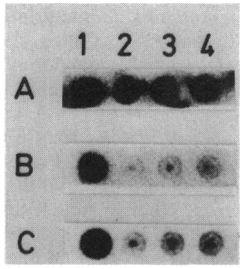

FIG. 6. Transcriptional regulation of the *R. meliloti glnT*, *fixABC*, and *glnA* genes. Autoradiogram of an RNA dot blot analysis. Samples (10 μg) of RNA purified from *R. meliloti* 1021 (wild type) (lanes 1 and 2) and *R. meliloti* 5001 (*ntrC*) (lanes 3 and 4) grown under nitrogen excess (lanes 2 and 4), or nitrogen-limited (lanes 1 and 3) conditions (61), were spotted onto nitrocellulose and hybridized with ^{32}P-labeled *glnT* (11.2-kb *Bam*HI fragment of pFB6162; B), *fixABC* (3.4-kb *Eco*RI-*Hin*dIII fragment of pCE116; C), and total *R. meliloti* DNA (positive control; A). For details, see Szeto et al. (61) and the text.

The *R. meliloti* 1021 *gln* locus cloned in plasmid pFB68 2 is analogous to *glnA* loci from enteric bacteria (2, 16) and members of the *Rhizobiaceae* (8, 20, 25, 57, 59). These *glnA* loci all share a high degree of DNA sequence conservation (as determined by Southern blotting and DNA sequence comparison), express a heat-stable GS, and encode a polypeptide of similar size (55,000 to 59,000 daltons; 2, 8, 16, 20, 25, 57, 59). In *R. leguminosarum* LPR1705, an open reading frame directly upstream of *glnA* encodes a protein of 12,000 to 14,000 daltons with homology to the *glnB* product (PII) of *E. coli* (12). We did not detect the production of a protein of similar size in the *R. meliloti* 1021 *glnA* region; however, the size of the *R. meliloti glnA* locus as determined by Tn*5* mutagenesis (Fig. 3) is large enough to accommodate a *glnBA* operon.

The *R. meliloti* 1021 *gln* locus cloned in plasmid pFB69 is analogous to the *glnII* loci of *B. japonicum* (9) and *A. tumefaciens* (57) in terms of DNA homology, molecular mass of the *glnII* gene product (monomer ~36,000 daltons), and phenotype of *glnII*::Tn*5* mutants (see below).

It is clear from these and other studies (8, 9, 57, 59) that distinct, unlinked genes encode GSI (*glnA*) and GSII (*glnII*). Moreover, studies on *A. tumefaciens* (57) and *R. meliloti* 1021 (this study; S. Rossbach, N. Heycke, and F. J. de Bruijn, unpublished observation) suggest that the *glnA*, *glnII*, and *glnT* loci are all located on the chromosome and not on Ti (tumor-inducing) or pSym (symbiotic plasmid) replicons.

In addition, no linkage between the *glnA* (or *glnII*) gene and the *ntrC* gene has been found in *A. tumefaciens* (56, 57), *R. meliloti* 1021 (61), or *A. caulinodans* (50), in contrast to the situation in enteric bacteria (2, 16, 21, 24).

Phenotypes of the *gln* mutants. The Gln$^+$ Asm$^+$ Fix$^+$ phenotype of *glnA*::Tn*5* *R. meliloti* 1021 mutants is analogous to that described for genetically well-defined *glnA* mutants of *R. meliloti* 104A14 (59) and *B. japonicum* (10). Similarly, the Gln$^+$ Asm$^+$ Fix$^+$ phenotype of the *glnII*::Tn*5* *R. meliloti* 1021 mutant that we constructed is the same as that of a *B. japonicum glnII* mutant strain (10) and of less well characterized *glnII* mutants of *R. meliloti* 41 (AK598 [34]) and *R. phaseoli* CFN42 (49). Thus, neither *glnA* or *glnII* appears to be essential for effective symbiotic nitrogen fixation, which parallels the situation in *A. tumefaciens*, in which *glnA* and *glnII* mutants are also Gln$^+$ and capable of tumor induction (virulent, or Vir$^+$ [57]).

The Gln$^-$ Fix$^+$ phenotype of *R. meliloti glnA glnII* double mutant strains was unexpected. Gln$^-$ mutants with a Fix$^-$ phenotype have been reported for *R. meliloti* 41 (37) and *Rhizobium* sp. strain 32HI (39, 42). Moreover, a *glnA glnII* (Gln$^-$) double mutant strain of *B. japonicum* is Nod$^-$ (10), and *glnA* (Gln$^-$) *A. caulinodans* strains are Nod$^+$ Fix$^-$ (19, 23). Assuming that the *glnT* locus is not expressed in bacteroids, in the case of *R. meliloti* 1021, rhizobial glutamine biosynthesis does not appear to be essential for symbiotic nitrogen fixation, suggesting that *Medicago* spp. may be capable of providing glutamine to Gln$^-$ *R. meliloti* bacteroids in planta, whereas *Glycine max* or *Sesbania* species may not be.

The *glnT* locus. The nature and function of the *glnT* locus, described here and by Rossbach et al. (57) for *A. tumefaciens*, remain an enigma. This locus is clearly capable of directing the synthesis of glutamine in *E. coli* when present on a multicopy plasmid (this study) or after fusion to a strong promoter or gene amplification (57). However, we could not detect GS transferase or biosynthetic activity in these strains. Moreover, *glnA glnII* double mutants of *R. meliloti*

1021 and *A. tumefaciens* C58 (57) are strict glutamine auxotrophs, suggesting that the *glnT* gene products may not function in the free-living state. We do not know whether the *glnT* locus is expressed in bacteroids; however, *R. meliloti* 1021 *glnT* mutants are Gln^+ Asm^+ Fix^+, suggesting that *glnT* does not play a special role in symbiotic interactions.

The *glnT* locus appears to consist of a complex operon, based on maxicell analysis of polypeptides encoded by *glnT* and *glnT*::Tn*5* plasmids. Tn*5* mutations at the beginning of the locus (A and B, Fig. 1C) are clearly polar on the expression of genes located downstream in the operon. It is unclear, however, exactly which enzymatic functions are encoded by *glnT*. No other enzymatic activity capable of catalyzing glutamine biosynthesis in *E. coli* except GS has been identified. We considered whether GS activity directed by *glnT* may be a "by-product" of an enzymatic (GS) reaction normally coupled to another biosynthetic pathway, such as purine biosynthesis (40). Purine biosynthesis in *E. coli* involves the entry of glutamine at the enzymatic steps catalyzed by *purM* and *purF* (36). However, plasmid pFB6162 did not hybridize to the cloned *purM* and *purF* (36) genes of *E. coli* (S. Rossbach and F. J. de Bruijn, unpublished observations).

It remains possible that *glnT* is expressed under physiological conditions not tested thus far. It will be interesting to determine the relationship between the presumptive *glnT* product(s) and a third form of rhizobial GS found by Kumar and Rao (38) and Tsuprun et al. (62) which appears to be expressed only in bacteroids. We are presently determining the DNA sequence of the *glnT* region to shed further light on the function of this unusual locus. Whatever its function, it is highly conserved in all *Rhizobiaceae* tested, including *A. caulinodans* (unpublished observations).

Regulation of the *gln* loci. Previous reports have indicated that GSI and GSII expression in the *Rhizobiaceae* is differentially regulated in response to the available nitrogen and carbon sources (14, 41), as well as to the oxygen concentration (1, 14, 51). *glnA* (GSI) gene expression in *B. japonicum* (1, 10) and *A. tumefaciens* (56, 57) appears to be constitutive. In fact, rhizobial GSI activity appears to be controlled only posttranslationally via (reversible) adenylation (13, 14, 39–41).

In this work we have shown that GSII expression in *R. meliloti* is regulated quite differently, via transcriptional control of *glnII* expression through the *ntr* system, in response to the cellular nitrogen status. Thus, the *ntrC* and *ntrA* genes, previously shown to be involved in the regulation of nitrogen fixation and nitrate utilization (*ntrA*, *ntrC*) as well as C_4-dicarboxylic acid transport (*ntrA* [55, 61]) also control *glnII* expression in *R. meliloti*. This conclusion is consistent with our previous studies and observations with *A. tumefaciens*, in which *ntrC* control of *glnII* expression was also documented (56). It is also consistent with conclusions drawn from studies with *B. japonicum*, in which *glnII* mRNA was only found under conditions of severe nitrogen limitation (10). Thus, whereas in *E. coli* the expression of a single *gln* gene (*glnA*) is regulated both transcriptionally (by the *ntr* system) (29, 54) and posttranslationally (by adenylylation) (44), in members of the *Rhizobiaceae* these regulatory circuits are separated and directed at two distinct *gln* genes.

Interestingly, although no direct physiological role for the *glnT* locus in *R. meliloti* could be identified, *glnT* is nevertheless actively transcribed, and its expression appears to be controlled in the same way as that of *glnII*, namely via the *ntr* system in response to the cellular N status. The reason for this coordinate expression remains to be elucidated.

In summary, our studies have revealed the presence of multiple, differentially regulated *gln* loci in *R. meliloti*. Their distinct roles in glutamine biosynthesis under the extremely variable physiological conditions that rhizobia are exposed to in the soil and in planta (in the bacteroid state) is only starting to be understood. However, contrary to expectation, we have shown that none of the three *R. meliloti gln* genes is essential for symbiotic nitrogen fixation.

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