The *Rhizobium meliloti* P_{II} protein, which controls bacterial nitrogen metabolism, affects alfalfa nodule development

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Symbiotic nitrogen fixation involves the development of specialized organs called nodules within which plant photosynthates are exchanged for combined nitrogen of bacterial origin. To determine the importance of bacterial nitrogen metabolism in symbiosis, we have characterized a key regulator of this metabolism in *Rhizobium meliloti*, the uridylylatable P_{II} protein encoded by *glnB*. We have constructed both a *glnB* null mutant and a point mutant making nonuridylylatable P_{II}. In free-living conditions, P_{II} is required for expression of the *ntrC*-dependent gene *glnII* and for adenylation of glutamine synthetase I. P_{II} is also required for efficient infection of alfalfa but not for expression of nitrogenase. However alfalfa plants inoculated with either *glnB* mutant are nitrogen-starved in the absence of added combined nitrogen. We hypothesize that P_{II} controls expression or activity of a bacteroid ammonium transporter required for a functional nitrogen-fixing symbiosis. Therefore, the P_{II} protein affects both *Rhizobium* nitrogen metabolism and alfalfa nodule development.

[Key Words: Symbiotic nitrogen fixation; *Rhizobium meliloti*; *glnB* gene]

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The soil bacterium *Rhizobium meliloti* establishes a specific symbiotic interaction with alfalfa. The symbiotic process begins by a molecular exchange (Peters et al. 1986) resulting in the synthesis of *Rhizobium* lipochitooligosaccharide Nod factors, which are highly potent and specific organogenetic factors responsible for the formation of root nodules (for review, see Carlson et al. 1995; Dénarié et al. 1996). During this organogenesis, *Rhizobium* infects the emerging nodule structure via root hairs and a network of infection threads that traverse cortical cells. Ultimately bacteria are released from infection threads into the cytoplasm of host cells, where they differentiate into nitrogen-fixing bacteroids (Vasse et al. 1990). This differentiation is accompanied by a rapid cessation of bacterial division, so that bacteroids appear functionally as nitrogen-fixing organelles exchanging ammonium for photosynthates. This implies a shift of bacterial nitrogen metabolism from ammonium

assimilation to ammonium export. In this paper we study the coupling between *Rhizobium* nitrogen metabolism and nodule development.

Assimilation of ammonium proceeds principally through a cycle involving glutamine synthetase (GS) and glutamine oxoglutarate aminotransferase (GOGAT) in *Rhizobium*. *R. meliloti* contains three GS genes, *glnA*, *glnII*, and *glnT*, encoding GSI, GSII, and GSIII, respectively (De Bruijn et al. 1989). GSI is homologous to enteric GS and its activity is regulated by enzyme adenylation like in enteric bacteria. Expression of GSI is regulated by combined nitrogen partly in an *ntrC*-dependent manner (Arcondéguy et al. 1996), whereas expression of GSII is strictly *ntrC*-dependent (De Bruijn et al. 1989). The GSIII gene *glnT* is cryptic and normally is not expressed in the presence of GSI or GSII (Shatters et al. 1993).

In many bacterial species regulation of GS involves a central regulatory protein called P_{II} (for review, see Merrick and Edwards 1995). It was therefore of interest to characterize the *R. meliloti* P_{II} gene *glnB* and to determine its involvement in nitrogen-fixing symbiosis. In enteric bacteria, P_{II} is a trimeric protein involved in two

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Figure 1. Western blot analysis of *R. meliloti* P_{II}. Fifteen percent SDS-PAGE gel probed with anti-P_{II} antibody. Five micrograms of protein extract was loaded in each lane. (Lanes 1,2) *R. meliloti* GMI708; (lanes 3,4) GMI3143 (*glnII::Tn5*); (lanes 5,6) GMI5995 (*ntrC::Tn5*); (lanes 7,8) GMI3107 (*ΔglnB10*); (lanes 9,10) GMI3109 (*glnBP5*). *R. meliloti* was grown on minimal medium with 75 mM ammonium (lanes 1,3,5,7,9) or 6 mM glutamate (lanes 2,4,6,8,10) as a nitrogen source.

different responses to a change in nitrogen status. The first response is a genetic response, by which P_{II} inhibits expression of the *ntr* regulon by stimulating NtrC~P dephosphorylation (Keener and Kustu 1988; Atkinson et al. 1994). The second is a metabolic response, by which P_{II} modulates adenylation of GS (Stadtman et al. 1980). In both responses P_{II} functions as an intracellular signal transducer. In nitrogen-limiting conditions, P_{II} is reversibly modified by uridylation of Tyr 51 (Adler et al. 1975). The degree of P_{II} uridylation is low in nitrogen excess and high under nitrogen deficiency, therefore reflecting the nitrogen status of the cell. In nitrogen-excess conditions, native unmodified P_{II} stimulates dephosphorylation of NtrC~P and adenylation of GS. This results in inhibited expression and activity of GS, respectively. Conversely under nitrogen-limiting conditions the modified form P_{II}-UMP stimulates deadenylation of GS without affecting NtrC phosphorylation, which allows maximal expression and full activity of GS (Ginsburg and Stadtman 1973; Atkinson et al. 1994).

Here we investigate the role of the P_{II} protein in the regulation of nitrogen metabolism in *R. meliloti* and show its involvement in GSI adenylation and GS expression. Therefore, like in enteric bacteria, P_{II} appears as an integrator of nitrogen metabolism that controls both metabolic and genetic responses to changes in the nitrogen status of *R. meliloti*. Moreover, the P_{II} protein was essential for an effective nitrogen-fixing symbiosis on alfalfa. Plant infection by *glnB* mutants was significantly impaired, and although the resulting nodules expressed high levels of nitrogenase, alfalfa plants inoculated with *glnB* mutants suffered nitrogen starvation and chlorosis in the absence of added combined nitrogen. These results indicate that bacterial nitrogen metabolism is coupled with alfalfa nodule infection and development and that this coupling involves the P_{II} protein of *R. meliloti*.

Results

Regulation of nitrogen metabolism by the P_{II} protein in *R. meliloti*

The *glnB* gene encoding P_{II} is linked tightly with the *glnA* gene encoding GSI in *R. meliloti* (Arcondéguy et al. 1996). To investigate the function of the P_{II} regulatory protein we constructed a null mutation by in-frame deletion within the *glnB*-coding sequence, therefore minimizing polar effects on the downstream *glnA* gene. This deletion was generated by PCR, subcloned into pLAFR3 to generate pTA26, then recombined into the *R. meliloti* genome using a sucrose counterselection procedure (see Materials and Methods). The resulting *ΔglnB10* strain GMI3107 did not express the P_{II} protein as verified by Western blot analysis (Fig. 1). However it expressed another strongly reacting 13.5-kD protein that might correspond to a second P_{II} protein as has been found in other organisms (De Zamaroczy et al. 1996; Van Heeswijk et al. 1996). The *ΔglnB10* mutant grew prototrophically slightly more slowly than the wild-type strain GMI708 on either rich or synthetic media ($\mu=0.22/\text{hr}$ vs. $\mu=0.26/\text{hr}$, respectively, on minimal medium with ammonium as a nitrogen source).

To test the involvement of the P_{II} protein in nitrogen metabolism in *Rhizobium*, we first investigated expression of the GSI gene *glnII* using a *glnII-lacZ* reporter plasmid (pFB691::MudIIPR48). The results showed that *glnII* expression is strictly *glnB*-dependent (Table 1). We further studied the role of P_{II} on expression and adenylation of GSI. Strains were made *glnII::Tn5* by N3 transduction so as to express GSI as the sole GS. Although P_{II} was not required for the basal expression of *glnA* in rich medium, it was required for *glnA* induction in nitrogen poor media (Table 2). Because both *glnII* expression and *glnA* induction are *ntrC*-dependent (De Bruijn et al. 1989; Arcondéguy et al. 1996), these results suggest that the P_{II} protein is involved in activation of NtrC in *R. meliloti*.

To test whether P_{II} is involved in the regulation of GSI adenylation, we measured GSI adenylation both before and after a nitrogen upshift (Table 3). Whereas in wild-type *R. meliloti*, GSI is adenylylated rapidly follow-

Table 1. Effect of *glnB* mutations on expression of a *glnII-lacZ* fusion

<i>R. meliloti</i> background	Relevant genotype	β-Galactosidase activity ^a (Miller units)	
		KNO ₃	(NH ₄) ₂ SO ₄
GMI708	wild type	4000	340
GMI3107	<i>ΔglnB10</i>	10	12
GMI3109	<i>glnBP5</i>	17	14

R. meliloti strains contained the *glnII-lacZ* reporter plasmid pFB691::MudIIPR48 (De Bruijn et al. 1989). Cultures were grown in minimal medium with 6 mM KNO₃ or 15 mM (NH₄)₂SO₄ as a nitrogen source.

^aStrain background β-galactosidase activity was <10 Miller units in the absence of a reporter plasmid.

Table 2. Expression of GSI activity in *glnB* mutants of *R. meliloti*

Strain	Relevant genotype	LB	GSI total activity (nmoles/min per mg)	
			glutamate	ammonium
GMI3143	<i>glnII::Tn5</i>	150	1200	540
GMI3146	Δ <i>glnB10 glnII::Tn5</i>	160	290	200
GMI3147	<i>glnBP5 glnII::Tn5</i>	150	140	180

γ GT activity reflects both native and adenylylated forms of GSI. Cultures were grown on rich medium (LB) or on minimal medium with 6 mM glutamate or 75 mM NH_4Cl as a nitrogen source.

ing addition of ammonium to a glutamate-grown culture (Arcondéguy et al. 1996), no such response was observed in the Δ *glnB10* background: GSI adenylylation remained uniformly low regardless of combined nitrogen. Therefore, in *R. meliloti*, P_{II} is required for the stimulation of GSI adenylylation similarly as in *Escherichia coli* (Ginsburg and Stadtman 1973).

The *R. meliloti* P_{II} protein possesses the Tyr51 residue that is the target for uridylylation in *E. coli*, and it was efficiently uridylylated in *E. coli* extracts in the presence of [α - ^{32}P]UTP (Fig. 2). This uridylylation depended on the *glnD*-encoded uridylyl transferase (Fig. 2, cf. lanes 1 and 4). To evaluate the relevance of P_{II} uridylylation in *R. meliloti*, we mutated Tyr51 into Phe, which cannot be uridylylated, and recombined this mutation into the *R. meliloti* genome using the same sucrose counterselection procedure as above (see Materials and Methods). The mutated Y51F P_{II} protein was also expressed in *E. coli* and found completely defective for uridylylation, as expected (Fig. 2, lane 3). The *glnB* allele carrying the Y51F mutation was termed *glnBP5*. The phenotype of *glnBP5 R. meliloti* mutant strains was similar to the phenotype of isogenic Δ *glnB10* null mutants in some respects—slow growth, absence of *glnII* expression (Table 1), and absence of GSI induction (Table 2). However GSI was constitutively adenylylated at a high level in a *glnBP5* background (Table 3), which was expected because of the lack of uridylylation of Y51F P_{II} . Nonu-

Table 3. Adenylylation of GSI in *glnB* mutants

Strain	Relevant genotype	LB	GSI adenylylation state ($\bar{n}$)		
			glutamate		ammonium
			(-N)	(+N)	
GMI3143	<i>glnII::Tn5</i>	11	2	10	7
GMI3146	Δ <i>glnB10 glnII::Tn5</i>	2	2	3	3
GMI3147	<i>glnBP5 glnII::Tn5</i>	10	8	8	7

Cultures were grown on rich medium (LB) or on minimal medium with 6 mM glutamate or 75 mM NH_4Cl as a nitrogen source. Where indicated (+N) glutamate-grown cultures were exposed to 15 mM ammonium sulfate for 30 min before measuring GSI adenylylation.

Figure 2. Uridylylation of *R. meliloti* P_{II} in *E. coli*. Fifteen percent SDS-PAGE gel of *E. coli* crude extracts (25 μ g of total protein) stained with Coomassie Blue (A) or labeled with [α - ^{32}P]UTP and autoradiographed (B). (Lane 1) RB9040(pTA2-4F); (lane 2) RB9040(pBSK+); (lane 3) RB9060(pTAJ3); (lane 4) RB9060(pTA2-4F); (lane 5) RB9060(pBSK+). The *glnD*⁻ strain RB9040 is defective for uridylyl-transferase, whereas the *glnB*⁻ strain RB9060 lacks P_{II} . Plasmids pTA2-4F and pTAJ3 overexpress *R. meliloti* P_{II} and the nonuridylylatable Y51F P_{II} protein (P_{II}^*), respectively.

ridylylated P_{II} locks GSI in an inactive form even at low nitrogen.

Expression of the P_{II} protein was followed under nitrogen-limiting and nitrogen-excess conditions by Western blot analysis (Fig. 1). This experiment showed a basal level of P_{II} expression in ammonium grown cells, above which level P_{II} expression could be further induced by nitrogen limitation. This induction was *ntrC*-dependent. Moreover, P_{II} uridylylation was required, as no *glnB* induction was observed in the *glnBP5* mutant background. On the other hand, expression of the cross-reacting 13.5-kD protein appeared constitutive and did not depend on *ntrC* nor on *glnB*.

The P_{II} protein is required for efficient infection of alfalfa

The symbiotic properties of the *glnB* mutants were tested on alfalfa, the homologous symbiont of *R. meliloti*. Nodulation kinetics clearly showed that the *glnB* mutations affect alfalfa nodulation, because nodulation was delayed for 3 days and final nodule number decreased (Fig. 3). Introduction of the *glnB*-containing plasmid pTACG32 into either *glnB* mutant restored normal nodulation kinetics, which showed that the delayed and decreased nodulation phenotype was not caused by secondary mutations outside the *glnB* locus.

This result prompted us to investigate the infection process with the *glnB* mutants. For this purpose, we introduced the constitutively expressed *hemA-lacZ* reporter plasmid pXLGD4 into the mutant strains to follow infection histochemically. Both *glnBP5* and Δ *glnB10* mutants exhibited similar symbiotic phenotypes, albeit somewhat stronger for *glnBP5* than for Δ *glnB10*. Five

Figure 3. Nodulation kinetics of *M. sativa* inoculated with *glnB* mutants. Nodule number was counted daily and averaged over 20 plants. (■) GMI708; (▲) GMI3109; (●) GMI3107.

days after inoculation, the *glnB* mutants induced various root hair deformations such as a tight curling at the tip of root hairs (Hac⁺ phenotype, Fig. 4A), a characteristic deformation observed on plants inoculated with wild-type *R. meliloti*, but also exhaustive deformations of long root hairs (Fig. 4B), a phenotype that was never observed on control plants. At this stage, starch accumulated either throughout the entire root (Fig. 4C) or in large nodule primordia (Fig. 4D). Such a starch accumulation was not observed on plants inoculated with wild-type *Rhizobium*.

A time-course analysis of the infection process showed obvious differences between plants inoculated with *glnB* mutants and wild-type *R. meliloti*. On control plants inoculated with wild-type *R. meliloti*, infection threads initiated in a curled root hair progressed rapidly toward the inner plant cortex, where the first divisions characteristic of the formation of a nodule primordium were

Figure 4. *R. meliloti glnB* mutants are affected in the infection process. Similar pictures were obtained with either the $\Delta glnB10$ mutant GMI3107 or the *glnBP5* mutant GMI3109. For conciseness and clarity, the above pictures are presented for either mutant. (A,B) Root hair deformations induced by the $\Delta glnB10$ mutant. The slight blue coloration reveals β -galactosidase activity of rhizobia bound to root hairs. In A, arrowheads point to curled root hairs. Bar, 50 μ m. (C,D) Histological localization of starch in cortical cells or in nodule primordia of plants inoculated with the *glnBP5* (C) or the $\Delta glnB10$ mutant (D). Bar, 100 μ m. (E–G) Infection phenotype observed 5 days after inoculation with wild-type *R. meliloti* GMI708 (E) or with the *glnBP5* mutant (F,G). In E, the arrowhead points to a dividing inner cortical cell. In G, an infected epidermal cell is marked with an asterisk. Bar, 50 μ m. (H–K) Developing nodules induced by wild-type *R. meliloti* (H,I) or $\Delta glnB10$ (J) and *glnBP5* (K) mutants. Note the lack of infection (6 days post inoculation, cf. H and I) or reduced infection by *glnB* mutants (7 days postinoculation, cf. J and K) of the nodule central regions. Also note the unusual photon density of the nodule meristem in I (star). Bar, 50 μ m.

often seen (Fig. 4E). On plants inoculated with *glnB* mutants, the development of infection threads was impaired significantly. Infection threads were frequently observed as abortive twisted structures remaining within the root hair (Fig. 4F) or in the epidermal cell (Fig. 4G). As a result of this incapacity of infection threads to progress normally into plant tissues, and contrary to control plants where the central part of growing nodules was occupied by an important network of infection (Fig. 4H), no noticeable infection was seen in the central part of many of the nodules elicited by *glnB* mutants (Fig. 4I). This infection was delayed and, when it occurred, it was significantly reduced when compared with control plants (Fig. 4, cf. J and K). Finally, it was noticed that many nodule primordia elicited by the *glnB* mutant strains appeared abnormally dense to photons, suggesting cell wall thickening (Fig. 4I). Another difference in the infection properties of *glnB* mutants versus the parental strain also appeared from quantitative data. Roots were stained with X-Gal 5 days after inoculation and infection threads were counted on 10 plants. The *glnB* mutants were found to infect alfalfa considerably less efficiently than the wild type. Whereas wild-type *R. meliloti* generated an average of 52 infection threads per plant, the $\Delta glnB10$ and *glnBP5* mutants averaged 12 and 8 infection threads per plant, respectively. These results indicate that the P_{II} protein, in its uridylylated form, is required for efficient infection of alfalfa by *R. meliloti*.

The defective infection phenotype described above prompted us to consider the possibility that *glnB* might regulate expression of *nod* genes, which are known to have a major role during infection (Debellé et al. 1986; Ardourel et al. 1994, 1995). To test this possibility, we introduced plasmids containing *lacZ* fusions in the structural gene *nodC* (pRmM57), or in the regulatory genes *nodD1* (pGMI930), *nodD2* (pGMI1003), *nodD3* (pGMI1004), or *syrM* (pGMI1005), into wild-type and *glnB* mutant backgrounds. The *glnB* mutations consistently caused a threefold decrease of *nodC-lacZ* expression from pRmM57 (Table 4). No effect was observed on expression of the regulatory genes *nodD1*, *nodD2*, *nodD3*, and *syrM* (data not shown). Therefore, the P_{II} protein is not stringently required for expression of *nod*

Table 4. Effect of *glnB* mutations on expression of *nodC*

<i>R. meliloti</i> background	Relevant genotype	β -Galactosidase activity ^a (Miller units)	
		–luteolin	+ luteolin
GMI708	wild type	29	290
GMI3107	$\Delta glnB10$	17	88
GMI3109	<i>glnBP5</i>	20	87

R. meliloti strains contained the *nodC-lacZ* reporter plasmid pRmM57 (Mulligan and Long 1985). Cultures were grown in minimal medium containing 14 mM succinate and 6 mM glutamate with or without 10 μ M luteolin.

^aStrain background β -galactosidase activity was <10 Miller units in the absence of a reporter plasmid.

Figure 5. *glnB* mutants exhibit a Nif⁺ Fix⁻ phenotype. Three-week-old alfalfa plants inoculated with *R. meliloti* GMI708 (WT), with GMI3109 (*glnBP5*), or not inoculated (H₂O). Where indicated, the growth medium had been complemented with 2 mM ammonium sulfate one week earlier. Corresponding ARA was measured on whole plants.

genes, but it does affect induction of the *nodABC* operon by luteolin.

P_{II} defective nodules contain an active nitrogenase but poorly support plant growth

Three-week-old alfalfa plants inoculated with either *glnB* mutant were stunted and chlorotic to various extents (Fig. 5). Dry weight measurements and nitrogen quantitation showed that these plants were nitrogen-starved (Table 5). Nodules appeared somewhat heterogeneous ranging from whitish and small to pink and normally sized. Because such a heterogeneity was observed among nodules on individual plants, it was not related to the genetic heterogeneity of alfalfa. The reinoculation on alfalfa of bacteria reisolated to single colonies from different types of nodules resulted in the same phenotype, that is, chlorotic plants bearing heterogeneous nodules. This result demonstrates that the normal-looking nodules were not attributable to suppressive mutations. Moreover, complementation of the *glnB* mutations with the *glnB*-containing plasmid pTACG32 restored the wild-type symbiotic phenotype, showing that the *glnB* mutations are responsible for plant nitrogen starvation and nodule heterogeneity. Unexpectedly, plants inoculated with *glnB* mutants did reduce acetylene to appreciable levels, with an average of 40% and 56% of the wild-type level for *glnBP5* and $\Delta glnB10$ mutant strains, respectively. This acetylene reduction activity (ARA) was quite variable, consistent with the nodule heterogeneity noted above. No strict correlation was observed between plant growth and ARA. For instance, the stunted plants shown in Figure 5, lane 3, exhibited 79% of wild-type ARA, whereas other better looking plants showed lower ARA. No correlation was either observed between plant growth and nodule aspect: We routinely observed stunted plants carrying normal-sized, pink nodules that, when excised, reduced acetylene at similar levels as wild-type excised nodules.

Table 5. Nitrogen deficiency of *glnB* mutant-inoculated alfalfa

Relevant genotype	Dry weight per plant (mg)	Total nitrogen per plant (μg)	Nitrogen content (%)
<i>aerial parts</i>			
Wild type	5.70 ± 0.48	263 ± 26	4.6 ± 0.2
<i>ΔglnB10</i>	3.50 ± 0.50	132 ± 39	3.8 ± 0.8
<i>glnBP5</i>	3.25 ± 0.87	110 ± 38	3.4 ± 0.5
<i>roots</i>			
Wild type	0.81 ± 0.07	22 ± 1	2.7 ± 0.2
<i>ΔglnB10</i>	1.05 ± 0.09	26 ± 2	2.5 ± 0.2
<i>glnBP5</i>	1.42 ± 0.17	30 ± 3	2.1 ± 0.2
<i>nodules</i>			
Wild type	0.40 ± 0.06	23 ± 2	5.7 ± 0.5
<i>ΔglnB10</i>	0.37 ± 0.02	19 ± 2	5.1 ± 0.6
<i>glnBP5</i>	0.37 ± 0.03	21 ± 3	5.5 ± 0.8
<i>whole plant</i>			
Wild type	6.91 ± 0.51	308 ± 28	4.4 ± 0.2
<i>ΔglnB10</i>	4.92 ± 0.43	177 ± 38	3.6 ± 0.6
<i>glnBP5</i>	5.04 ± 0.85	161 ± 41	3.2 ± 0.5

R. meliloti strains GMI708, GMI3107 (*ΔglnB10*), and GMI3109 (*glnBP5*) were inoculated on alfalfa, and nitrogen content was assayed 3 weeks later. Standard deviations were calculated on five batches of four plants.

The high nitrogen fixation activity of *glnB* mutants was directly confirmed by ¹⁵N-labeling experiments, which showed 66% and 64% of the wild-type level for *glnBP5* and *ΔglnB10* mutant strains, respectively (Table 6). Nodule nitrogenase specific activities were 71% and 69% of wild-type, respectively, showing that *R. meliloti glnB* mutants are Nif⁺. Consistent with the presence of an active nitrogenase, the *glnB* gene was found not to be required for expression of *nifA*, *fixK*, and *fixN* as monitored with the *lacZ* fusion reporter plasmids pCHK57, pJJ5, and pGMI931, respectively (data not shown). However, and consistent with the nitrogen-starved phenotype noted above, the flux of combined nitrogen from nodules toward the aerial parts of the plants was markedly reduced in *glnB* mutant inoculated plants (Table 6). On addition of ammonium sulfate, plant growth resumed normally and chlorosis reverted (Fig. 5, lane 4),

which confirmed that plants had suffered nitrogen starvation (Fix⁻ phenotype) despite the high level of nitrogenase activity (Nif⁺ phenotype). This experiment also ruled out the alternative possibility that chlorosis could be attributable to a toxic effect of *glnB* mutants on alfalfa. Therefore, *glnB* mutants induce the formation of nodules that contain active nitrogenase but support plant growth poorly in the absence of added combined nitrogen.

Histological examination of *glnB* mutant induced nodules confirmed the heterogeneity mentioned above. The clearing of entire nodules with sodium hypochlorite, followed by staining with potassium iodide, revealed that starch that is deposited only in the cells of the discrete interzone II–III in wild-type nodules (Fig. 6A; Vasse et al. 1990) accumulated heavily in the central regions of most of the nodules induced by *glnB* mutants (Fig. 6B). Moreover, 80-μm thick longitudinal sections of three-week-old nodules showed that nodules differed from each other in terms of invasion of the central cells. Occupancy of nodule central tissue ranged from high to scarce, with various intermediates in between (Figs. 6C–6F). Methylene blue/toluidine blue counterstaining of nodule sections that were poorly invaded stained a few other central cells, indicating that these cells were not optically empty (arrows in Fig. 6, D and E). This latter staining could correspond to cells occupied by either viable bacteria that had lost the pXLGD4 reporter plasmid or by bacteroids undergoing precocious senescence. Histology of the nodules also led to two conclusions (Figs. 6C–6F). (1) Infection threads were observed on nodule sections whatever the level of invasion of central cells. Therefore, low invasiveness was not attributable to lack of infection threads. (2) Starch accumulation in nodule cells varied considerably among the nodules elicited by the *glnB* mutant strains.

Electron microscopic examination confirmed the data described above and showed that bacteroid differentiation was similar to that of the wild type, including differentiation into mature nitrogen-fixing type IV bacteroids (Vasse et al. 1990; Fig. 7). This is consistent with the ARA measured with these nodules. However, it was also noticed that *glnB* bacteroids senesced more rapidly than wild-type bacteroids and that this senescence could start in the most distal cells of the nitrogen-fixing zone III (Fig. 7B).

Table 6. Partitioning of fixed nitrogen in *glnB* mutant inoculated alfalfa

Relevant genotype	Nitrogen fixed per plant (nmoles of N ₂)				Nodule nitrogenase sp. ac. (nmoles of N ₂ /hr per mg of dry nodule weight)
	aerial parts	roots	nodules	whole plant	
Wild type	30.8 ± 4.7	3.9 ± 0.5	33.9 ± 6.1	68.7 ± 5.7	86 ± 17
<i>ΔglnB10</i>	13.6 ± 4.5	4.4 ± 1.2	25.8 ± 2.3	43.8 ± 5.1	59 ± 7
<i>glnBP5</i>	11.0 ± 4.7	5.6 ± 0.8	28.5 ± 7.3	45.1 ± 11.4	61 ± 14

The same alfalfa plants as in Table 5 were exposed for 2 hr to ¹⁵N₂-labeled air and assayed for ¹⁵N incorporation. Standard deviations were calculated on five batches of four plants.

Figure 6. Histology of *glnB* mutant induced nodules. (A,B) Histological localization of starch (brown color) in whole nodules elicited by wild-type *R. meliloti* (A) or the *glnBP5* mutant (B). The arrowhead in A shows the starch deposit in the few cell layers of interzone II-III in control nodules. Dark-field microscopy. Bar, 200 μ m. (C-F) Various nodules elicited by *glnB* mutants. Similar pictures were obtained with either the Δ *glnB10* or the *glnBP5* mutant. Note the differences between individual nodules in terms of cell occupancy by rhizobia (blue color) and starch deposit (arrowheads). Arrows in D and E point to methylene blue/toluidine blue counter-stained cells. Eighty-micrometer-thick sections viewed by bright-field microscopy. Bar, 100 μ m.

Discussion

Functionality of a nitrogen-fixing nodule requires that both plant and rhizobial symbiotic partners mutually adapt to meet the physiological requirements of nitrogen fixation and to allow for assimilation of fixed nitrogen. On the plant side, this implies a need to provide bacteroids with the low oxygen tension necessary for nitrogenase expression and activity and to fuel nitrogen fixing bacteroids with appropriate photosynthates for their en-

ergy metabolism. On the bacterial side, this implies the routing of fixed nitrogen into plant amino acid metabolism. It has therefore been long expected that the development of a functional symbiosis should be coupled with an appropriate regulation of bacterial nitrogen metabolism. For instance, expression of *E. coli* glutamate dehydrogenase in *Rhizobium etli* interferes with nodulation (Mendoza et al. 1995) and nitrogen fixation (Bravo et al. 1988). On the other hand *R. meliloti* mutants defective for GSI or GSII (De Bruijn et al. 1989), or for the

Figure 7. Ultrastructure of *glnB* mutant bacteroids. Distal part of the nitrogen fixing zone III of nodules induced by wild-type *R. meliloti* (A) or the *glnBP5* mutant (B). Note the nitrogen-fixing type IV bacteroids (white stars). Features of premature nodule degeneration in B are electron-dense bacteroids (white asterisks) and senescent rhizobia enclosed within a single peribacteroid membrane (black star). Electron microscopy. Bar, 1 μ m.

global nitrogen regulator NtrC (Szeto et al. 1987), develop normal nitrogen-fixing nodules on alfalfa. In the present work we identify the P_{II} protein of *R. meliloti* as a key regulatory component involved in both bacterial nitrogen regulation and nodule development. Not only is the P_{II} protein required for proper plant assimilation of nitrogen fixed by *Rhizobium* bacteroids. It is also involved in plant infection. Therefore, the P_{II} protein integrates the regulation of a wide array of functions related to bacterial nitrogen metabolism, nodule development, and symbiotic nitrogen fixation.

The P_{II} protein as an integrator of nitrogen metabolism in *Rhizobium*

In enteric bacteria the P_{II} protein has a central role in nitrogen regulation because it is involved in both genetic regulation of the *ntr* regulon and in metabolic regulation of GS activity. It is an integrator of the cellular nitrogen status, responding to α -ketoglutarate and glutamine pools via reversible uridylation (Atkinson et al. 1994). Native trimeric P_{II} binds α -ketoglutarate and stimulates NtrB-catalyzed dephosphorylation of NtrC-P (Kamberov et al. 1995), thereby affecting expression of GS and of other *ntrC*-dependent genes. Fully uridylylated P_{II}, on the other hand, does not affect phosphorylation of NtrC in *E. coli* (Atkinson et al. 1994).

In *R. meliloti*, P_{II} also affects the *ntr* regulon, albeit somewhat differently (Fig. 8). As in enteric bacteria, nonuridylylated P_{II} (expressed in the *glnBP5* mutant) prevents expression of *ntrC*-dependent genes such as *glnII*, presumably by stimulating the dephosphorylation of NtrC-P. Unlike in enteric bacteria however, the *R. meliloti* Δ *glnB* null mutant is *ntr*-deficient as judged from the absence of *glnII* expression and *glnA* induction. We therefore suggest that in *R. meliloti*, the P_{II} protein, in its uridylylated form, is required for NtrB-dependent activation of NtrC by phosphorylation, or possibly for expression of the *ntrC* gene itself. Thus, absence of P_{II}-UMP signals nitrogen excess for the *ntr* regulon in *R. meliloti*.

The other characterized function of P_{II} in enteric bacteria is the modulation of GS activity by reversible covalent enzyme adenylation. In *R. meliloti* GSI is regulated similarly by reversible adenylation in response to the cellular nitrogen status (Arcondéguy et al. 1996). Like in enteric bacteria, GSI adenylation is stimulated by the native P_{II} protein whereas P_{II}-UMP promotes GSI deadenylation (Table 3). Therefore, absence of P_{II} signals nitrogen deficiency for the regulation of GSI activity by adenylation, whereas nonuridylylated P_{II} signals nitrogen excess for both genetic *ntr* regulation and metabolic regulation of GSI activity (Fig. 8).

Expression of the P_{II} protein appeared to be partly nitrogen-regulated in *R. meliloti* (Fig. 1). P_{II} was expressed at a low level in nitrogen-rich conditions and was induced on nitrogen limitation in an *ntrC*-dependent manner. Consistent with this regulation, a characteristic σ^{54} -dependent promoter sequence is conserved

Figure 8. Current model for the regulation of nitrogen assimilation in *R. meliloti*. Uridylyltransferase catalyzes uridylation (UTase) and deuridylylation (UR) of the P_{II} protein (the *glnB* gene product). Adenylyltransferase (ATase) catalyzes adenylation and deadenylation of GSI (the *glnA* gene product). NtrB catalyzes phosphorylation and dephosphorylation of NtrC. The phosphorylated form of NtrC activates various promoters, including the *glnB* and *glnII* promoters. On the basis of the *Ntr*⁻ phenotype of the Δ *glnB10* mutant we propose that P_{II}-UMP stimulates NtrC phosphorylation in *R. meliloti*, contrary to what is observed in enteric bacteria. Note that the *glnBP5* mutant making nonuridylylatable P_{II} will be locked in a high GSI adenylation, low NtrC phosphorylation mode.

upstream of *glnB* in *R. meliloti* and *Rhizobium leguminosarum* bv. *viciae* (Chiurazzi and Iaccarino 1990), and the downstream gene *glnA* is also partly inducible under the control of *ntrC* (Arcondéguy et al. 1996). In addition, the *glnB* gene appears to be autoregulated as it was not induced in the *glnBP5* background (Fig. 1). This autoregulation of *glnB*, together with its *ntrC*-dependence, is consistent with the role of P_{II}-UMP in *ntr* regulation as noted above: Uridylylation of P_{II} is required for activation of NtrC, hence for induction of *glnB* (Fig. 8).

Coupling between *Rhizobium* nitrogen metabolism and plant infection

A striking and unexpected finding is that the P_{II} protein of *R. meliloti* strongly interferes with the plant infection process. *glnB* mutants are unable to infect the plant normally. Although they elicit proper root hair curling, they form a reduced number of infection threads which often abort (Fig. 4). Therefore, the *glnB* mutant developmental bottleneck appears to lie after infection thread initiation, at the level of infection thread progression. The lowered infectivity of *glnB* mutants is accompanied by strong root hair deformations and unusual starch deposition in

the root. These symbiotic defects are observed with either the $\Delta glnB10$ null mutant or the $glnBP5$ mutant unable to uridylylate P_{II} . This indicates that the uridylylated form P_{II} -UMP is required for a normal establishment of the symbiotic relationship, including the infection step, in alfalfa. In some respects the early plant responses to $glnB$ mutants resemble those that are elicited on alfalfa by various *nod* mutants such as *nodF*, *nodE*, *nodL*, *noeA*, and *noeB* mutants (Debellé et al. 1986; Ardourel et al. 1994, 1995), and by exopolysaccharide deficient mutants (*exo* mutants; Hirsch et al. 1984; Niehaus et al. 1993). All these mutants impaired in plant infection also elicit unusually intense root hair deformations and starch deposition at the root level (G. Truchet, unpubl.), as do $glnB$ mutants (this study). Therefore, the P_{II} protein couples plant infection with the regulation of nitrogen metabolism in *R. meliloti*.

One distinct possibility that could account for the defective infection phenotype of $glnB$ mutants is the regulation of *nod* gene expression by the P_{II} protein. Although not essential, the $glnB$ gene is required for full induction of the *nodABC* operon by the specific flavonoid inducer luteolin (Table 4). In this respect, it is worth recalling that the *nodABC* operon is also nitrogen-regulated (Dusha et al. 1989, 1993). It is not unprecedented that altered regulation of *nod* gene expression may alter infection and nodulation properties of *Rhizobium*. For instance, overexpression of the regulatory gene *nodD3* is known to perturb the spectrum of Nod factors synthesized and their symbiotic properties (Demont et al. 1994). $glnB$ mutations might act in a similar way, by changing the balance of *nod* gene expression and Nod factor synthesis, resulting in defective infection. However it should be noted that infection by $glnB$ mutants was not stimulated by exogenous addition of purified Nod factors (10^{-7} M; data not shown). An alternative possibility that cannot be ruled out presently would be a reduced development of bacteria within infection threads due to an altered utilization of plant nitrogenous compounds, therefore impairing the development of infection threads.

Uncoupling between nitrogen fixation and nitrogen assimilation

Remarkably, plants inoculated with $glnB$ mutants present strong nitrogen starvation symptoms despite a high nitrogenase content as assayed by acetylene reduction activity (ARA) and ^{15}N incorporation (Fig. 5; Table 6). The $glnBP5$ mutant induces slightly more severe plant symptoms than the $\Delta glnB10$ null mutant. This can be interpreted in terms of the model shown on Figure 8: The $glnBP5$ mutant makes a P_{II} protein that cannot be uridylylated, therefore locking nitrogen regulation in the OFF mode, whereas the $\Delta glnB10$ mutant makes no P_{II} protein at all, therefore letting P_{II} -regulated systems reach intermediate steady-states. Consistent with the high ARA, $glnB$ mutants are not affected in expression of *nif* and *fix* genes that are required for nitrogenase expression or ac-

tivity. $glnB$ mutants also develop into mature nitrogen-fixing type IV bacteroids (Fig. 7), which are not found with *nif* or *fix* mutants of *R. meliloti* (Vasse et al. 1990). In addition, nodules induced by $glnB$ mutants accumulate large amounts of starch (Fig. 6B), which indicates an imbalance of carbon over nitrogen metabolites consistent with the overall nitrogen starvation symptoms noted above.

We have considered several possibilities to explain this paradoxical $\text{Nif}^+ \text{Fix}^-$ phenotype. First, it might have resulted from defective expression of the *ntr* regulon. However, because *ntrC* mutants of *R. meliloti* are Fix^+ (Szeto et al. 1987), expression of the *ntr* regulon is clearly not essential for symbiosis. The second possibility we have considered is altered regulation of GS expression. However, *R. meliloti* bacteroids contain no GSII and only low levels of GSI (De Bruijn et al. 1989; Arcondéguy et al. 1996). Moreover, *glnA*, *glnII*, and *glnAglnII* double mutants of *R. meliloti* are Fix^+ , which shows that GSI and GSII are dispensable for symbiosis (De Bruijn et al. 1989). The third possibility concerns GSI adenylation, which is affected by P_{II} (Table 3). However, a *glnA1* mutant making an adenylation-defective GSI is Fix^+ , which shows that GSI adenylation is not required for symbiosis (Arcondéguy et al. 1996). Moreover, although the $\Delta glnB10$ and $glnBP5$ mutations lock GSI in opposite adenylation modes (Table 3), they cause very similar symbiotic phenotypes. Therefore, GSI adenylation is not involved. The fourth possibility is that the conjunction of delayed infection and slightly reduced nitrogenase activity causes the seedling to be nitrogen starved at a critical stage. Root growth is stimulated in response to this starvation (Table 5), which is a well-known response to nutrient limitation (Clarkson 1985). The resulting increased demand for nitrogen in roots (Table 6) would in turn further aggravate nitrogen starvation of the aerial parts. Although we are not aware of precedents for such a nutritional dead-lock, we cannot rule it out presently. The fifth possibility is that $glnB$ mutants could be affected in expression or activity of a bacteroid ammonium transporter. It has long been thought that the intensive flux of ammonium between bacteroids and the host cell cytoplasm could be achieved by passive diffusion of uncharged ammonia across both bacteroid and peribacteroid membranes. However, recently, Tyerman et al. (1995) used patch-clamp techniques to demonstrate the existence of a specific ammonium transporter in the peribacteroid membrane, therefore questioning the role of passive ammonia diffusion in symbiosis. In addition, a number of ammonium transporters have now been identified in plants (Ninneman et al. 1994), yeast (Marini et al. 1994), and bacteria (Wray et al. 1994). The existence of an ammonium transporter in the peribacteroid membrane hints toward the existence of a matching transporter in the bacteroid membrane, which would allow for high ammonium efflux at physiological pH. Therefore, we hypothesize that the *R. meliloti* P_{II} protein is involved in the regulation of a bacteroid ammonium transporter important for a functional nitrogen-fixing symbiosis.

Materials and methods

Bacterial strains, media and microbiological techniques

Strains and plasmids used are listed in Table 7. *R. meliloti* was grown at 30°C in Luria broth in the presence of appropriate antibiotics or in minimal V medium (Arcondéguy et al. 1996) with 0.4% glucose and nitrogen sources as indicated. Plasmids were introduced into *R. meliloti* strains by conjugation. To make *R. meliloti* strains deficient for GSII, the *glnII::Tn5* mutation from strain 2-37 was transduced using phage N3 (Martin and Long 1984).

glnB mutant construction

The point mutation replacing Tyr-51 with Phe was constructed by PCR with pTA2 as a template, the M13 -20 primer and the

mutagenic oligonucleotide 5'-CGGAGCTCTACCGCGGCG-CAGAATTTGTCGTCG containing a naturally occurring *SacI* site (italicized) just upstream of the mutated codon (bold). In-frame deletion of *glnB* was constructed by PCR with pTA2 as a template, the M13 -20 primer and the mutagenic oligonucleotide 5'-CGGAGCTCTTCGTATCTAATG that provides a new *SacI* site 138 nucleotides downstream of the original *SacI* site in *glnB*. The 780-bp (point mutation) and 642-bp (deletion) PCR products were made blunt-end with T4 DNA polymerase, cloned into the *SmaI* site of pBluescript, and sequenced. The *SacI*-*EcoRI* inserts containing the point mutation or the 138 nucleotide deletion were cloned into pTA9 to replace the wild-type sequence, which generated pTA20 and pTA21, respectively. The 6.44- and 6.31-kb *HindIII*-*BamHI*-mutated fragments from pTA20 and pTA21 were then recloned into the

Table 7. Strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
<i>Rhizobium meliloti</i>		
GMI708	Rif ^r derivative of strain 2011	Batut et al. (1985)
GMI3063	<i>glnB::sacB</i> , Rif ^r , Gm ^r , Nm ^r	this work
GMI3107	<i>ΔglnB10</i> , Rif ^r , Gm ^r	this work
GMI3109	<i>glnBP5</i> , Rif ^r , Gm ^r	this work
GMI3143	<i>glnII::Tn5</i> Sm ^r , Nm ^r , Rif ^r	Arcondéguy et al. (1996)
GMI3146	GMI3107 transductant, <i>ΔglnB10 glnII::Tn5</i> , Sm ^r , Nm ^r , Rif ^r	this work
GMI3147	GMI3109 transductant, <i>glnBP5 glnII::Tn5</i> , Sm ^r , Nm ^r , Rif ^r	this work
GMI5995	<i>ntrC21.2::Tn5</i> , Sm ^r , Nm ^r , Rif ^r	Arcondéguy et al. (1996)
<i>Escherichia coli</i>		
RB9040	<i>glnD99::Tn10</i> , Tc ^r	Bueno et al. (1985)
RB9060	<i>ΔglnB2306</i>	Bueno et al. (1985)
Plasmids		
pCHK57	Tc ^r , <i>nifA-lacZ</i> fusion	Ditta et al. (1987)
pFB682	Ap ^r , <i>glnA</i> -containing pBR322 derivative	De Bruijn et al. (1989)
pFB691::MudIIIPR48	Tc ^r , <i>glnII-lacZ</i> fusion	De Bruijn et al. (1989)
pGMI930	Tc ^r , <i>nodD1-lacZ</i> fusion	Faucher et al. (1988)
pGMI931	Tc ^r , <i>fixN-lacZ</i> fusion	David et al. (1988)
pGMI1003	Tc ^r , Nm ^r , <i>nodD2::Tn5</i> -B20 pMH902 derivative	F. Maillat (pers. comm.)
pGMI1004	Tc ^r , Nm ^r , <i>nodD3::Tn5</i> -B20 pMH903 derivative	F. Maillat
pGMI1005	Tc ^r , Nm ^r , <i>syrM::Tn5</i> -B20 pMH904 derivative	F. Maillat
pIB279	Ap ^r , Nm ^r , <i>sacB-neo</i> cassette	Blomfield et al. (1991)
pIJ5	Tc ^r , <i>fixK-lacZ</i> fusion	Batut et al. (1989)
pMH902	Tc ^r , <i>nodD2</i> -containing pRK290 derivative	M.A. Honma (pers. comm.)
pMH903	Tc ^r , <i>nodD3</i> -containing pWB5A derivative	Honma et al. (1990)
pMH904	Tc ^r , <i>syrM</i> -containing pWB5A derivative	Honma et al. (1990)
pPH1JI	Sm ^r , Gm ^r , Mob ⁺ IncP	Hirsch and Beringer (1984)
pRK2073	Spc ^r , Tp ^r , Tra ⁺ , Mob ⁺	Leong et al. (1982)
pRmM57	Tc ^r , <i>nodC-lacZ</i> fusion	Mulligan and Long (1985)
pTA2	Ap ^r , <i>EcoRI-HindIII</i> fragment of pFB682 in pKS	Arcondéguy et al. (1996)
pTA2-4F	Exo III deletion of pTA2	this work
pTA3	Ap ^r , <i>PvuII-HindIII</i> fragment of pFB682 in pKS	this work
pTA5	Ap ^r , <i>EcoRI-EcoRV</i> fragment of pTA2 in pKS	Arcondéguy et al. (1996)
pTA8	Ap ^r , pUC18-Not deleted of <i>SacI</i> , <i>KpnI</i> , and <i>EcoRI</i> sites	this work
pTA9	Ap ^r , <i>HindIII-BamHI</i> fragment of pTA3 in pTA8	this work
pTA13	Ap ^r , <i>sacB</i> cassette inserted into <i>SacI</i> site of pTA9	this work
pTA14	Tc ^r , <i>NotI</i> fragment of pTA13 in pLAFR3	this work
pTA20	Ap ^r , pTA9 with <i>glnBP5</i>	this work
pTA21	Ap ^r , pTA9 with <i>ΔglnB10</i>	this work
pTA25	Tc ^r , <i>HindIII-BamHI</i> fragment of pTA20 in pLAFR3	this work
pTA26	Tc ^r , <i>HindIII-BamHI</i> fragment of pTA21 in pLAFR3	this work
pTACG32	Tc ^r , <i>EcoRI-BamHI</i> fragment of pTA5 in pLAFR3	this work
pTAJ3	pTA2-4F-containing <i>glnBP5</i>	this work
pUC18-Not	Ap ^r	Herrero et al. (1990)
pXLGD4	Tc ^r , <i>hemA-lacZ</i>	Leong et al. (1985)

pLAFR3 vector, which generated pTA25 and pTA26, respectively.

glnB mutations were recombined into the *R. meliloti* genome using a sucrose-sensitive strain, GMI3063, carrying a *sacB-neo* cassette in the *glnB* gene, which was constructed as follows. The *sacB-neo* cassette was excised with *Bam*HI from pB279 (Blomfield et al. 1991), made blunt-end with T4 DNA polymerase, cloned into the unique *Sac*I site of pTA9, generating pTA13. The 7.7-kb *Not*I insert from pTA13 was made blunt-end and recombined into the pLAFR3 *Eco*RI site. The resulting plasmid pTA14 was mobilized into *R. meliloti* by triparental conjugation using the helper plasmid pRK2073. Recombination of the *sacB-neo* cassette into the chromosomal copy of *glnB* was selected for by chasing pTA14 with the incompatible plasmid pPH1JI in the presence of gentamycin (50 µg/ml), rifampicin (200 µg/ml), and neomycin (100 µg/ml). The resulting sucrose-sensitive strain contained the *sacB-neo* cassette in *glnB* as confirmed by Southern blot analysis.

The mutant plasmids pTA25 and pTA26 were mobilized into *R. meliloti* GMI3063. Recombination of the relevant mutations into the chromosomal copy of *glnB* was selected for by chasing with the incompatible plasmid pPH1JI in the presence of sucrose (5%), gentamycin, and rifampicin (Fig. 9). Sucrose-resistant colonies were screened by genomic PCR with the following primers: 5'-AAGCGGAGAGACATT-3' and 5'-CGAGGATTG-GAAAGA-3'. The size of the amplified product (278 bp) demonstrated the deletion within *glnB* in strain GMI3107, whereas sequencing of the 416-bp PCR product demonstrated the presence of the *glnBY51F* mutation in strain GMI3109. The structure of the *glnBA* locus in these mutant strains was further confirmed by Southern blot analysis using as a probe the *glnB*-containing 1.7-kb *Eco*RV-*Eco*RI fragment from pTA2.

Western blot analysis

Protein extracts from *R. meliloti* (5 µg) were loaded onto a 15% SDS-polyacrylamide gel. After electrophoresis the gel was blot-

ted onto HybondC extra membrane (Amersham), probed with anti- P_{II} antiserum (1/5000), (a kind gift of Dr. M. de Zamaroczy; antiserum 1, de Zamaroczy et al. 1996), and detected using the ECL system (Amersham).

For protein quantitation, cells were harvested, resuspended in 20 mM Tris-Cl (pH 7.2), 0.1 mM DTT, and 6 M guanidinium chloride, and lysed for 3 min at 90°C. After centrifugation for 2 min at 6000g, the supernatant was assayed for protein using the Bio-Rad assay system.

GS assays

GS activity was measured using the γ -glutamyl transferase (γ GT) assay. Cell suspensions (10 ml) were brought to 0.25 mg/ml of hexadecyltrimethylammonium bromide (Fluka), chilled on ice, harvested in the cold, washed once with 0.1% KCl, and resuspended in 0.5 ml 0.1% KCl, and γ GT activity was measured at isoactivity pH 7.0 as described in Arcondéguy et al. (1996). The average GSI adenylation state was estimated as: $\bar{n} = 12 - 12b/a$, where a is the total transferase activity in the presence of Mn^{2+} (corresponding to both unadenylylated and adenylylated forms), and b is the transferase activity in the presence of added 60 mM Mg^{2+} (reflecting unadenylylated GSI). To determine γ GT-specific activity we used $\epsilon = 0.532 A_{540}/\mu\text{mole}$ for glutamyl-hydroxamate.

P_{II} overexpression and uridylylation

The *R. meliloti* P_{II} and P_{II} Y51F proteins were overexpressed in *E. coli* containing plasmids pTA2-4F and pTAJ3, respectively, and grown in Luria broth in the presence of 400 µM IPTG. Cultures (25 ml) were harvested, resuspended in 1 ml of 50 mM 2-methylimidazole-Cl (pH 7.6), containing 200 mM KCl and 0.1 mM $MnCl_2$. Cells were sonicated and overexpression of P_{II} was verified on a 15% SDS-polyacrylamide gel. Uridylylation assays were performed at 28°C with 25 µg protein in the presence of 10 mM

Figure 9. Construction of *glnB* mutants. (A) Map of the *glnB* locus indicating plasmids used. Polylinker sites are italicized. Abbreviations: (B) *Bam*HI; (E) *Eco*RI; (H) *Hind*III; (N) *Nco*I; (Pv) *Pvu*II; (S) *Sac*I; (V) *Eco*RV. (B) Schematic view of the construction of the *glnBP5* mutant GMI3109. (1) The *glnBP5* mutant plasmid pTA25 was conjugated into the sucrose-sensitive *R. meliloti* strain GMI3063. (2) Double recombination events were selected for by chasing pTA25 with the incompatible plasmid pPH1JI while selecting for sucrose resistance (loss of the *sacB-neo* cassette).

MgCl₂, 0.1 mM ATP, 2 μ M UTP, 10 μ Ci [α -³²P]-UTP (800 Ci/mmol), and 18 mM α -ketoglutarate to start the reaction. The reaction was stopped after 15 min by the addition of SDS sample buffer, and samples were heated for 3 min at 90°C and loaded onto a 15% SDS-polyacrylamide gel. After electrophoresis the gel was vacuum dried and autoradiographed.

Plant assays

Seeds of *Medicago sativa* cv. Europe were surface sterilized, germinated, inoculated (5×10^5 bacteria per tube), and grown in test tubes on nitrogen-free agar slants. Nitrogenase activity was assayed by the acetylene reduction technique (Turner and Gibson 1980) on three-week-old plants. Ethylene produced was assayed 2 hr after addition of 10% acetylene in the atmosphere. Bacteria were reisolated from crushed nodules as already described (Faucher et al. 1988).

Incorporation of ¹⁵N-enriched dinitrogen into plant tissues was measured on 3-week-old plants as follows. Plants were exposed for 2 hr to ¹⁵N₂-labeled air (approximate atom % ¹⁵N: 10%). The exact atom % ¹⁵N in the atmosphere was determined using mass spectrometry. Batches of four plants were harvested, and shoots, roots, and nodules were rapidly excised, then dried for two days at 70°C. Dry weights were measured on a Sartorius M500P microscale. Shoot fractions were milled (MM2 mixer mill, Retsch GmbH, Germany) and dried again for 24 hr at 70°C. Total N and ¹⁵N contents were measured on a Roboprep CN analyzer (Europa Scientific) followed by mass spectrometry (Tracermass, Europa Scientific), and N fluxes were calculated as described previously (Clarkson et al. 1996).

Histology

Light microscopy was performed either on whole plants or on 80 μ m-thick sections. Hair deformations, infection, and early nodulation steps were studied on whole plants inoculated with wild-type or mutant strains carrying the *hemA-lacZ* reporter plasmid pXLGD4 (Leong et al. 1985). β -Galactosidase was detected as described in Boivin et al. (1990) using X-Gal as a substrate. Histochemical staining of starch was performed according to Ardourel et al. (1994). Histology of mature nodules was studied on 80- μ m sections of nodules collected from plants treated as described above. These sections were obtained from fixed nodules using a Micro-cut apparatus (H1200, Bio-Rad) and counter-stained with 0.2% methylene blue and 1% toluidine blue. Processed plants or nodule sections were viewed with an Olympus Vanox microscope (Olympus Optical Co. Ltd., Tokyo, Japan) using dark-field or bright-field microscopy. For electron microscopy, 3 week-old nodules were fixed with glutaraldehyde (2.5% in 0.15 M sodium cacodylate at pH 7.2), dehydrated in an ethanol series, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with an Hitachi EM600 electron microscope (Hitachi Ltd., Tokyo, Japan).

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