The Rhizobium meliloti $P_\Pi$ 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 $P_\Pi$ protein encoded by $glnB$. We have constructed both a $glnB$ null mutant and a point mutant making nonuridylylatable $P_\Pi$. In free-living conditions, $P_\Pi$ is required for expression of the ntrC-dependent gene $glnII$ and for adenylylation of glutamine synthetase I. $P_\Pi$ 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 $P_\Pi$ controls expression or activity of a bacteroid ammonium transporter required for a functional nitrogen-fixing symbiosis. Therefore, the $P_\Pi$ 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; Dénatre 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 adenylylation 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 $P_\Pi$ [for review, see Merrick and Edwards 1995]. It was therefore of interest to characterize the R. meliloti $P_\Pi$ gene $glnB$ and to determine its involvement in nitrogen-fixing symbiosis. In enteric bacteria, $P_\Pi$ is a trimeric protein involved in two
These results indicate that bacterial nitrogen metabolism in R. meliloti is coupled with alfalfa nodule infection and development and that this coupling involves the PIIL protein of R. meliloti.

Figure 1. Western blot analysis of R. meliloti PIIL. Fifteen percent SDS-PAGE gel probed with anti-PIIL antibody. Five micrograms of protein extract was loaded in each lane. [Lanes 1,2] R. meliloti GMI708; [lanes 3,4] GMI3143 (glnlI::Tn5); [lanes 5,6] GMI5995 (ntrC::Tn5); [lanes 7,8] GMI3107 (AglnBlO); [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.

Results

Regulation of nitrogen metabolism by the PIIL protein in R. meliloti

The glnB gene encoding PIIL is linked tightly with the glnA gene encoding GSI in R. meliloti (Arcondégy et al. 1996). To investigate the function of the PIIL 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 pLABF3 to generate pTA26, then recombined into the R. meliloti genome using a sucrose counterselection procedure (see Materials and Methods). The resulting AglnBlO strain GMI3107 did not express the PIIL 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 PIIL protein as has been found in other organisms (De Zamaroczy et al. 1996, Van Heeswijk et al. 1996). The AglnBlO mutant grew prototrophically slightly more slowly than the wild-type strain GMI708 on either rich or synthetic media (μ=0.22/hr vs. μ=0.26/hr, respectively, on minimal medium with ammonium as a nitrogen source).

To test the involvement of the PIIL protein in nitrogen metabolism in Rhizobium, we first investigated expression of the gSSI gene glnII using a glnII-lacZ reporter plasmid [pFB691::MudlPR48]. The results showed that glnII expression is strictly glnB-dependent (Table 1). We further studied the role of PIIL on expression and adenyllylation of GSI. Strains were made glnII::Tn5 by N3 transduction so as to express GSI as the sole GS. Although PIIL 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 and glnA induction are ntrC-dependent (De Bruijn et al. 1989; Arcondégy et al. 1996), these results suggest that the PIIL protein is involved in activation of NtrC in R. meliloti.

To test whether PIIL is involved in the regulation of GSI adenyllylation, we measured GSI adenyllylation both before and after a nitrogen upshift (Table 3). Whereas in wild-type R. meliloti, GSI is adenyllylated rapidly follow-

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

| R. meliloti background | Relevant genotype | glnA induction [Miller units] | glnB induction [Miller units] |
|-----------------------|------------------|-------------------------------|-------------------------------|
| GMI708 | wild type | 4000 | 340 |
| GMI3107 | AglnBlO | 10 | 12 |
| GMI3109 | glnBP5 | 17 | 14 |

R. meliloti strains contained the glnII-lacZ reporter plasmid pFB691::MudlPR48 [De Bruijn et al. 1989]. Cultures were grown in minimal medium with 6 mM KNO₃ or 15 mM (NH₄)₂SO₄ as a nitrogen source.

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

Symbiotic role of Rhizobium PIIL protein

Different responses to a change in nitrogen status. The first response is a genetic response, by which PIIL 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 PIIL regulates modulates adenyllylation of GS (Stadtman et al. 1975). The degree of PIIL uridylylation is low in nitrogen excess and high under nitrogen deficiency, therefore reflecting the nitrogen status of the cell. In nitrogen-excess conditions, native unmodified PIIL stimulates dephosphorylation of NtrC-P and adenyllylation of GS. This results in inhibited expression and activity of GS, respectively. Conversely under nitrogen-limiting conditions the modified form PIIL-UMP stimulates deadenylylation 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 PIIL protein in the regulation of nitrogen metabolism in R. meliloti and show its involvement in GSI adenyllylation and GS expression. Therefore, like in enteric bacteria, PIIL 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 PIIL 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 PIIL protein of R. meliloti.
uniformly low regardless of combined nitrogen. Therefore, in R. meliloti, P

| Strain         | Relevant genotype | GSI total activity (nmol/min per mg) |
|---------------|------------------|-------------------------------------|
| GMI3143       | glnH::Tn5        | 150 1200 540                         |
| GMI3146       | ΔglnB10 glnH::Tn5| 160 290 200                           |
| GMI3147       | glnBP5 glnH::Tn5 | 150 140 180                           |

γGT 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 NH₄Cl as a nitrogen source.

Strain Relevant genotype LB glutamate ammonium
GMI3143 glnH::Tn5 150 1200 540
GMI3146 ΔglnB10 glnH::Tn5 160 290 200
GMI3147 glnBP5 glnH::Tn5 150 140 180

Expression of GSI activity in glnB mutants of R. meliloti

| Strain | Relevant genotype | GSI adenylylation state (B) |
|--------|------------------|----------------------------|
|        |                  | glutamate                  |
|        |                  | LB (−N) (−N) ammonium      |
| GMI3143| glnH::Tn5        | 11 2 10 7                  |
| GMI3146| ΔglnB10 glnH::Tn5| 2 2 3 3                   |
| GMI3147| glnBP5 glnH::Tn5 | 10 8 8 7                   |

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

Table 3. Adenylylation of GSI in glnB mutants

γGT 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 NH₄Cl as a nitrogen source. Where indicated (−N) glutamate-grown cultures were exposed to 15 mM ammonium sulfate for 30 min before measuring GSI adenylylation.

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Symbiotic role of Rhizobium \( P_{\mu} \) protein

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
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 △glnB10 and glnBP5 mutants averaged 12 and 8 infection threads per plant, respectively. These results indicate that the PIII 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 [Debelle 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 PIII protein is not stringently required for expression of nod genes, but it does affect induction of the nodABC operon by luteolin.

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 △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 4. Effect of glnB mutations on expression of nodC

| R. meliloti background | Relevant genotype | β-Galactosidase activity* (Miller units) |
|-----------------------|------------------|----------------------------------------|
|                       |                  | -luteolin     | + luteolin  |
| GMI708                | wild type        | 29            | 290         |
| GMI3107               | △glnB10          | 17            | 88          |
| GMI3109               | glnBP5           | 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 μM luteolin.

*Strain background β-galactosidase activity was <10 Miller units in the absence of a reporter plasmid.
were calculated on five batches of four plants. 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.

Table 5. Nitrogen deficiency of glnB mutant-inoculated alfalfa

| Relevant genotype | Dry weight per plant (mg) | Total nitrogen content per plant (µg) | Nitrogen content (%) |
|------------------|---------------------------|--------------------------------------|----------------------|
| Wild type        |                           |                                      |                      |
| ΔglnB10          | 5.70 ± 0.48               | 263 ± 26                             | 4.6 ± 0.2            |
| glnBP5           | 3.50 ± 0.50               | 132 ± 39                             | 3.8 ± 0.8            |
| Wild type        |                           |                                      |                      |
| ΔglnB10          | 0.81 ± 0.07               | 22 ± 1                               | 2.7 ± 0.2            |
| glnBP5           | 1.05 ± 0.09               | 26 ± 2                               | 2.5 ± 0.2            |
| Wild type        |                           |                                      |                      |
| ΔglnB10          | 0.40 ± 0.06               | 23 ± 2                               | 5.7 ± 0.5            |
| glnBP5           | 0.37 ± 0.02               | 19 ± 2                               | 5.1 ± 0.6            |
| Wild type        |                           |                                      |                      |
| ΔglnB10          | 6.91 ± 0.51               | 308 ± 28                             | 4.4 ± 0.2            |
| glnBP5           | 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, pl5, and pGM931, 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).

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

| Relevant genotype | Nitrogen fixed per plant (nmoles of N2) | Nodule nitrogenase sp. ac. (nmoles of N2/hr per mg of dry nodule weight) |
|------------------|----------------------------------------|---------------------------------------------------------------|
| Wild type        |                                       |                                                               |
| ΔglnB10          | 30.8 ± 4.7                            |                                                               |
| glnBP5           | 11.0 ± 4.7                            |                                                               |
| ΔglnB10          | 3.9 ± 0.5                              |                                                               |
| glnBP5           | 5.6 ± 0.8                              |                                                               |
| ΔglnB10          | 33.9 ± 6.1                             |                                                               |
| glnBP5           | 5.6 ± 0.8                              |                                                               |
| ΔglnB10          | 28.5 ± 7.3                             |                                                               |
| glnBP5           | 45.1 ± 11.4                            |                                                               |

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.
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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 μm. [C-F] Various nodules elicited by glnB mutants. Similar pictures were obtained with either the 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 μm.

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 energy 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 degenerescence in B are electron-dense bacteroids (white asterisks) and senescent rhizobia enclosed within a single peribacteroid membrane (black star). Electron microscopy. Bar, 1 μm.
global nitrogen regulator NtrC [Szeto et al. 1987], develop normal nitrogen-fixing nodules on alfalfa. In the present work we identify the PII protein of R. meliloti as a key regulatory component involved in both bacterial nitrogen regulation and nodule development. Not only is the PII protein required for proper plant assimilation of nitrogen fixed by Rhizobium bacteroids. It is also involved in plant infection. Therefore, the PII protein integrates the regulation of a wide array of functions related to bacterial nitrogen metabolism, nodule development, and symbiotic nitrogen fixation.

The PII protein as an integrator of nitrogen metabolism in Rhizobium

In enteric bacteria the PII 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 \( \alpha \)-ketoglutarate and glutamine pools via reversible uridylylation [Atkinson et al. 1994]. Native trimeric PII binds \( \alpha \)-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 PII on the other hand, does not affect phosphorylation of NtrC in E. coli [Atkinson et al. 1994].

In R. meliloti, PII also affects the ntr regulon, albeit somewhat differently [Fig. 8]. As in enteric bacteria, nonuridylylated PII (expressed in the glbBP5 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 \( \Delta glnB \) null mutant is ntr-deficient as judged from the absence of glnII expression and glnA induction. We therefore suggest that in R. meliloti, the PII 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 PII–UMP signals nitrogen excess for the ntr regulon in R. meliloti.

The other characterized function of PII in enteric bacteria is the modulation of GS activity by reversible coregulated enzyme adenylylation. In R. meliloti GSI is regulated similarly by reversible adenylylation in response to the cellular nitrogen status [Arcondéguy et al. 1996]. Like in enteric bacteria, GS is adenylated by the native PII protein whereas PII–UMP promotes GSI deadenylation [Table 3]. Therefore, absence of PII signals nitrogen deficiency for the regulation of GS activity by adenylylation, whereas nonuridylylated PII signals nitrogen excess for both genetic ntr regulation and metabolic regulation of GS activity [Fig. 8].

Expression of the PII protein appeared to be partly nitrogen-regulated in R. meliloti (Fig. 1). PII 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 \( \sigma^{34} \)-dependent promoter sequence is conserved upstream of glb 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 glbBP5 background [Fig. 1]. This autoregulation of glnB, together with its ntrC-dependence, is consistent with the role of PII–UMP in ntr regulation as noted above: Uridylation of PII 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 PII protein of R. meliloti strongly interferes with the plant infection process. glb 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 ΔglnB10 null mutant or the glnBP5 mutant unable to uridylylate Pn. This indicates that the uridylylated form Pn-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 [Debelle 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 effective infection phenotype of Rhizobium. These symbiotic defects are observed with either the ΔglnB10 null mutant or the glnBP5 mutant [this study]. Therefore, the Pn 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 Pn 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 ¹⁵N incorporation (Fig. 5; Table 6). The glnBP5 mutant induces slightly more severe plant symptoms than the ΔglnB10 null mutant. This can be interpreted in terms of the model shown on Figure 8: The glnBP5 mutant makes a Pn protein that cannot be uridylylated, therefore locking nitrogen regulation in the OFF mode, whereas the ΔglnB10 mutant makes no Pn protein at all, therefore letting Pn-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 activity. 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 Nif⁺ 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; Arcondeguy et al. 1996]. Moreover, glnA, glnI, and glnAglnI double mutants of R. meliloti are Fix⁺, which shows that GSI and GSII are dispensible for symbiosis [de Bruijn et al. 1989]. The third possibility concerns GSI adenylylation, which is affected by Pn (Table 3). However, a glnA mutant making an adenylylation-defective GSI is Fix⁺, which shows that GSI adenylylation is not required for symbiosis [Arcondeguy et al. 1996]. Moreover, although the ΔglnB10 and glnBP5 mutations lock GSI in opposite adenylylation modes (Table 3), they cause very similar symbiotic phenotypes. Therefore, GSI adenylylation is not involved. The fourth possibility is that the conjuction 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 ammonium 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 Pn protein is involved in the regulation of a bacteroid ammonium transporter important for a functional nitrogen-fixing symbiosis.
Symbiotic role of *Rhizobium* P1 protein

**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 (Arcondégy 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 *glnB*-i5 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-CAGAAATTGTTCGTCG 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'-CGGAGCTCTACCGCGGCG-CAGAAATTGTTCGTCG 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'-CGGAGCTCTACCGCGGCG-CAGAAATTGTTCGTCG containing a naturally occurring SacI site (italicized) just upstream of the mutated codon [bold].

**Materials and methods**

**Table 7. Strains and plasmids used**

| Strain or plasmid | Relevant characteristics | Source or reference |
|-------------------|--------------------------|---------------------|
| **Rhizobium meliloti** |             |                     |
| GM1078            | RifR derivative of strain 2011 | Batut et al. [1985] |
| GM103065          | *glnB*-sacB, RifR, GmR, NmR | this work           |
| GM103107          | ΔglnBl0, RifR, GmR | this work           |
| GM103109          | *glnBP5*, RifR, GmR | this work           |
| GM103143          | *glnII*:Tn5 SmR, NmR, RifR | Arcondégy et al. [1996] |
| GM103146          | GM103107 transductant, ΔglnBl0 *glnII*:Tn5, SmR, NmR, RifR | this work           |
| GM103147          | GM103109 transductant, *glnBP5* *glnII*:Tn5, SmR, NmR, RifR | this work           |
| GM105995          | *intrCI2*:Tn5, SmR, NmR, RifR | Arcondégy et al. [1996] |
| **Escherichia coli** |             |                     |
| RB9040            | *glnD99*:Tn10, TcR | Bueno et al. [1985] |
| RB9060            | ΔglnBl206 | Bueno et al. [1985] |
| **Plasmids**      |             |                     |
| pCHK57            | TcR, *nisA*-lacZ fusion | Ditta et al. [1987] |
| pFB682            | ApR, *glnA*-containing pBR322 derivative | De Bruijn et al. [1989] |
| pFB691::MudIIPR48 | TcR, *glnII*-lacZ fusion | F. Maillet (pers. comm.) |
| pGM1020           | TcR, *nodD1*-lacZ fusion | F. Maillet |
| pGM1031           | TcR, *fixN*-lacZ fusion | F. Maillet |
| pGM10003          | TcR, NmR, *nodD2*:Tn5-B20 pMH902 derivative | Batut et al. [1989] |
| pGM10004          | TcR, NmR, *nodD3*:Tn5-B20 pMH903 derivative | De Bruijn et al. [1988] |
| pGM10005          | TcR, NmR, *syrM*:Tn5-B20 pMH904 derivative | Batut et al. [1989] |
| pFB279            | ApR, NmR, sacB-neo cassette | F. Maillet |
| pKS4              | TcR, *fixX*-lacZ fusion | F. Maillet |
| pMH9023           | TcR, *nodD2*-containing pRK290 derivative | Batut et al. [1989] |
| pMH909            | TcR, *nodD3*-containing pWB5A derivative | De Bruijn et al. [1988] |
| pMH904            | TcR, *syrM*-containing pWB5A derivative | De Bruijn et al. [1988] |
| pPH11             | SmR, GmR, MobR IncP | F. Maillet |
| pRK2073           | SpR, TpR, TraR, MobR | F. Maillet |
| pRM57             | TcR, *nodC*-lacZ fusion | F. Maillet |
| pTA2              | ApR, *EcoRI*-HindIII fragment of pFB682 in pKS | Arcondégy et al. [1996] |
| pTA2-4F           | Exo III deletion of pTA2 | this work           |
| pTA3              | ApR, *PvuII*-HindIII fragment of pFB682 in pKS | Arcondégy et al. [1996] |
| pTA5              | ApR, *EcoRI*-EcoRV fragment of pTA2 in pKS | Arcondégy et al. [1996] |
| pTA8              | ApR, pUC18–Not deleted of SacI, KpnI, and EcoRI sites | this work           |
| pTA9              | ApR, HindIII-BamHI fragment of pTA3 in pTA8 | this work           |
| pTA13             | ApR, sacB cassette inserted into SacI site of pTA9 | this work           |
| pTA14             | TcR, NotI fragment of pTA13 in pLAFR3 | this work           |
| pTA20             | ApR, pTA9 with *glnBP5* | this work           |
| pTA21             | ApR, pTA9 with ΔglnBl0 | this work           |
| pTA25             | TcR, HindIII-BamHI fragment of pTA20 in pLAFR3 | this work           |
| pTA26             | TcR, HindIII-BamHI fragment of pTA21 in pLAFR3 | this work           |
| pTA6              | TcR, HindIII-BamHI fragment of pTA21 in pLAFR3 | this work           |
| pTA32             | TcR, EcoRI-BamHI fragment of pTA5 in pLAFR3 | this work           |
| pTA33             | TcR, EcoRI-BamHI fragment of pTA5 in pLAFR3 | this work           |
| pUC18-Not         | ApR | Herrero et al. [1990] |
| pXLD18            | TcR, *hemA*-lacZ | Leong et al. [1985] |
Arcondéguy et al.

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 BamHI from pPB279 (Blomfield et al. 1991), made blunt-end with T4 DNA polymerase, cloned into the unique SacI site of pTA9, generating pTA13. The 7.7-kb NotI insert from pTA13 was made blunt-end and recloned into the pLAFR3 EcoRI 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 pH1JI 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 pH1JI 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.

Western blot analysis

Protein extracts from R. meliloti (5 μg) were loaded onto a 15% SDS-polyacrylamide gel. After electrophoresis the gel was blotted onto HybondC extra membrane (Amersham), probed with anti-Pn 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 μg 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 (γGT) 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 γGT activity was measured at isoactivity pH 7.0 as described in Arcondéguy et al. (1996). The average GSI adenylylation 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 γGT-specific activity we used \( c = 0.532A_{\text{abs}}/\mu \text{mole for glutamyl-hydroxamate.} \)

\( P_{\text{H}} \) overexpression and uridylylation

The R. meliloti Pn and Pn 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 Pn 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) BamHI, (E) EcoRI, (H) HindIII, (N) NcoI, (Pv) PvuII, (S) SacI, (V) EcoRV. (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 pH1JI while selecting for sucrose resistance [loss of the sacB-neo cassette].

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buffer, and samples were heated for 3 min at 90°C and loaded. 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. The gel was vacuum dried and autoradiographed.

Plant assays

Seeds of Medicago sativa cv. Europe were surface sterilized, germinated, inoculated [5 x 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 µm-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 pXLDG4 [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-µm sections of nodules collected from plants inoculated with the reporter, grown 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].

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