The *Rhizobium* *GstI* Protein Reduces the NH_4^+ Assimilation Capacity of *Rhizobium leguminosarum*

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We show that the protein encoded by the glutamine synthetase translational inhibitor (*gstI*) gene reduces the NH_4^+ assimilation capacity of *Rhizobium leguminosarum*. In this organism, *gstI* expression is regulated by the *ntr* system, including the P_{II} protein, as a function of the nitrogen (N) status of the cells. The *GstI* protein, when expressed from an inducible promoter, inhibits glutamine synthetase II (*glnII*) expression under all N conditions tested. The induction of *gstI* affects the growth of a glutamine synthetase I (*glnA*[−]) strain and a single amino acid substitution (W48D) results in the complete loss of *GstI* function. During symbiosis, *gstI* is expressed in young differentiating symbiosomes (SBs) but not in differentiated N_2 -fixing SBs. In young SBs, the P_{II} protein modulates the transcription of *NtrC*-regulated genes such as *gstI* and *glnII*. The evidence presented herein strengthens the idea that the endocytosis of bacteria inside the cytoplasm of the host cells is a key step in the regulation of NH_4^+ metabolism.

Additional keywords: histochemical localization, PCR mutagenesis, posttranscriptional regulation, protein purification, *Vicia hirsuta*.

The regulation of ammonium (NH_4^+) metabolism has a key role in the *Rhizobium*–legume symbiosis. During development of root nodules, *Rhizobium* bacteria differentiate inside the invaded plant cells into N_2 -fixing symbiosomes (SBs) that release most of the NH_4^+ produced therein by the nitrogenase complex. Several independent lines of evidence demonstrate that the uncoupling between NH_4^+ assimilation and N_2 -fixation activities is a prerequisite for an efficient symbiotic interaction (Bravo et al. 1988; Mendoza et al. 1995; Patriarca et al. 1996; Tatè et al. 1999). The molecular mechanisms acting to reduce the NH_4^+ assimilation ability of N_2 -fixing SBs, however, are largely unknown.

To assimilate NH_4^+ under free-living conditions of growth, *Rhizobium leguminosarum* bv. *viciae* (hereafter called *R. leguminosarum*) expresses two different glutamine synthetase (GS) enzymes: GSI and GSII. GSI, encoded by the *glnA* gene, is a dodecameric enzyme that is posttranslationally regulated by an adenylation–deadenylation mechanism (Rossi et al. 1989). The *glnA* gene, which is part

of the *glnBA* operon, is expressed either from its own −35/−10 promoter, *glnAp*, or from a −24/−12 (σ^{54} -dependent) N-regulated promoter (*glnBAp*) that is situated immediately upstream of *glnB* (Chiurazzi and Iaccarino 1990). GSII encoded by the *glnII* gene is a heat-sensitive octameric enzyme (Manco et al. 1992). The *glnII* gene is transcribed as a monocistronic unit from a single −24/−12 (σ^{54} -dependent) promoter that is activated by the two-component regulators, *NtrB* and *NtrC*, under N-limiting conditions of growth (Patriarca et al. 1992; Patriarca et al. 1993; Patriarca et al. 1994). Transcription of *glnII* is constitutive in a *glnB* mutant strain (Amar et al. 1994), whereas the induction of the *glnII* promoter is abolished in a *glnD* mutant (Schluter et al. 2000). Therefore, in contrast to the situation reported in *Rhizobium meliloti* (Arcondeguy et al. 1997), the regulatory protein P_{II} (encoded by the *glnB* gene) of *R. leguminosarum* is essential to inactivate, by dephosphorylation, *NtrC* under N-excess conditions. Its absence, however, does not abolish the capacity of the protein kinase *NtrB* to activate *NtrC* by phosphorylation. The *glnD* gene product uridylyltransferase (UTase) is required to inactivate the P_{II} protein, by uridylylation, under N-limiting conditions (Schluter et al. 2000), allowing the activation of *NtrC*. Thus, whereas GSI is subject to transcriptional and posttranslational regulation, GSII appears to be regulated only at the transcriptional level.

The glutamine synthetase translational inhibitor (*gstI*) gene encodes a 63 amino acid protein and is situated immediately upstream of *glnII* (Spinosa et al. 2000). The gene is transcribed divergently with respect to *glnII*, and a preliminary analysis showed that its transcription is regulated negatively by *NtrC*. *GstI* was identified by means of a deletion–complementation analysis performed in *Klebsiella pneumoniae* and, in this organism, when *gstI* is present, it is possible to detect *glnII*-specific mRNA but not GSII activity nor the GSII protein. These data suggest that *gstI* may be part of a previously unrecognized mechanism acting to reduce the NH_4^+ assimilation capacity of *R. leguminosarum*. Thus, to gain more information about the role of *gstI*, we tested its expression and function in *R. leguminosarum* under free-living growth conditions. Because bacterial NH_4^+ assimilation is expected to be repressed during differentiation of nitrogen-fixing symbiosomes, *gstI* expression also was studied during the symbiotic interaction between *R. leguminosarum* and the legume *Vicia hirsuta*.

RESULTS

The influence of nitrogen status on *gstI* promoter activity.

To test *gstI* promoter (*gstIp*) activity, plasmid pAR212a (Fig. 1B) carrying a *gstIp-lacZ* transcriptional fusion was introduced into the wild-type strain LPR1105 of *R. leguminosarum*. The resulting bacteria were grown in minimal medium (RMM), with different N and C sources, and β -galactosidase activity was measured (Fig. 1A). In the presence of glucose, *gstIp* activity is lower with either glutamine or glutamate as the sole N source than it is with glutamine plus NH_4Cl . The highest activity coincides with the utilization of glutamine (or glutamate) as the C source in the presence of NH_4Cl . When glutamine is used as the sole C source, NH_4^+ is excreted by *Rhizobium* cells (our unpublished data). Thus, the *gstI* promoter is regulated by the N status of the cell and, in particular, a low C:N intracellular ratio (C-limiting and N-excess conditions) is necessary to induce the *gstI* promoter. *glnII* promoter activity (plasmid pAR35), however, is very low in bacteria growing under N-excess conditions (glutamine plus NH_4Cl), whereas under N-limiting conditions (i.e., with glutamate as the sole N source), *glnII* transcription is induced severalfold. Therefore, the growth conditions at which *glnIIp* is the least active coincide with conditions in which *gstIp* is most active.

To evaluate the effect of the Ntr system on the *gstI* promoter, a *glnB* (P_{II}) mutant strain, BS11, of *R. leguminosarum* (Amar et al. 1994) was transformed with plasmid pAR212a (*gstIp-lacZ*), and the resulting strain was assayed for β -galactosidase activity (Fig. 1A). As a control, strain BS11 was transformed with plasmid pAR35 (*glnIIp-lacZ*). Independent of the N source used, strain BS11-pAR212a showed a low β -galactosidase activity (50 to 150 units), whereas strain BS11-pAR35 had a high activity (750 to 900 units) under all N conditions tested. Thus, under N-excess growth conditions, P_{II} is essential to activate *gstI* transcription and inactivate *glnII* transcription. Moreover, under N-limiting conditions such as

RMM-glutamate, *glnIIp* activity is twofold higher in strain BS11 compared with strain LPR1105 (wild type). Therefore, because P_{II} modulates the protein kinase activity of NtrB, thus regulating NtrC activity, we conclude that NtrC is not fully active (phosphorylated) in the wild-type cells incubated in RMM-glutamate.

Influence of GstI on *glnII* expression in *R. leguminosarum*.

To test the effect of the GstI protein on *glnII* expression, we put *gstI* under the control of an inducible promoter, namely the *dctA* promoter of *R. meliloti* that is induced by succinate (the *dctA* gene codes for a succinate transporter). In *R. leguminosarum*, the *dctA* promoter (plasmid *dctAp-lacZ*) is induced more than 20 times by the addition of succinate to bacterial cells incubated under N-limiting or N-excess conditions (Fig. 2A). Surprisingly, *dctAp* is stimulated by the presence of glucose in the medium, even if the bacteria growth rate increases by adding glucose to RMM-succinate. On the basis of these results, a plasmid carrying a *dctAp-gstI* transcriptional fusion (plasmid pAR237) was constructed (Fig. 2B).

The wild-type strain LPR1105 of *R. leguminosarum* was transformed with either plasmid pAR231 (carrying *dctAp*) or pAR237 (*dctAp-gstI*). The resulting strains were grown in RMM containing glucose as the C source and glutamine plus NH_4Cl as the N sources (N excess). The cells were then shifted to RMM-glutamate plus glucose (N limiting), with or without added succinate (to induce *gstI*). After 3 h of incubation, aliquots of cells were harvested, crude extracts were prepared, and GSII activity was measured (Fig. 2B). Control strain LPR1105-pAR231 showed 1,500 to 2,000 units of GSII activity after the cells were shifted to RMM, with or without succinate, whereas strain LPR1105-pAR237 showed 1,750 units when shifted to RMM without succinate but only 180 to 200 units when *gstI* expression was induced by adding succinate. The inhibitory effect of *gstI* on *glnII* expression was expressed as the ratio of GSII activity obtained in the absence

Fig. 1. A, N regulation of *gstI* and *glnII* promoters. Strains LPR1105 (wild type) and BS11 (*glnB*) of *Rhizobium leguminosarum* were transformed with either plasmid pAR212a (*gstIp*) or pAR35 (*glnIIp*). Cells of the resulting strains were grown in TYR medium and shifted to RMM medium containing 1% glucose, 10 mM glutamine, 10 mM glutamate, and/or 18 mM NH_4Cl , as indicated. Cells were incubated for 3 h at 30°C (initial optical density at 590 nm of 0.2) and assayed for β -galactosidase activity, according to the method of Miller (1972). Values reported are the average of at least three independent assays, and errors are indicated. **B,** Scheme of the *gstI-glnII* intergenic region. NtrC binding sites, transcription start sites of both genes, and insert of plasmids pAR35 and pAR212a are indicated.

and presence of succinate (–Suc/+Suc). A ratio of one or lower indicates no inhibitory effect, whereas higher values such as those obtained with strain LPR1105-pAR237 (*dctAp–gstI*) indicate inhibition of GSII expression.

The inhibition of *glnII* expression mediated by *gstI* also was tested in a strain lacking GSI. For this purpose, strain AR7 (*glnA::km*) of *R. leguminosarum* (Amar et al. 1994) was transformed with either plasmid pAR231 (*dctAp* alone) or with pAR237 (*dctAp–gstI*). The resulting cells were grown in

RMM-glutamine plus NH_4Cl as N sources (N excess) and then shifted for 3 h to RMM-glutamate (N limiting), with or without added succinate. A ratio of 1.2 (GSII activity –Suc/+Suc) was obtained with strain AR7-pAR231, whereas a ratio of 10.5 was obtained with strain AR7-pAR237 (Fig. 2D). Thus, increasing the intracellular level of GstI under N-limiting conditions prevents the induction of *glnII* expression, even in the absence of GSI. Surprisingly, the maximal GSII activity values measured with strain AR7 are at least five

Fig. 2. GstI-mediated inhibition of *glnII* expression. **A**, Regulation of the *dctA* promoter activity. Strain LPR1105 (wild type) of *Rhizobium leguminosarum* was transformed with plasmid pAR231 carrying a *dctAp–lacZ* fusion. The resulting bacteria were grown in RMM medium containing, when indicated, glucose (Glc), succinate (Suc), glutamate (Glt), or NH_4Cl (NH_4^+) and used to assay β -galactosidase activity. **B**, Top: activity of GstI and its mutant derivatives on *glnII*(GSII) expression. Strain LPR1105 was transformed with the indicated plasmids described in text. Cells were grown in TYR medium and shifted, after 3 h, to RMM–glutamate (N-limiting condition), with or without added succinate (Suc). Crude extracts were prepared, and the GS transferase activity (nmol of γ -glutamylhydroxamate per min per mg of protein) was determined as described elsewhere (Spinosa et al. 2000). Bottom: scheme of a plasmid used to induce *gstI* expression. **C**, Top: GstI expression. Crude extracts (1 mg in each lane) prepared from cells of strain LPR1105 (wild type) or LPR1163 (Δ *gstI–glnII*) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a nitrocellulose sheet, and probed with an antiserum raised against the purified GstI protein. Bottom: strains LPR1105(pAR237) and LPR1105(pAR253) were grown as in **B**, with or without added succinate (S), and GstI was assayed by immunoblot (100 μ g in each lane) as described above. **D**, GstI activity in different genetic backgrounds. Strains AR7 (*glnA*[–]) and BS11 (*glnB*[–]) of *R. leguminosarum* harboring the indicated plasmids were grown in TYR medium. Strains AR7-pAR231 and AR7-pAR237 were shifted after 3 h to RMM–glutamate (N-limiting condition), with or without added succinate (Suc), whereas strain BS11-pAR237 was shifted after 3 h RMM–glutamine plus ammonium, with or without added succinate (Suc). Crude extracts were prepared and assayed for GSII activity as described in **B**. **E**, Effect of GstI induction on bacterial growth. Strains LPR1105 (wild type) and AR7(GSI[–]) carrying the indicated plasmids were grown in TYR medium, washed, and streaked on RMM–agar containing glucose and glutamate, with (+Suc) or without (–Suc) added succinate. **F**, *glnA* (GSI[–]) mutant strain UNF1827 of *Klebsiella pneumoniae* was cotransformed with plasmid pAR18 (*glnII*) and either plasmid pBR329 (vector alone), pMS25 (GstI), pAR230 (W48D), or pAR232 (R59A). Cells from the resulting strains were grown in TY medium, washed, and streaked on NFDM minimal medium containing glutamine (Gln) or glutamate (Glt) as the sole nitrogen source.

times lower than those showed by the wild-type strain LPR1105. In fact, cells of strain AR7-pAR231, shifted for 3 h to RMM-glutamate, showed a GSII activity value of 250 to 300 units (Fig. 2D), whereas strain LPR1105 had values of 1,500 to 2,000 units under the same condition (Fig. 2B). Therefore, during the adaptation of *Rhizobium* cells to an imposed condition of N limitation, GSI activity is apparently required to allow the expression of the GSII protein.

In the *glnB* mutant strain BS11, unlike the wild-type strain, *glnIIp* is highly active, independent of the N status of the cells (Fig. 1A). Thus, we exploited this transcriptional deregulation to test GstI activity under N-excess conditions. For this purpose, it also is important to note that *dctAp* is inducible in bacteria growing in minimal medium with glutamine plus NH_4Cl as N sources (Fig. 2A). An inhibitory ratio of 5.5 was obtained with strain BS11-pAR237 (Fig. 2D), indicating that GstI is able to inhibit *glnII* expression under N-limiting and N-excess conditions. The activity of GstI is apparently lower in the *glnB* mutant than in the wild-type strain. *glnIIp* activity, however, is twofold higher in strain BS11 incubated in RMM-glutamate than it is in the wild-type strain incubated under the same conditions (Fig. 1A). Hence, titration of the GstI protein by a higher intracellular level of *glnII* transcripts may explain the lower inhibitory ratio observed.

Isolation of GstI mutant derivatives.

A "remote modeling" strategy was used to predict a three-dimensional structure for the GstI protein (Spinosa et al. 2000). On the basis of this model, two mutant derivatives of GstI carrying a single amino acid substitution were obtained by polymerase chain reaction (PCR) mutagenesis of the structural gene (see below). To avoid differences in protein levels resulting from a different context, the DNA fragments carrying the mutated *gstI* genes were cloned into the same vector with the same restriction enzymes and, consequently, under the same regulatory signals (promoter and ribosome binding site). The resulting plasmids were used to transform strain LPR1105, and inhibition of *glnII* expression was tested by measuring GSII activity. When compared with the wild-type protein, activity of the W48D GstI (plasmid pAR253) was drastically reduced (Fig. 2B). An inhibitory ratio of 5.1 was obtained with the R59A GstI (plasmid pAR254), indicating that this mutation causes a partial loss of activity.

To test the presence of the GstI protein, we used immunoblot assays (Fig. 2). For this purpose, the GstI protein was purified, after its overexpression in *Escherichia coli*, and used to induce the production of an antiserum in rabbit (see below). As shown (Fig. 2C), the GstI protein was detected in a crude extract of strain LPR1105 (wild type), whereas no cross-reacting material was detected in a crude extract prepared from strain LPR1163 (carrying a *gstI*-*glnII* deletion). Moreover, the GstI protein was detected in crude extracts of strains LPR1105-pAR237 (wild type) and LPR1105-pAR253 (W48D) when incubated in the presence of the inducer succinate (Fig. 2C). The protein also was detected, although at lower levels in crude extracts prepared from cells incubated in the absence of the inducer. The mutated R59A protein was detected in crude extracts of strain LPR1105-pAR254 (data not shown). On the basis of these results, we conclude that the expression of GstI proteins was induced from the *dctAp* in wild-type and mutant derivatives and the absence of any in-

hibitory effect is not correlated with the lack of expression of the mutated proteins. In the case of strain LPR1105-pAR253 (coding for the W48D mutant), however, the level of cross-reacting material was approximately fivefold greater than that detected in the wild-type strain LPR1105-pAR237 (Fig. 2C). The reason for this higher level is unknown presently, but it is possible that the W48D mutation may increase GstI stability.

Growth effects mediated by the induction of *gstI*.

To test the effect of the induction of GstI on bacterial growth, plasmids bearing *gstI* (or the two mutant derivatives), under the control of the *dctA* promoter, were used to transform two different strains of *R. leguminosarum*, LPR1105 (wild type) and AR7 (GSI⁻). Bacteria were plated on RMM-agar containing glucose and glutamate (Fig. 2E). To activate the *dctA* promoter and induce *gstI* expression, one set of plates was supplemented with succinate. Transconjugant derivatives of strain LPR1105 carrying pAR237(wt), pAR253(W48D), or pAR254(R59A), did not exhibit growth differences in the presence or absence of the *gstI* inducer (Fig. 2E). Growth of strains AR7-pAR237 and AR7-pAR254 in the presence but not the absence of succinate, however, was inhibited significantly compared with pAR253-transformed cells. Thus, under N-limiting conditions, the GstI-mediated inhibition of *glnII* expression apparently is compensated by a mechanism requiring GSI. Moreover, the residual inhibitory activity of the R59A mutated protein (Fig. 2B) was sufficient to affect bacterial growth when it was expressed in the GSI⁻ mutant strain AR7 (Fig. 2E).

Previously, it was observed that the *R. leguminosarum glnII* gene expressed from plasmid pAR18 is able to complement the glutamine auxotrophy of *glnA* mutant strain UNF1827 of *Klebsiella pneumoniae* (Patriarca et al. 1992). It also has been observed that *gstI* (carried on a second plasmid, pMS25) is able to inhibit *glnII* expression from plasmid pAR18 and, therefore, the growth of strain UNF1827 when incubated in minimal medium (NFDm) without added glutamine (Spinosa et al. 2000). With the use of pBR329 as a vector, two plasmids carrying the mutated versions (W48D or R59A) of GstI were constructed. Strain UNF1827 cotransformed with plasmid pAR18 (*glnII*) plus either pBR329 (vector alone) or pAR230 (W48D) could grow on NFDm-glutamate, whereas UNF1827-pAR18/pMS25 (wild-type GstI) and UNF1827-pAR18/pAR232 (R59A) could not grow on NFDm-glutamate (Fig. 2F). This indicates that, similar to what occurs in *R. leguminosarum*, the R59A mutant derivative of GstI rather than the W48D mutant is able to inhibit *glnII* expression in *K. pneumoniae*.

Expression of the *gstI* promoter in *V. hirsuta* nodules.

Mature root nodules of *V. hirsuta* induced by *R. leguminosarum* show all the developmental zones in longitudinal sections (Vasse et al. 1990). Zone I is the most distal zone, with respect to the root, and corresponds to the apical nodule meristem. Zone II, the invasion zone, is characterized by the presence of infection threads and plant cells containing young symbiosomes (SBs). Interzone II-III showed a sharp developmental switch in starch accumulation (Vasse et al. 1990) and nitrogenase (*nif*) gene expression (Yang et al. 1991), continuing into the N-fixing zone (zone III). Young SBs, which occasionally divide, are observed in the root-distal portion of zone

II, whereas nondividing, elongating SBs are observed in the root-proximal portion.

V. hirsuta plantlets were inoculated with *R. leguminosarum* transconjugants containing plasmid pAR212a (carrying a *gstIp-lacZ* fusion). The *gstI* promoter is active in SBs released in the first invaded cells of the inner cortex during primordium formation (Fig. 3A and B). In 1-week-old nodules, a decreasing distal-proximal gradient begins to be observed (Fig. 3C) and, later, the activity is restricted to SBs present in the younger invaded cells of zone II (Fig. 3D and E). In 3-week-old nodules, the *gstI* promoter is switched off sharply in a single cell layer of interzone II-III, where transcription of *nif* genes is activated (i.e., nitrogenase becomes active) and starch granules accumulate (Fig. 3F). The *gstI* promoter is inactive (or its activity is undetectable) in nondividing differentiated SBs located inside the host cells of either zone III (fixing zone) or in degrading SBs located in the host cells of the senescence zone (the most proximal invaded zone).

The *ntr* system is operative in symbiosomes.

To evaluate whether the *ntr* system senses the nitrogen status of the SBs, the *glnB* mutant strain BS11 of *R. leguminosarum* was transformed with plasmid pAR212a (carrying a *gstIp-lacZ* transcriptional fusion). In contrast to nodules induced by the wild-type strain, LPR1105 (Fig. 4A), *gstIp* is not detectable in SBs of nodules induced by strain BS11-pAR212a (Fig. 4B). This indicates that the regulatory protein P_{II} is necessary for the derepression of the *gstI* promoter (presumably through dephosphorylation of NtrC), not only under free-living conditions but also in younger SBs. The *glnII* promoter (carried on plasmid pAR35) is active in dividing bacteria located inside the infection threads (data not shown), whereas it is inactive in younger SBs of nodules formed by strain LPR1105 (Fig. 3C). In contrast, *glnIIp* is clearly active in young SBs of nodules elicited by the *glnB* mutant strain BS11 (Fig. 3D). Moreover, the *nifH* promoter is strongly activated in SBs of interzone II-III in nodules formed by strain LPR1105 (Fig. 3E), whereas it is only weakly active (undetectable in some nodules) in nodules induced by strain BS11, although it does maintain the correct expression pattern (Fig. 3F). In fact, *nifHp* is inactive in young SBs of zone II, whereas it becomes active in a single cell layer of interzone II and III.

DISCUSSION

Why a minority of bacteria, including *R. leguminosarum*, expresses at least two GS isozymes, remains an unresolved question (De Bruijn et al. 1989; Fuchs and Keister 1980; Merrick and Edwards 1995). The recognition of a GstI-mediated mechanism able to inhibit *glnII* expression, however, may help to provide an understanding as to the function of each GS. We observe that the induction of GstI inhibits the expression of GSII and reduces the NH₄⁺ assimilation capacity of *R. leguminosarum*. In fact, the induction of *gstI* delays the growth of strain AR7 (GSI⁻), thus indicating that, in the absence of GSI, GSII is essential for bacterial growth in minimal medium without added glutamine. In strain AR7, however, *glnII* is at least five times less induced than in the wild-type strain, indicating that, after shifting the cells from N-excess to N-starvation, GSI activity is essential to allow for the normal

expression of *glnII*. After *gstI*-mediated inhibition of *glnII* expression, cells of the wild-type strain showed no relevant growth defects. Thus, to maintain the glutamine pool, the absence (or lower levels) of GSII must be compensated by the activity or the intracellular level of GSI. The *glnA* gene is transcribed from the NtrC-regulated *glnBA* promoter and thus is able to respond to the N status of the cells (Chiurazzi and Iaccarino 1990; Ercolano et al. 2001). We observed (*unpublished data*) that *glnBAp* is more active in a *glnII* mutant strain of *R. leguminosarum*.

The *gstI-glnII* intergenic region contains two NtrC binding sites that appear to play a key role in the control of both promoters (Fig. 1B). In fact, it has been demonstrated that the NtrC protein binds to this DNA fragment and that the binding affinity depends upon the nitrogen status of the cell (Patriarca et al. 1994). Here, by measuring the activities of *gstIp* and *glnIIp* in cells subjected to a different N status, we found that both promoters are N regulated, although in the opposite sense: the binding of NtrC activates *glnIIp* and simultaneously represses *gstIp*. Consequently, the maximal expression of *gstI* occurs under N-excess conditions and coincides with the presence of a low intracellular level of *glnII* mRNA and vice versa. Thus, the stoichiometric ratio between *glnII* mRNA and GstI protein may be important because it has been proposed that GstI binds to the 5' untranslated leader region of *glnII* transcripts and inhibits their translation (Spinosa et al. 2000). Therefore, the induction of *glnIIp* after a shift from N-excess to N-limiting conditions may allow, by titration of the GstI protein, translation of *glnII* transcripts.

The results presented herein suggest that GstI activity depends upon its expression and is not influenced by the N status of the cells. These data, however, do not permit us to exclude the presence of a mechanism acting to regulate GstI activity under physiological conditions. In fact, when *gstI* is expressed from an inducible promoter, any molecule(s) involved in a putative mechanism of posttranslational modification of GstI such as inactivation or degradation could be titrated.

It has been established that, for an effective *Rhizobium*-legume symbiotic interaction, NH₄⁺ assimilation activities, including NH₄⁺ transport activity, must be switched off in the bacterial partner, whereas they are induced in the plant host cells (Bravo et al. 1988; Carvalho et al. 2000; Tatè et al. 1998; Tatè et al. 1999). To evaluate a putative role of GstI in maintaining low GS activity in the SBs, the activity of the *gstI* promoter was followed during the *R. leguminosarum*-*V. hirsuta* symbiotic interaction. We observe that the *gstI* promoter is active in SBs located inside the younger invaded host cells (zone II). Therefore, the expression of *gstI* may explain why *glnII* transcripts were detected in bacterial cells isolated from nodules, whereas neither GSII activity nor the GSII protein were not (Martin et al. 1988; Shatters et al. 1989).

We observe that *gstIp* is switched off (i.e., repressed or inactivated) in SBs located inside the host cells of interzone II-III. This pattern of *gstI* expression was unexpected because i) in SBs of interzone II-III, the nitrogenase complex is expressed and NH₄⁺ is produced; ii) it has been observed previously (Patriarca et al. 1996) that NtrC, the transcriptional repressor of *gstIp*, is expressed in the young SBs of zone II, whereas it is not expressed in N₂-fixing SBs of zone III; iii) in bacteria growing with NH₄⁺ as the nitrogen source, the *gstIp*

promoter is strongly activated as a result of the inactivation of NtrC; iv) *gstIp* is at least tenfold more active in a *ntrC* mutant than in its wild-type parent (not shown). Therefore, because NH_4^+ is present as a result of nitrogenase activity and NtrC is absent, *gstIp* is expected to be derepressed in N_2 -fixing SBs of zone III. Why the *gstIp* promoter is down regulated in interzone II-III is presently unknown, but the same developmental transcriptional pattern was observed when the *ORF1-ntrBC* (Patriarca et al. 1996), *glnBA* (Ercolano et al. 2000), *lipA* (Tatè et al. 1997), and *ropA* genes (De Maagd et al. 1994) were analyzed. These results support the notion that, in SBs located in invaded host cells of interzone II-III, a developmental switch involving the transcriptional machinery such as a change in RNA polymerase specificity mediated by an alteration in the sigma factor population takes place.

We also observe that, in nodules induced by the *glnB* (P_{II}) mutant (strain BS11), *gstIp* is inactive in all types of SBs, including the younger SBs located in the last invaded host cell of zone II (the cell layers most closely related to zone I; Fig. 4A). *glnIIp*, which is inactive in the SBs of wild-type nodules, however, is active in the young SBs of nodules induced by strain BS11, where the induction of *nif* gene transcription is strongly reduced. Moreover, the expression of NtrC and P_{II} in young SBs of nodules induced by *R. leguminosarum* has been observed previously (Ercolano et al. 2000; Patriarca et al. 1996). Taken together, these data strongly suggest that i) the

P_{II} -mediated regulation of NtrC activity is the first step in the molecular mechanism leading to the uncoupling of NH_4^+ assimilation and N_2 fixation in differentiated SBs; ii) this event occurs early during host cell invasion, at a time when bacteria surrounded by the symbiosome membrane inside the host cells rather than in the SBs of interzone II-III are released (Fig. 4); and iii) the faint (in some nodules undetectable) activation of *nif* gene transcription in nodules formed by strain BS11 supports the idea (Tatè et al. 1999) that repression of NH_4^+ assimilation activities is a prerequisite for an efficient symbiotic interaction.

MATERIALS AND METHODS

Bacterial strains and media.

Strains of *R. leguminosarum* were grown at 30°C on either TYR-rich medium (Beringer 1974) or RMM, a chemically defined minimal medium (Hooykaas et al. 1977). Strains of *K. pneumoniae* were grown on TY-rich medium or NFDM, a chemically defined minimal medium (Dixon et al. 1977). *E. coli* strains were grown on TY medium. Antibiotics used, per ml: 5 µg of tetracycline; 30 µg of kanamycin; 100 µg of rifampicin; 100 µg of ampicillin; and 250 µg of carbenicillin. Nitrogen sources used, per ml: 1 mg of glutamine and 1 mg of glutamate or NH_4Cl . All media were solidified with 1.5% agar (Difco, Detroit, MI, U.S.A.).

Fig. 3. Histochemical localization of β -galactosidase activity in different developmental stages of *Vicia hirsuta* nodules induced by *Rhizobium leguminosarum* biovar *viciae* carrying pAR212a (*gstIp*). Developing nodules were harvested and stained as described elsewhere (Patriarca et al. 1996). β -galactosidase activity expressed from *gstIp* is evident in the first invaded cells located at **A** and **B**, the inner cortex of early emergent nodules. In a 10-day-old nodule, **C**, a decreasing apical-proximal gradient of β -galactosidase activity is observed. Later, the β -galactosidase activity begins to be evident in the infection zone II, just behind the meristem (zone I), and is not detectable in **D–F**, the cell layers of zone III. **F**, Section also is stained with I_2 -KI so that starch granules appear stained in brown. Blocking the staining reaction at the same time (30–60 min), the nodules obtained from plantlets inoculated with LPR1105 (pMP220; the promoterless vector) showed no detectable activity. **F**, Spatial development (zones I, II, III, and IV) of a mature nodule, including interzone II-III (white bar), are indicated.

Purification of GstI protein and antiserum preparation.

The coding region of *gstI* was amplified by PCR with a specific synthetic oligonucleotide (5'-TCTCCATATGCCCA-CCGGTTTCCACCG-3' and SP6 primer (Promega, Milan, Italy) with plasmid pAR205 (Spinosa et al. 2000) as a template. The resulting DNA fragment was eluted, digested with *NdeI*-*HindIII* restriction enzymes, and ligated with the *NdeI*-*HindIII*-digested pET22b(+) plasmid (Novagen, Madison, WI, U.S.A.). The resulting plasmid, pAR228, was used to transform competent cells of strain JM109(DE3) of *E. coli*. Strain JM109-pAR228 was grown in complete SB medium to an optical density at 590 nm of 2.4. Then 1 mM isopropyl- β -D-thiogalactopyranoside was added to the culture and, after 3 h, bacterial cells were harvested by centrifugation and disrupted by a cycle of freezing and thawing in a buffer containing 50 mM Tris-HCl, pH 7.5; 50 mM NaCl; and 1% Triton X-100. The insoluble fraction of the crude extract was collected by centrifugation, solubilized in 6M guanidine-HCl, and the proteins were reduced fully by the addition of 0.1 M dithiothreitol. The resulting crude extract was loaded onto a high-pressure liquid chromatography (HPLC) C8 column (Applied Biosystems, Foster City, CA, U.S.A.). A fraction containing a protein with the predicted molecular weight was then loaded onto a Bondapack HPLC C18 column (Millipore, Bedford,

MA, U.S.A.). The N-terminal amino acid sequence was determined by automated Edman degradation with a Model 477-A gas phase sequencer (Applied Biosystems) equipped with an on-line 120 A phenylthiohydantoin analyzer. Under the conditions described, approximately 8 mg of GstI protein were obtained from 1 liter of cell culture. The purified protein was used to obtain an antiserum in rabbit (BioPat, Telese, Italy).

Western blots.

Electrophoretic runs were performed with a Mini-Protean II cell unit (Bio-Rad Laboratories, Richmond, CA, U.S.A.) at room temperature. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with gels prepared with a discontinuous gradient (18, 15, and 12.5%) of polyacrylamide. Proteins were transferred onto an immunoblot polyvinylidene difluoride (PVDF) membrane (0.2 mm; Bio-Rad) in 0.375 M Tris-glycine buffer (pH 8.3) containing 10% methanol with the trans-blot apparatus (Bio-Rad). After 1 h at 50 V, the PVDF sheet was removed, soaked in blocking buffer (1 $\times$ phosphate-buffered saline, 5% milk, and 0.05 % Tween 20), and incubated for 1 h with anti-GstI antiserum from rabbit diluted 1:1000 in blocking buffer. Thereafter, the membrane was washed three times for 15 min with the blocking buffer

Fig. 4. Left: histochemical localization of β -galactosidase activity in developing nodules of *Vicia hirsuta* induced by *Rhizobium leguminosarum* A, C and E, strain LPR1105 or by B, D, and F, the *glnB* (P_{II}) mutant strain BS11. Before inoculation, by *R. leguminosarum* was transformed either with A and B, plasmid pAR212a (*gstI*p-lacZ), C and D, pAR35 (*glnI*p-lacZ), or E and F, pAR24 (*nifH*p-lacZ). Developing nodules were harvested and stained as described elsewhere (Patriarca et al. 1996). Right: scheme of the process of endocytosis by which *R. leguminosarum* bacteria (in red) are released from an infection thread (blue) inside the cytoplasm of a host invaded cell (yellow). The symbiosomes are surrounded by the symbiosome membrane (SM), which initially showed the characteristic of the plasma membrane (green).

and incubated for 1 h with horseradish peroxidase-linked whole antibody from donkey, as a second antibody, diluted 1:15000 in blocking buffer. The filters were washed and developed with the ECL Western blotting system (Amersham Pharmacia, Milan, Italy).

Expression of *gstI* from an inducible promoter.

Standard molecular cloning techniques were utilized (Sambrook et al. 1989). To obtain plasmid pAR237, a *XbaI*–*NsiI* (380 bp) DNA fragment obtained from plasmid pAR236 was purified and ligated into *XbaI*–*PstI*-digested pAR231. Plasmid pAR236, a *XbaI*–*HindIII* (380-bp) DNA fragment carrying *gstI* was obtained from pAR228 and cloned into *XbaI*–*HindIII*-digested pGEM7Zf(+). Plasmid pAR231 carrying a *dctAp*–*lacZ* transcriptional fusion was constructed by cloning an *EcoRI*–*XbaI* fragment from plasmid pAR143 into *EcoRI*–*XbaI*-digested pMP220 (Spaink et al. 1987). Plasmid pAR143 was obtained by cloning an 0.8-kb *PstI* fragment filled with T4 DNA polymerase from plasmid pCU700 (Wang et al. 1993) into *SmaI*-digested pGEM3Zf (Promega).

Site-directed mutagenesis.

The mutant derivatives of *gstI* were prepared by the overlap extension method (Ho et al. 1989) with PCR. Plasmid pAR205 (Spinosa et al. 2000) was used as template for amplification reactions carried out with an expanded high-fidelity PCR system (Roche-Boehringer, Milan, Italy), with two end primers and two complementary mutagenic primers for each site-specific mutation. Oligonucleotide sequences used (mismatched sites for site-directed mutagenesis are underlined): Trp48(+), 5'-AGCTGCGATTACCATGACGAGG-3'; Trp48(-), 5'-CTCGTCATGGATATCGCAGC-3'; Arg59(+), 5'-GAATCCGACGCCCGACGAAG-3'; Arg59(-), 5'-CTTCGTCGGGGCGTCCGATTTC-3'. Each amplification reaction was performed with 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The final amplified products were cut appropriately with restriction enzymes, purified with the Quiaex II agarose gel-extraction kit (M-Medical, Milan, Italy), and ligated into *SphI*–*HindIII*-digested pGEM7Zf+ (Promega). The cloned fragments were sequenced to confirm the presence of the mutation and to rule out the possibility that replication errors were introduced during amplification. The mutated DNA was amplified and cloned into pET22b(+), as described to obtain pAR228. Mutant derivatives of *gstI* were then cloned under the control of the *dctA* promoter by the same cloning strategy followed to obtain plasmid pAR237.

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