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The *Rhizobium leguminosarum glnB* gene is down-regulated during symbiosis

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Abstract Symbiotic nitrogen fixation involves the development, on the legume plant root, of specialised organs called nodules, within which plant photosynthates are exchanged for combined nitrogen of bacterial origin. The *glnB* gene encodes a signal transduction protein (P_{II}) which is a component of the bacterial nitrogen regulation (Ntr) system and an essential regulator of ammonium assimilation. We demonstrate that in *Rhizobium leguminosarum* the *glnB* promoter is strongly regulated by nitrogen and NtrC, but still shows a significant level of activity in conditions of nitrogen excess. Expression of genes involved in nitrogen assimilation has been shown to be absent in nitrogen-fixing bacteroids, and, in agreement with this, we find that the *glnB* promoter is down-regulated during bacteroid differentiation at a time coincident with the arrest of bacterial division in the nodule. This pattern is common to other bacterial genes involved in nitrogen assimilation and it is noteworthy that the zone where the *glnB* promoter is active is coincident with the region in which NtrC is expressed.

Key words Nitrogen metabolism · Symbiosis · Nitrogen fixation · P_{II}

Introduction

The symbiotic interaction of bacteria of the genus *Rhizobium* with legume plants requires changes in

nitrogen metabolism for both partners. During the development of legume root nodules rhizobia penetrate the curled root hairs and invade a nodule primordium through tubular structures called infection threads. Ultimately, bacteria surrounded by a plant-derived membrane are released into the cytoplasm of plant cells, where they differentiate into morphologically distinct forms called bacteroids. This differentiation process is accompanied by a rapid cessation of bacterial division, so that bacteroids are functionally nitrogen-fixing organelles that exchange ammonium for photosynthates. This implies a shift of bacterial nitrogen metabolism from ammonium assimilation to ammonium export.

Free-living rhizobia in the soil assimilate nitrogen, obtained by direct uptake of ammonium salts or by nitrate reduction. When nitrogen sources become limiting, nitrogen assimilation is enhanced by the increased synthesis and activity of a high-affinity ammonium carrier, of nitrate reductase, and of glutamine synthetase (GS). Rhizobia synthesise two forms of GS, GSI encoded by *glnA* and GSII encoded by *glnII*, and as in other Proteobacteria transcription of genes coding for GS is controlled by the global nitrogen regulatory (*ntr*) system.

The P_{II} protein, encoded by *glnB*, is an intracellular signal transducer which, in enteric bacteria such as *Escherichia coli*, facilitates regulation of GS via two distinct responses to a change in nitrogen status (for review see Merrick and Edwards 1995). P_{II} can reduce transcription of genes in the Ntr regulon, that includes *glnA*, by stimulating dephosphorylation of the transcriptional activator NtrC-P (Atkinson et al. 1994), and at a metabolic level P_{II} modulates adenylation and consequent inactivation of GSI (Stadtman et al. 1980). P_{II} itself can be reversibly modified by uridylylation of Tyr51 (Adler et al. 1975); the degree of uridylylation being low in nitrogen excess and high under nitrogen deficiency, thereby reflecting the nitrogen status of the cell. In nitrogen-excess conditions, unmodified P_{II} stimulates dephosphorylation of NtrC-P and adenylation of GSI, thereby reducing both the expression and activity of GSI. Conversely, under nitrogen-limiting

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conditions P_{II} -UMP stimulates deadenylation of GSI without affecting NtrC phosphorylation, thereby allowing maximal expression and full activity of GSI (Atkinson et al. 1994).

In all proteobacteria of the α subgroup so far studied, *glnB* and *glnA* are clustered on the chromosome, and in many cases *glnB* transcription appears to be controlled by tandem promoters (Martin et al. 1989; Foster-Hartnett and Kranz 1994; Michel-Reydellet et al. 1997). In three members of the Rhizobiaceae – *Rhizobium leguminosarum*, *R. meliloti* and *Azorhizobium caulinodans*, –24/–12 promoter sequences have been identified upstream of *glnB*, and in *R. meliloti* and *A. caulinodans* *glnB* expression is induced upon nitrogen limitation in an NtrC-dependent manner (Arcondeguy et al. 1997; Michel-Reydellet et al. 1997). Analysis of the expression of *R. leguminosarum glnB* in a heterologous background, namely *Klebsiella pneumoniae*, indicated that it was also NtrC dependent, but did not require an upstream activator sequence (Chiurazzi and Iaccarino 1990).

Characterization of *glnB* mutants in these organisms confirmed a central role for P_{II} in regulation of nitrogen metabolism, though the effects differed in each organism (Amar et al. 1994; Arcondeguy et al. 1997; Michel-Reydellet et al. 1997). In free-living growth conditions, a *R. meliloti glnB* mutant was impaired in GSI adenylation, whilst an *A. caulinodans glnB* mutant was not (Arcondeguy et al. 1997; Michel-Reydellet et al. 1997). In both *R. leguminosarum* and *A. caulinodans glnB* mutations cause constitutive expression of NtrC-dependent promoters such as *glnBp* and *glnIIp* (Amar et al. 1994; Michel-Reydellet et al. 1997), whereas *glnIIp* is inactive in the *R. meliloti glnB* mutant (Arcondeguy et al. 1997). The phenotype of the *R. leguminosarum* and *A. caulinodans* mutants is consistent with that seen in *E. coli*, such that in the absence of P_{II} , NtrB phosphorylates NtrC regardless of the N status. Consequently, in high-N conditions NtrC is probably fully phosphorylated and the *glnII* or *glnBA* promoters are expressed. A similar model can also account for the phenotype of a *Rhodobacter capsulatus glnB* mutant (Kranz and Haselkorn 1988). On the other hand in *R. meliloti* P_{II} appears to act somewhat differently. As in enteric bacteria, a non-uridylylatable P_{II} mutant prevents expression of NtrC-dependent genes such as *glnII*; however, the null mutant is Ntr deficient, as judged by the absence of *glnII* expression (Arcondeguy et al. 1997).

There is also a striking difference between *R. leguminosarum* and *R. meliloti* or *A. caulinodans* in the symbiotic role of P_{II} . *A. caulinodans glnB* mutants produced chlorotic plants and although normal numbers of nodules were formed and *nifH* transcription was normal, they did not show nitrogenase activity (Michel-Reydellet et al. 1997). This may reflect a role of P_{II} in post-translational regulation of nitrogenase activity (Michel-Reydellet and Kaminski 1999). Plant infection by *glnB* mutants of *R. meliloti* was also significantly impaired, nodulation kinetics were delayed and final nodule number decreased (Arcondeguy et al. 1997). The nodules formed were very

heterogeneous, and although some nodules appeared normal and were capable of nitrogen fixation, the plants suffered nitrogen starvation and chlorosis developed in the absence of added combined nitrogen. In contrast, a *glnB* mutant of *R. leguminosarum* is Nod⁺Fix⁺ and consequently P_{II} does not appear to be essential for symbiotic nitrogen fixation (Amar et al. 1994).

In order to relate *glnB* promoter activity to the phenotypes of the *R. leguminosarum glnB* mutant we have now analysed the expression of the *R. leguminosarum glnB* promoter during both free-living and symbiotic growth. Analysis of appropriate *lacZ* fusions indicated a marked but not complete dependence of *glnB* promoter activity on NtrC during free-living growth, with a significant level of *glnB* transcription occurring in nitrogen-excess conditions. An alternative transcriptional activator of the same family as NtrC, namely *K. pneumoniae* NifA, also induces *glnBp* activity in a heterologous *E. coli* system. During bacteroid differentiation the *glnB* promoter was also found to be markedly down-regulated at a time coincident with the arrest of bacterial division in the nodule.

Materials and methods

Bacterial strains and plasmids

R. leguminosarum bv. *viciae* wild-type strain 1004, a Rif^R derivative of RCR1001 (Hooymaas et al. 1977); *Rhizobium etli* wild type strain CE3 and its *ntrC::Tn5* derivative strain CFN2012 (Moreno et al. 1992) were grown at 30 °C in TYR medium (Tatè et al. 1998). *E. coli* ET8894 and *K. pneumoniae* UNF 931 were grown in NFD medium (Dixon et al. 1977) at 30 °C. Conjugative crosses between *E. coli* and *Rhizobium* strains were performed as described by Amar et al. (1994).

To obtain pMPCH1 the 632-bp *PstI* fragment from pCH12 (Chiurazzi and Iaccarino 1990) was ligated into the *PstI* site of pMP220 (Spaink et al. 1987). To obtain pMPCH2 the 324-bp *XhoI-PstI* fragment from pCH7A (Chiurazzi and Iaccarino 1990) was first ligated into *XhoI + PstI*-digested pGEM4 (Promega) and then subcloned as an *EcoRI-PstI* fragment into *EcoRI + PstI*-digested pMP220. To obtain pMPCH3 the *AatII-PstI* fragment from pCH14 (Chiurazzi and Iaccarino 1990) was blunt-end ligated into *SmaI + PstI*-digested pGEM4 and then subcloned as a *EcoRI-PstI* fragment into *EcoRI + PstI*-digested pMP220. pMPCH4 was obtained by ligation of the 139-bp *EcoRV-PstI* fragment from pCH11 (Chiurazzi and Iaccarino 1990) into *SmaI + PstI*-digested pGEM4 followed by ligation of the *EcoRI-PstI* fragment into *EcoRI + PstI*-digested pMP220.

Strains and plasmids used are listed in Table 1.

RNA extraction and primer extension analysis

RNA was extracted and primer extension analysis was performed as previously described (Chiurazzi and Iaccarino 1990). In each case primers *glnII* and *glnB* (Chiurazzi and Iaccarino 1990; Patriarca et al. 1992) were annealed with RNA samples in a single reaction mix.

β -Galactosidase assay

β -Galactosidase activity was assayed according to the method of Miller (1972).

Table 1 Strains and plasmids used

Strains and plasmids	Description	Source/reference
<i>E. coli</i> ET8894	$\Delta(rhaA\ glnA\ ntrB\ ntrC)1703::Muets\ rbs\ gyrA$	MacNeil (1981)
<i>K. pneumoniae</i> UNF 931	$hisD2, \Delta lac2002, recA56, sb1300::Tn10, hsdR1$	Tuli and Merrick (1988)
<i>R. leguminosarum</i> 1004	Rif ^R derivative of RCR1001	Hooykaas et al. (1977)
<i>R. etli</i> CE3	Wild type	Noel et al. (1984)
CFN2012	$ntrC::Tn5$	Moreno et al. (1992)
Plasmids		
pMM14	<i>Kamp-ntrC</i> fusion in pACYC184	Merrick (1983)
pMC71A	<i>Tcp-nifA</i> fusion in pACYC184	Buchanan-Wollaston et al. (1981)
pMB163	<i>lacp-NifA</i> C-terminal deleted fusion in pACYC184	Morett et al. (1988)
pAR66	ORF1- <i>ntrBCp</i> in pMP220	Patriarca et al. (1996)
pMP220	Tc ^r <i>lacZ</i> promoter trap	Spaink et al. (1987)

Nitrogenase activity assay

Nitrogenase activity was determined by measuring acetylene reduction in the *K. pneumoniae* UNF931 strain transformed with the different *glnBp* fragments. *K. pneumoniae* cells from a single colony were grown for 8 h in LB medium and then diluted and grown for 12 h in NFD medium with serine (130 mg/l) as nitrogen source. Acetylene (1/10 vol/vol) was added to the rubber-capped tubes and the ethylene produced was quantified with a Sigma 3B gas chromatograph (Perkin-Elmer, Foster City, Calif.).

Plant growth and histochemical localization of β -galactosidase activity

Vicia hirsuta seeds were germinated and inoculated as previously described (Amar et al. 1994). The histochemical analysis was performed according to Patriarca et al. (1996). Nodule sections were cleared by immersion for 5 min in a mixture of benzyl benzoate and benzyl alcohol (2:1) and then observed with a light microscope by means of dark- and bright-field optics. Nodules induced by *Rhizobium* strains carrying the pMP220 vector showed no background X-Gal staining. The stained nodules were photographed on a Nikon microscope using bright-field and epipolarization optics.

Results

Functional analysis of the *glnB* regulatory region in *R. leguminosarum*

To identify the *glnB* promoter region, defined fragments of the sequence upstream of the *glnB* gene were cloned into the promoter-probe plasmid pMP220 carrying the *E. coli lacZ* reporter gene (Spaink et al. 1987). The derived plasmids were conjugated into wild-type *R. leguminosarum* bv. *viciae* (strain 1004) and tested for β -galactosidase activity. Plasmid pMPCH1, which carries a DNA fragment extending 511 nt upstream of the *glnB* transcription initiation site (Chiurazzi and Iaccarino 1990), yielded a β -galactosidase activity that was 4.8-fold higher in the presence of KNO₃ compared to NH₄Cl as sole nitrogen source (Fig. 1). This level of nitrogen-dependent regulation is consistent with the results obtained by primer extension analysis (Chiurazzi

and Iaccarino 1990), indicating that pMPCH1 contains the entire upstream region needed for the physiological N-dependent regulation of the *glnB* promoter.

The promoter region was then delimited by testing plasmids pMPCH2, pMPCH3 and pMPCH4, which carry progressively larger deletions in the *glnB* upstream region. The induced promoter activity seen in KNO₃-grown cells decreased by 75% when the region between positions -511 and -203 was deleted (compare values obtained with pMPCH1 and pMPCH2 in strain 1004). Hence *glnB* transcriptional activation appears to be mainly dependent on an upstream activating sequence (UAS) in this region, but the level of activity obtained with cells grown in the presence of NH₄Cl is not affected by deletion of this region. The β -galactosidase activity was further reduced (to 5% of the full promoter activity) both in KNO₃ and NH₄Cl, with plasmid pMPCH3, indicating that the sequence mapped between positions -203 and -53 also plays a role in *glnB* promoter regulation. Finally, the promoter activity was reduced to background with pMPCH4, in which the 5' (-18) region of the -24/-12 promoter sequence is deleted.

In order to test whether the NtrC gene product is involved in transcriptional regulation of *glnB* during free-living conditions, the pMP220 derivative plasmids were introduced, by conjugation, into wild-type (CE3) and *ntrC*⁻ (CFN2012) strains of *R. etli*. Although the values for β -galactosidase activity obtained in this genetic background were slightly lower (Fig. 1), the level of nitrogen-dependent regulation was maintained, indicating conservation of the regulatory properties of *glnBp* in *R. etli*. This result was reinforced by a primer extension analysis performed with the same oligonucleotide used to map the +1 site in *R. leguminosarum* (Chiurazzi and Iaccarino 1990), which identified the same +1 in *R. etli* (data not shown).

The β -galactosidase activity of strain CFN2012 (pMPCH1) grown in the presence of KNO₃ was only 11% of the wild-type activity (Fig. 1). Hence, although, as previously reported, analysis of the *glnB* upstream region does not reveal any obvious NtrC-binding sites

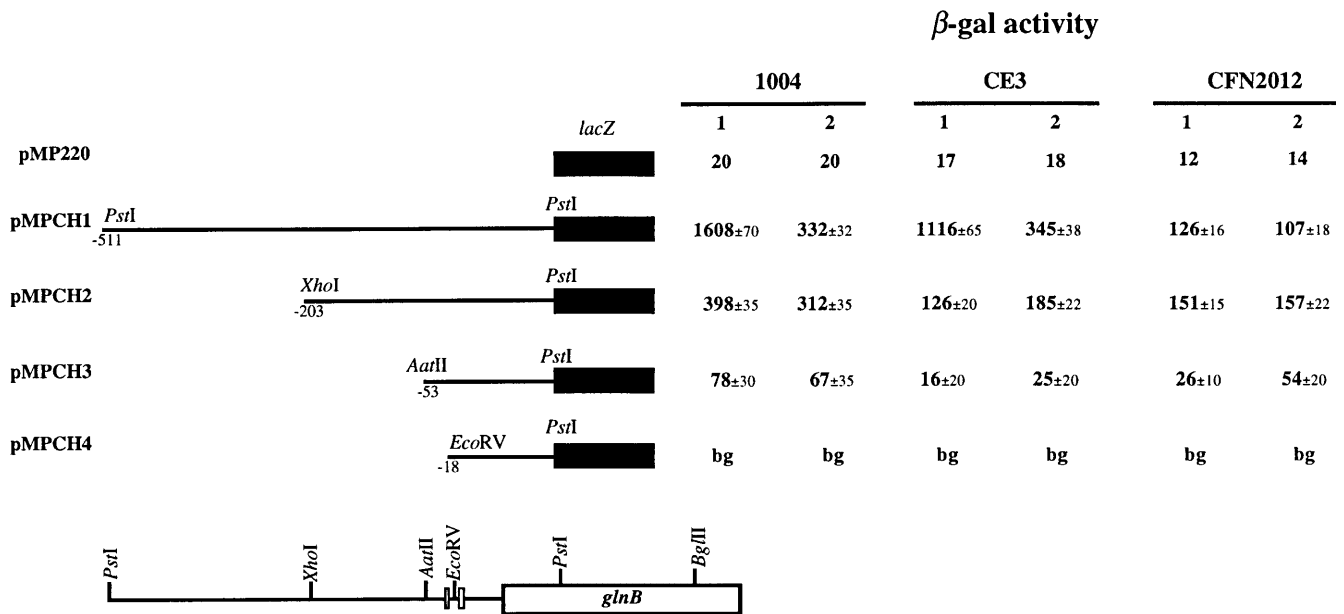


Fig. 1 Functional analysis of the *glnB* upstream region in the *R. leguminosarum* 1004 wild type and *R. etli* wild type CE3 and *ntrC*⁻ CFN2012 strains. The *lacZ* fusions are described in Materials and methods. Numbers indicate the β -galactosidase activity (Miller 1972) of bacteria grown in RMM minimal medium with either 10 mM KNO₃ (1) or 10 mM NH₄Cl (2) as sole nitrogen source. The background levels obtained with pMP220 in the different growth conditions have been subtracted. Values are averages of three different experiments and standard errors are indicated. A partial restriction map of the *glnB* genomic region is shown at the bottom. The open rectangles indicate the -24/-12 promoter sequence

(Chiurazzi and Iaccarino 1990), *glnB* expression is largely NtrC dependent. The level of activity observed in CFN2012 under the same growth conditions was not affected by the deletion of the UAS in pMPCH2, indicating that NtrC-dependent activation requires sequences between the *PstI* and *XhoI* sites.

Interestingly, with NH₄Cl as the nitrogen source, the β -galactosidase activity obtained in CE3 with pMPCH1 (345 U) was higher than with CFN2012 (107 U), suggesting that this reduced level of expression is also NtrC dependent. As expected, such NtrC-dependent, nitrogen-independent regulation was not observed with pMPCH2. As in *R. leguminosarum* 1004 strain, in CE3 and CFN2012, deletion of the *XhoI*-*AatII* fragment (pMPCH3 in Fig. 1) caused a further reduction in the promoter activity in both low and high N. The residual β -galactosidase activity in the *ntrC*⁻ background is peculiar to the *glnB* promoter when compared with the complete NtrC dependence observed with other -24/-12 promoters of genes involved in nitrogen metabolism, such as *glnII* or *amtB* (Patriarca et al. 1992; Tatè et al. 1998). We performed a primer extension analysis with specific *glnB* and *glnII* oligonucleotides and compared the relative levels of the cognate transcripts in the different N conditions. As shown in Fig. 2, the level of *glnB* and *glnII* transcripts

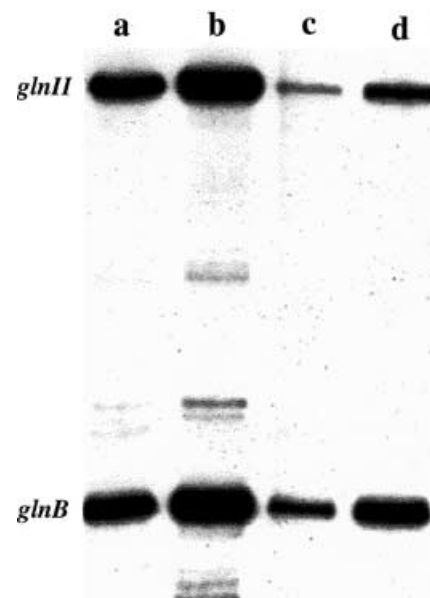


Fig. 2 Comparative primer extension analysis of the *glnB* and *glnII* transcripts. Primer extension analysis was carried out with 8 and 16 μ g of RNA extracted from *R. leguminosarum* grown in presence of 10 mM KNO₃ as the sole nitrogen source (lanes a and b), and with 8 and 16 μ g of RNA extracted from *R. leguminosarum* grown in presence of 10 mM NH₄Cl as the sole nitrogen source (lanes c and d). The products derived from the *glnB* and *glnII* transcripts are indicated

in the presence of KNO₃ was comparable, whereas in the presence of NH₄Cl the relative amount of *glnB* transcript was threefold higher. Thus, under conditions where NtrC is completely or partially dephosphorylated, the *glnB* promoter shows a level of activity which is unusual for *rpoN*-dependent promoters regulated by NtrC.

The effect of NifA on *glnB* promoter activity

The observed NtrC-independent activity in *R. etli*, and the previous analysis of *glnBp* performed in the heterologous genetic background of *K. pneumoniae* (Chiurazzi and Iaccarino 1990), prompted us to investigate whether NifA, a σ^{54} -dependent transcriptional activator which is a central regulatory component for nitrogen fixation, is also capable of activating the *glnB* promoter.

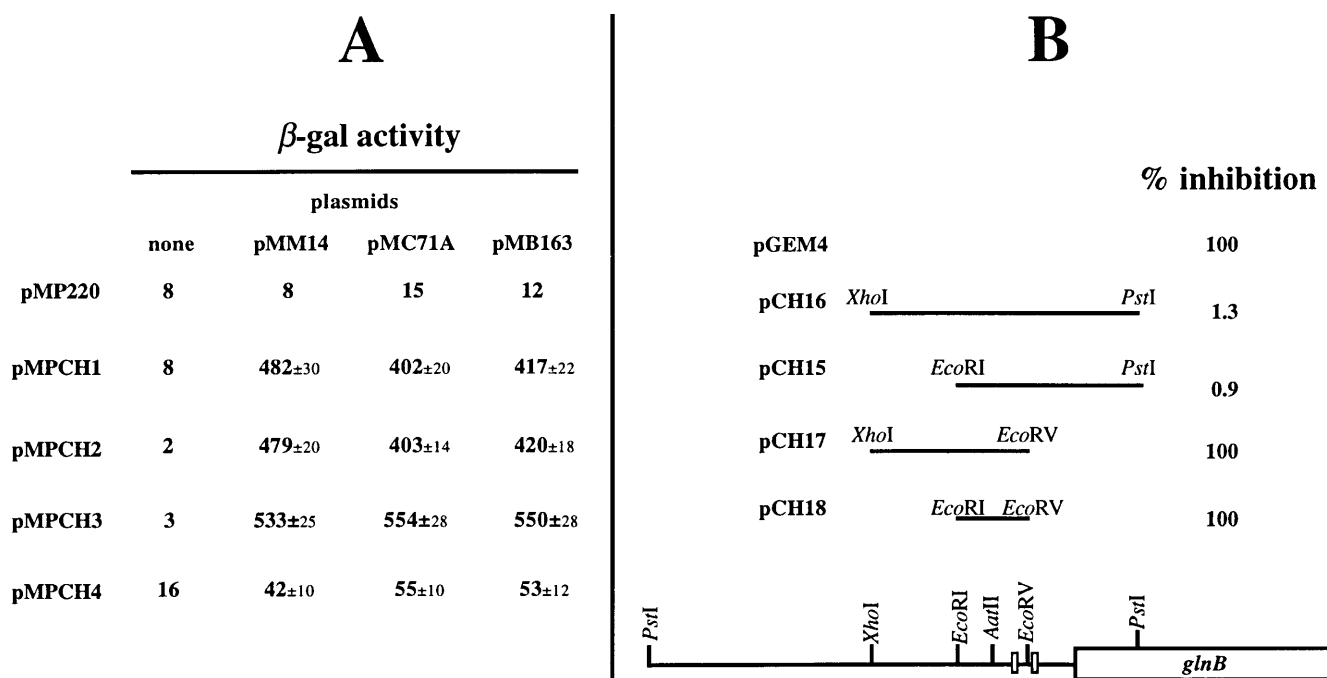
NifA-dependent activation of *glnBp* was tested by two different approaches. First, the pMP220 derivative plasmids used in the analysis shown in Fig. 1 were transferred into the heterologous genetic background of *E. coli* strain ET8894, which carries a chromosomal deletion of the *glnAntrBC* operon. Induction of β -galactosidase activity was detected when the *KpNifA*, encoded by plasmid pMC71A, was present, and this

Fig. 3A Comparison of the transcriptional activation of *glnBp* by NtrC, NifA and NifA*. The plasmids pMPCH1, pMPCH2, and pMPCH3 (Fig. 1) were transformed into the *E. coli* ET8894 strain containing plasmids pMM14 expressing the *K. pneumoniae* NtrC (Merrick 1983), pMC71A expressing the *K. pneumoniae* NifA (Buchanan-Wollaston et al. 1981) and pMB163 expressing a C-terminal deleted derivative of the *K. pneumoniae* NifA* (Morett et al. 1988). Values indicate the β -galactosidase activity (Miller 1972) expressed by bacteria grown in NFDM minimal medium. The background levels obtained in absence of transcriptional activation have been subtracted. **B** Inhibition of *K. pneumoniae* nitrogenase activity by *R. leguminosarum glnBp* sequences. The *glnB* upstream sequences were subcloned in the high-copy-number pGEM4 plasmid. Values represent the percentage inhibition of the nitrogenase activity (expressed as mmol of C_2H_2 reduced per h per ml of culture) of bacteria grown in NFDM minimal medium with serine (200 μ g/ml) as sole nitrogen source and are the average of three independent determinations. A partial restriction map of the *glnB* genomic region is shown at the bottom. The open rectangles indicate the $-24/-12$ promoter sequence

activity was comparable to that obtained using NtrC (pMM14) as transcriptional activator (Fig. 3A). NifA-dependent activation of the *glnB* promoter was also tested in ET8894 harbouring derivative plasmids carrying deletions of the *glnB* upstream region. Comparable high levels of β -galactosidase activity were obtained with plasmids pMPCH1, and pMPCH2, indicating that in *E. coli* the promoter activity is not dependent on the UAS mapped in *R. leguminosarum*.

Activation of *glnBp* was also examined in ET8894 cells containing plasmid pMB163. This plasmid expresses an altered form of *KpNifA* (NifA*) that is defective in DNA binding due to the deletion of its C-terminal region containing the helix-turn-helix DNA-binding motif. As shown in Fig. 3A, NifA* promoted an equivalent level of *lacZ* activation to that seen with the wild-type NtrC and NifA proteins. These results indicate *glnBp* can be activated by NtrC or NifA, despite the fact that no sequences resembling NtrC (GCAC-N₇-GTGC) or NifA (TGT-N₁₀-ACA; Morett and Buck 1988) binding sites are found in or around the *glnB* upstream region. This suggests that the expression observed in conditions of overexpression of NtrC, NifA and NifA* is due to direct interaction of the activator with the RNA polymerase- σ^{54} complex bound at the $-24/-12$ sequence. The activation observed in this situation can not, however, be related directly to *glnB* regulation in *Rhizobium*, because different levels of NtrC and different physiological conditions will exist in this organism.

An alternative explanation for the NifA-dependent transcriptional activation of *glnBp* is that, even in the heterologous *E. coli* host, NifA induces another transcriptional activator, which in turn activates *glnBp*. This possibility was ruled out by performing multicopy *nif* inhibition experiments. When promoters containing



NifA-binding sites are introduced into *K. pneumoniae*, *nif* gene expression and nitrogenase activity are inhibited, most probably due to titration of NifA, and this observation has been used in the past to define sequences important for NifA binding (Buck et al. 1987). If NifA activates transcription from *glnBp*, titration of NifA should result in decreased *nif* gene expression and nitrogenase activity (Buchanan-Wollaston et al. 1981). To test this hypothesis, restriction fragments containing *glnBp* cloned in the multicopy pGEM4 plasmid (Promega) were introduced into *K. pneumoniae* UNF931 and tested for inhibition of nitrogenase. Plasmids pCH15 and pCH16 caused complete inhibition of nitrogenase activity, whereas pCH17 and pCH18 carrying only sequences upstream of the -24/-12 promoter did not show any inhibition (Fig. 3B). The inhibition of nitrogenase activity seen in the presence of the *EcoRI-PstI* fragment on pCH15 is most probably not due to binding of NifA to this fragment but rather to a strong interaction between NifA and the holoenzyme which is expected to be efficiently bound to the -24/-12 promoter sequence.

Expression of *glnBp* during nodule development

The symbiotic activity of *glnBp* was tested in the *R. leguminosarum*-vetch system, in which indeterminate nodules develop. In a longitudinal section of a mature indeterminate nodule it is possible to recognize all the developmental zones (Vasse et al. 1990). Proceeding from the apex of the nodule toward the root, the apical zone (zone I) corresponds to the nodule meristem, the invasion zone (zone II) is characterized by the presence of infection threads and of plant cells containing young bacteroids, and the interzone II-III shows a sharp developmental switch coincident with starch accumulation and nitrogenase gene (*nif*) induction by NifA. This pattern of expression continues in the whole nitrogen-fixing zone (zone III).

Roots of *V. hirsuta* plantlets were inoculated with *R. leguminosarum* 1004 containing pMPCH1. Roots were harvested at different times after inoculation and in situ expression of β -galactosidase was observed after staining with X-Gal. The *glnB* promoter is active in bacteria growing inside the infection threads (data not shown) and in bacteroids released in the first invaded cells of the inner cortex during primordium formation (Fig. 4A). In a more mature nodule (7 days post infection, d.p.i.) a decreasing distal-proximal gradient begins to be observed (Fig. 4A), and at 21 d.p.i. switch-off of the promoter is observed in the N_2 -fixing zone and the activity is restricted to bacteroids present in the younger invaded cells of the invasion zone (Fig. 4C). As shown in Fig. 4D, the pattern of *glnB* promoter activity is comparable to that of the down-regulated ORF1-*ntrBC* promoter (Patriarca et al. 1996). On the other hand, as a control, a *R. etli nifHp-lacZ* fusion was switched on in the interzone II-III and was expressed throughout zone III (data not shown).

Discussion

We report here an analysis of the transcriptional regulation of the *R. leguminosarum glnB* gene during both free-living and symbiotic growth. In free-living, nitrogen-starved cells the promoter was induced around five-fold in an NtrC-dependent manner, but a significant level of activity, some of which was still NtrC-dependent, remained under conditions of nitrogen-sufficient growth. We also report the first histological localization of *glnB* promoter activity in nodular tissue, and we find that expression of *glnB* is significantly down-regulated during nodule development.

Expression of *glnBp* in free-living cells

The β -galactosidase activities reported in Fig. 1 indicate a strong dependence of the *glnB* promoter on NtrC when it is expressed in *Rhizobium*, and identify a UAS located between positions -511 and -203. The lack of an obvious NtrC binding site in this upstream sequence suggests that expression could be due either to the binding of NtrC to a sequence which is not sufficiently similar to the consensus to be easily identified, or to the involvement of a different activator, which is dependent on NtrC for its own synthesis. Promoters that are naturally activated by NtrC in the absence of any UAS have been reported in *Salmonella typhimurium* (*argT*, Schmitz et al. 1988) and *K. oxytoca* (*nasR*, Wu et al. 1999).

The *glnB* UAS detected in *Rhizobium* was not apparent when the promoter was analysed in a heterologous *K. pneumoniae* system in an earlier study, and in that case a deletion extending up to the *AatII* site (pMPCH3 in Fig. 1) retained full activity (Chiurazzi and Iaccarino 1990). Those data, together with the comparable analysis reported here in the heterologous *E. coli* background – where NtrC and NifA are equally effective as activators, suggest that in both heterologous systems promoter activation occurs by recruitment of the activator from solution to a preformed, DNA-bound RNA polymerase- σ^{54} complex. Activation of σ^{54} -dependent promoters from solution is a characteristic of promoters in which the -24/-12 sequence shows a high degree of homology to the consensus sequence (Morett and Buck 1989) and, as shown in Fig. 5, the *glnB* promoter sequence is such a promoter which shows essentially complete agreement with the consensus.

Hence, as the levels of expression in the heterologous systems are only around 25% of that seen in *Rhizobium*, it seems that the second hypothesis, i.e. that in *Rhizobium* the *glnB* promoter is normally regulated by a novel activator protein, is the more likely. Such a situation has also been reported for the *glnB* promoter of *Azospirillum brasilense*, which is subject to nitrogen control but is not dependent for its expression on NtrBC (de Zamaroczy et al. 1993). In that case mutations in *glnB* and *glnA*

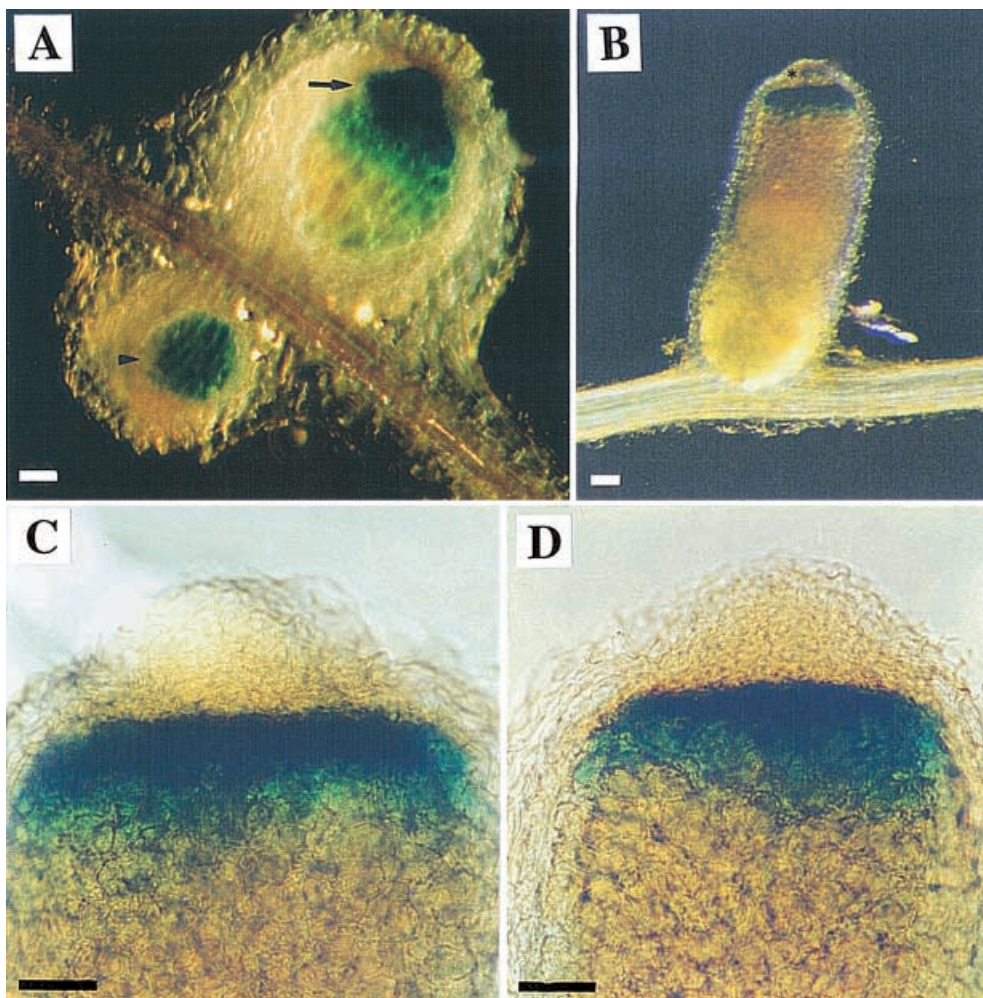


Fig. 4A–D Histochemical localization of β -galactosidase activity at different developmental stages in *V. hirsuta* nodules induced by *R. leguminosarum* bv. *viciae* carrying either pMPCH2 or pAR66 (ORF1-*ntrBC* promoter region fused to the *lacZ* described in Patriarca et al. 1993). **A** β -Galactosidase activity expressed from the *glnBp* (plasmid pMPCH1) is evident in the first invaded cells of an early emergent nodule (*arrowhead*). In a slightly older nodule a decreasing apical-proximal gradient of β -galactosidase activity is observed (*arrow*). **B** In 21-day-old nodules β -galactosidase activity becomes evident in the infection zone just behind the meristem (*asterisk*). **C** Higher magnification of the zone of *glnBp* activity. **D** β -Galactosidase activity expressed from the ORF1-*ntrBCp* in a comparable mature nodule. Nodules obtained from plantlets inoculated with *R. leguminosarum* bv. *viciae* carrying the pMP220 plasmid show no activity

nitrogen metabolism (Pawlowski et al. 1991). NtrX is also present in *R. etli* (Patriarca, Merrick and Nogueira, unpublished).

Whilst the data from *R. leguminosarum* and *R. etli* indicate that the response of the *glnB* promoter to the availability of fixed nitrogen is directly or indirectly dependent on NtrC, the level of expression in the presence of NH_4Cl is relatively high (around 30% of the fully induced level) compared to other NtrC-dependent promoters, e.g. *glnIp*. This may reflect the sensitivity of *glnBp* (or the promoter controlling the expression of an alternative transcriptional activator) to a low level of the

affected *glnBp* expression and it was suggested that glutamine synthetase might act by modulating the intracellular N status, and P_{II} might modify the properties of an unidentified regulator which could be a functional homologue of NtrC. A comparable activator could also be required for expression of the *R. etli* *amtB* promoter, which is NtrC dependent, but does not contain any strong upstream NtrC-binding site (Tat  et al. 1998). A putative candidate for this activator is the NtrC-like protein NtrX, which was originally identified in *A. caulinodans*, where it plays a role in the regulation of

consensus	R Y T G G C A Y R R N N N Y T G C W N N R	% of similarity
<i>R. leg. glnB</i>	A C T G G C A C G A T A T C T G C A T C A	100
<i>S. typ. argT</i>	A A T G G C A T A A G A C C T G C A T G A	94
<i>K. oxy. nasR</i>	T A A G G C A C G G T T A T T G C T T G G	81

Fig. 5 Comparison of the *R. leguminosarum* *glnB*, *S. typhimurium* *argT* and *K. oxytoca* *nasR* -24/-12 promoter sequences with the consensus sequence. The conserved positions are *underlined* and the percentage identity is indicated. R, purines; Y, pyrimidines; W, A or T

NtrC-P present in NH_4Cl -grown cells. The primer extension analysis shown in Fig. 2 confirms that, by comparison with the *glnIIp*, the low level of the N-dependent regulation of the *glnIIp* is due to a higher level of activity under conditions of nitrogen excess. Such a phenomenon is not without precedent, as similar data for NtrC-dependent promoters have been reported in other organisms, e.g. the *K. pneumoniae* and *A. caulinodans glnK-amtB* promoters (Jack et al. 1999; Michel-Reydellet and Kaminski 1999). Finally there is also a low level (around 10%) of expression from *glnBp* that is apparently independent of NtrC or of the cellular nitrogen status. This expression is dependent on promoter sequences between positions -203 and -53 and could potentially be controlled by another different transcriptional activator.

Expression of *glnBp* during symbiosis

The experiments reported in Fig. 4 demonstrate that transcription of *glnB* is switched off during the development of indeterminate *V. hirsuta* nodules. This identifies a correlation between promoter activity and the physiology of the different developmental zones within this type of nodule. The *glnBp* is expressed in zone II and down-regulation takes place in zone III. The pattern of *glnBp* activity is identical to that of the down-regulated ORF1-*ntrBCp* (Fig. 4C, D) (Patriarca et al. 1996). Since in zone II of a mature nodule the O_2 concentration is partially lowered (Soupeine et al. 1995) we can not exclude the possibility that the oxygen tension is the signal responsible for promoter down-regulation. Furthermore, an identical pattern of down-regulation of *glnBp* was observed in a *R. leguminosarum nifA*⁻ (Fix⁻) strain (data not shown), ruling out the possibility that the appearance of ammonia synthesized through nitrogenase activity is responsible for this pattern of activity.

The pattern of *glnBp* activity is consistent with the proposed model of uncoupling between nitrogen assimilation and nitrogen fixation during symbiotic growth of the bacteria (Tatè et al. 1999). Expression of P_{II} within the infection zone should be required to facilitate dephosphorylation of NtrC and consequent down-regulation of *ntr*-regulated genes, including *amtB* and *glnII*, whose expression must be switched off to achieve maximum efficiency of symbiotic nitrogen fixation (Tatè et al. 1999; Patriarca et al., unpublished data). This model presumes the maintenance of P_{II} in its deuridylylated form, thereby stimulating dephosphorylation of NtrC-P (Patriarca et al. 1994) and adenylation of GSI (Espin et al. 1994) – with the consequent inhibition of GS expression and activity. However, the presence of deuridylylated P_{II} presumes a nitrogen-excess condition in zone II of the nodule, or alternatively an earlier disappearance of uridylyltransferase, the *glnD* gene product. The expression of *glnBp* in bacteroids within young infected cells and in zone II of mature nodules could be

NtrC dependent or NtrC independent, but the lack of *glnBp* activity in zone III of the nodule, where nitrogen fixation takes place, indicates that *R. leguminosarum* NifA does not induce *glnBp* activity in the nodular tissue, despite the fact that *K. pneumoniae* NifA, when overexpressed, can activate the promoter in free-living cells. This could be explained by some functional difference between the *K. pneumoniae* and *R. leguminosarum* NifA proteins (Morett and Segovia 1993) or by the presence of a specific transcriptional repressor that is switched on in interzone II–III. Alternatively, down-regulation of *glnBp* in zone III could be explained by the presence in *R. leguminosarum* of two different σ^{54} proteins, as has been reported for *R. etli* (Michiels et al. 1998) and *Bradyrhizobium japonicum* (Kullik et al. 1991). In this case a σ^{54} with a specific affinity for the *glnB* promoter sequence could be down-regulated, whilst a second σ^{54} responsible for *nif* gene activation could be maintained in the zone where nitrogen fixation takes place.

However, the observed pattern of down-regulation for *R. leguminosarum glnBp* is consistent with the reported Nod⁺ Fix⁺ phenotype of the *glnB* mutant (Amar et al. 1994) and with the lack of any early or late symbiotic phenotype. The constitutive expression of NtrC-dependent promoters observed in an *R. leguminosarum glnB*⁻ mutant (Amar et al. 1994) would not give any significant symbiotic phenotype, as it has been shown by immunolocalization analysis that NtrC protein disappears abruptly from bacteroids within one cell layer (Patriarca et al. 1996).

On the basis of a number of studies a dual pattern of down-regulation of bacterial gene expression within the nodule is now apparent. Some genes, such as *amtB* (Tatè et al. 1998) and *glnII* (Patriarca et al. unpublished data), are active in bacteria growing inside infection threads but are inactive in bacteroids. Other genes, such as *glnB*, *nod*, *ropA* and *ntrBC* (Sharma and Signer 1990; de Maagd et al. 1994; Patriarca et al. 1996) and *lipA* (Patriarca et al., unpublished data) show a different pattern of expression; their promoters are active in young bacteroids but inactive in differentiated N-fixing bacteroids (zone III). Analysis of the features of those *Rhizobium* promoters whose activity has so far been characterised during symbiosis does not yet allow us to define a general scheme for transcriptional regulation of rhizobial genes in zones I, II and II–III of the nodule. Furthermore, the two known patterns of early or late switch-off are observed for both -35/-10 and -24/-12 promoters. This suggests that the respective sigma factors are not limiting or inactive in these zones of the nodule. However, *glnBp* is the first -24/-12 promoter to show a down-regulated pattern in interzone II–III.

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