

The *Rhizobium etli amtB* Gene Coding for an NH_4^+ Transporter Is Down-Regulated Early During Bacteroid Differentiation

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During development of root nodules, *Rhizobium* bacteria differentiate inside the invaded plant cells into N_2 -fixing bacteroids. Terminally differentiated bacteroids are unable to grow using the ammonia (NH_3) produced therein by the nitrogenase complex. Therefore, the nitrogen assimilation activities of bacteroids, including the ammonium (NH_4^+) uptake activity, are expected to be repressed during symbiosis. By sequence homology the *R. etli amtB* (ammonium transport) gene was cloned and sequenced. As previously shown for its counterpart in other organisms, the *R. etli amtB* gene product mediates the transport of NH_4^+ . The *amtB* gene is cotranscribed with the *glnK* gene (coding for a P_{II} -like protein) from a nitrogen-regulated σ^{54} -dependent promoter, which requires the transcriptional activator NtrC. Expression of the *glnKamtB* operon was found to be activated under nitrogen-limiting, free-living conditions, but down-regulated just when bacteria are released from the infection threads and before transcription of the nitrogenase genes. Our data suggest that the uncoupling between N_2 -fixation and NH_3 assimilation observed in symbiosomes is generated by a transcriptional regulatory mechanism(s) beginning with the inactivation of NtrC in younger bacteroids.

Additional keywords: *Phaseolus vulgaris*.

The soil bacterium *Rhizobium etli* has the ability to colonize the roots of common beans (*Phaseolus vulgaris*) and to induce therein specialized organs, the nitrogen-fixing nodules. From the point of view of the bacterial partner, this symbiotic interaction is a multistage process including bacterial multiplication in the rhizosphere, infection of the root hairs, and bacterial growth inside a network of infection threads that traverse cortical cells. Subsequently, bacteria from the tip of infection threads are extruded into unwallied infection droplets and are then released into the plant cells through a mechanism similar to endocytosis, becoming engulfed in a membrane of plant

origin, called the peribacteroidal membrane (PBM). Within the symbiosomes (consisting of bacteroids enclosed by the PBM), the bacteria, termed bacteroids, arrest division and then differentiate into their mature form, which are organelle-like structures able to reduce atmospheric dinitrogen (for a review see Mylona et al. 1995). In summary, nodules are very complex and specialized structures designed to accommodate the requirements of symbiotic N_2 fixation.

Since most of the nitrogen fixed in bacteroids is liberated to the host plant to satisfy its demands for nitrogen, N_2 fixation and NH_4^+ metabolism (transport and assimilation) are expected to be uncoupled in bacteroids. Microorganisms that fix N_2 under free-living conditions assimilate the NH_4^+ produced by nitrogenase activity through the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway (Merrick and Edwards 1995) and also activate ammonium transporter(s), most probably in order to retrieve NH_3 escaping by diffusion (Kleiner 1993). In contrast, it has been shown that in N_2 -fixing bacteroids NH_4^+ assimilation activities are either absent or are expressed at a very low level (Bergersen and Turner 1967; Brown and Dilworth 1975; Glenn and Dilworth 1984). However, since the material used in these assays most probably contained bacteroids at different stages of differentiation, it is very difficult (or impossible) to draw unambiguous conclusions from these experiments (e.g., a low enzymatic activity may correspond to a high activity in only a subpopulation of bacteroids). In this context almost nothing is currently known about the mechanism(s) of gene regulation acting during bacteroid differentiation to uncouple N_2 fixation and NH_4^+ assimilation activities.

In *R. etli* (formerly *Rhizobium leguminosarum* bv. *phaseoli*; Segovia et al. 1993), transcription of genes involved in NH_4^+ assimilation, such as *glnII*, which codes for glutamine synthetase II (GSII), is controlled in response to fixed nitrogen availability by the global nitrogen regulatory system (*ntr* system), including the protein kinase NtrB and the nitrogen transcriptional regulator NtrC (Moreno et al. 1992; Patriarca et al. 1993, 1994). Furthermore, it was recently demonstrated that the *R. etli* NtrC protein disappears from bacteroids during their differentiation, thus suggesting that NtrC-dependent promoters, such as that controlling expression of GSII, may be active in bacteria growing inside infection threads and in younger dividing-bacteroids but inactive in nondividing, terminally differentiated bacteroids (Patriarca et al. 1996). Nev-

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ertheless, this hypothesis should be tested, since alternative transcriptional activators, which in symbiosis are able to activate promoters that require NtrC under free-living conditions, may be present in N₂-fixing bacteroids.

While genes coding for GS and their regulation are well described, nothing is currently known about genes involved in NH₄⁺ transport in the family *Rhizobiaceae*. The presence of an energy-dependent transport system for NH₄⁺, determined by methylammonium [¹⁴C]-MA uptake assays, was previously demonstrated in many different *Rhizobium* spp. (Kleiner 1985, 1993). In *Bradyrhizobium* sp. ANU289, the expression of a (methyl)ammonium transport system was found to be dependent on NtrC and strongly regulated by nitrogen availability (Udvardi et al. 1992). Genes coding for high-affinity (methyl)ammonium transporters have been cloned from *Saccharomyces cerevisiae* (Marini et al. 1994), *Arabidopsis thaliana* (Ninnemann et al. 1994), *Corynebacterium glutamicum* (Siewe et al. 1996), and *Escherichia coli* (van Heeswijk et al. 1996). These genes encode highly homologous membrane proteins of around 54 kDa, with 10 to 12 predicted transmembrane domains, and they potentially constitute a new family of transporters (Marini et al. 1994).

We report here the cloning and sequencing of the *glnKamtB* operon of *R. etli* and the demonstration that the product of the *amtB* gene mediates transport of (methyl)ammonium in *R. etli*. The promoter of the *glnKamtB* operon was identified and found to be subject to nitrogen control exerted through the modulation of NtrC activity. Consequently the intracellular level of *glnKamtB* mRNA was regulated by nitrogen availability. Moreover, strain CFN2012 (*ntrC*⁻) showed no detectable NH₄⁺ uptake activity, as measured by [¹⁴C]-MA uptake assays and, in correlation, the absence of *glnKamtB* transcripts as determined by RNase protection assays. Furthermore, by using histochemical localization of β-galactosidase activity expressed from *lacZ* fusions, it was observed that the promoter of the *glnK-amtB* operon is active in bacteria growing inside infection threads but is inactive just as bacteria are released within the invaded plant cells.

RESULTS

Identification of the *R. etli amt* locus.

To clone the *amt* locus of *R. etli*, two oligonucleotides, 5'-CCGGGATTAGCGTTATTT-3' and 5'-GTTAAAGCCGAAC CAGCC-3', based on the sequence of the *nrgA* gene of *B. subtilis*, were synthesized and used in polymerase chain reaction experiments with *B. subtilis* DNA as template. The resultant DNA fragment (about 600 bp) was ³²P-labeled and probed in a Southern blot experiment against *R. etli* DNA digested with *Hind*III. A hybridizing band of about 1.3 kb was revealed, electroeluted from a parallel lane of the gel, and ligated to *Hind*III-digested pGEM7Zf+ DNA. By colony hybridization several positive clones were isolated and the specificity of the cloned DNA fragments was confirmed by Southern blot analysis.

For one of these plasmids, pAR124, a restriction map was determined (Fig. 1) and in order to clone the 5'-flanking region the leftmost *Hind*III/*Sac*I fragment from pAR124 (pAR129) was ³²P-labeled and probed in a Southern blot experiment against *R. etli* DNA double-digested with *Sac*I and a second restriction enzyme. From the *Aat*II/*Sac*I double digestion a distinct hybridizing band of about 1.5 kb was identified,

electroeluted from a parallel lane of the gel, and ligated to *Aat*II/*Sac*I-digested pGEM7Zf+ DNA. After colony hybridization several positive clones were isolated and the specificity of the cloned DNA fragments was confirmed by Southern blot analysis. For one plasmid, pAR131, a restriction map was determined (Fig. 1) and the DNA sequence of the region between the *Hind*III and *Sac*I restriction sites in pAR131 was shown to be identical to the equivalent region in pAR124. Colinearity with the genome of *R. etli* was confirmed with different DNA fragments from either pAR124 or pAR131 as probes in Southern blot experiments against DNA digested with different restriction enzymes (data not shown).

Sequence analysis and codon usage frequency analysis of the DNA region covered by pAR124 and pAR131 revealed three open reading frames (ORFs) (Fig. 2). The first of these extended from the *Pst*I site of clone pAR131 (Fig. 1) to nucleotide 198 (65 codons) of the sequence shown in Figure 2. The deduced protein sequence showed significant homology (53 to 66% identical residues) to that of the C-terminal portion of the acyl CoA thioesterase II (the *tesB* gene product) of various organisms.

The second ORF is of 351 bp (117 codons) with an ATG start codon at position 409 of the nucleotide sequence and a stop codon (TAA) at position 759. A potential ribosome binding site was identified just upstream of this ORF and no potential Rho-independent transcriptional terminator was identified immediately downstream of it. The deduced protein sequence, with a predicted molecular weight of 12,564, is very similar to that of members of the P_{II} protein family (encoded by *glnB*, and also by *glnK*, in *E. coli*), which is a component of the signal transduction pathway of the nitrogen regulation (*ntr*) system found in many eubacteria. In *Rhizobiaceae* the *glnB* gene is located immediately upstream of the structural gene (*glnA*) for glutamine synthetase I (GSI) (Colonna-Romano et al. 1987; Martin et al. 1989; Arcondéguy et al. 1996). A sequence comparison suggests that this ORF encodes a P_{II}-like protein more closely related to *E. coli* GlnK than to *E. coli* GlnB. Given these similarities and the linkage of this *glnB*-like gene to a homologue of *E. coli amtB* (see below) we propose to designate this gene *glnK*.

The third ORF is of 1,293 bp (431 codons) with an initiating ATG at position 921 of the nucleotide sequence and a stop codon (TGA) at position 2211 (Fig. 2). A potential Rho-independent transcriptional terminator (a GC-rich sequence with dyad symmetry followed by a T-rich region) is present just downstream of the coding sequence. The ORF encodes a

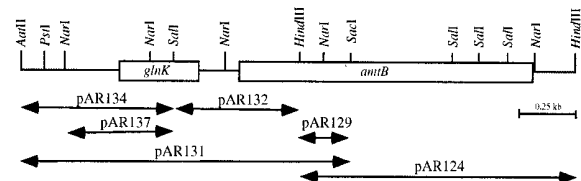


Fig. 1. Physical map of the *amt* locus of *Rhizobium etli*, with partial restriction map and localization of the *glnK* and *amtB* genes (boxed regions). Plasmids indicated below (all cloned into pGEM7Zf+) are as follows: pAR124 (1.3-kb *Hind*III fragment), pAR129 (0.24-kb *Hind*III/*Sac*I fragment), pAR131 (1.5-kb *Aat*II/*Sac*I fragment), pAR132 (0.53-kb *Hind*III/*Sall* fragment cloned in the *Hind*III/*Xho*I sites), pAR134 (0.7-kb *Aat*II/*Sall* fragment cloned in the *Aat*II/*Xho*I sites) and pAR137 (0.43-kb *Nar*I/*Nar*I fragment cloned in the *Cla*I site).

protein with a predicted molecular weight of 45,520 and comparison of the deduced protein sequence with sequences in the GenPept data base revealed significant similarity only with the previously reported ammonium transporter Amt-like sequences. We therefore propose this ORF to be the *R. etli amtB* gene, the deduced product of which shares 46% identical residues with the product of the *E. coli amtB* gene, 43% identical residues with the product of *B. subtilis nrgA*, 30% identical

residues with the product of the *S. cerevisiae* MEP1 gene, and 28% identical residues with the product of the *A. thaliana* AMT1 gene. Computer-based predictions of secondary structure (Top Pred; von Heijne 1992) suggest that the protein may have 10 or 11 membrane-spanning helices (see Figure 2). This sequence shows no marked similarities to other known ion transport proteins, suggesting that the AMT proteins constitute a new class of membrane-bound transport proteins.

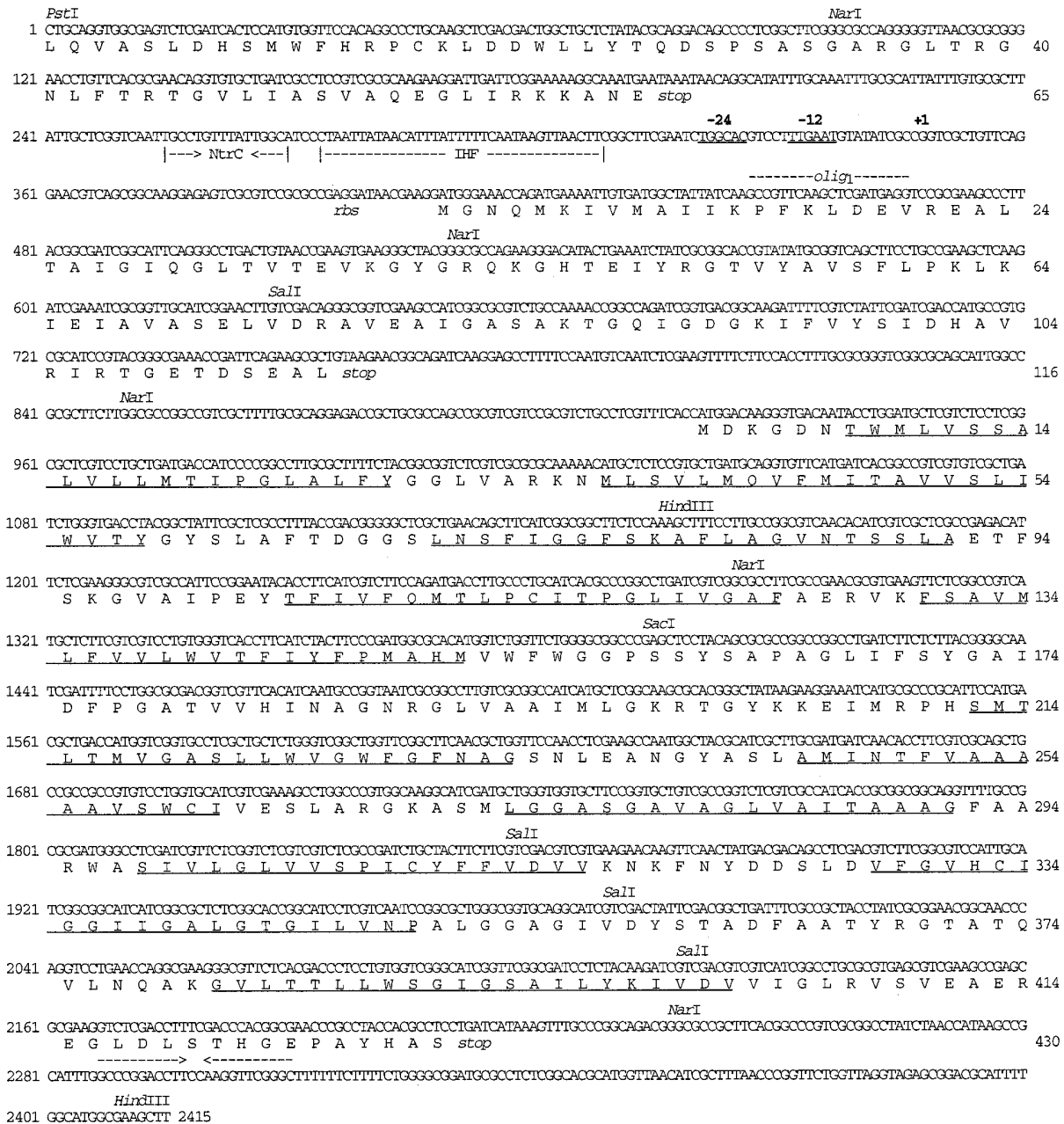


Fig. 2. DNA sequence of *glnK* and *amtB* and its 5' flanking region. Sequence of 2,415 nucleotides begins at leftmost *PstI* site shown in Figure 1. Translated sequences of *glnK* and *amtB* are shown in single-letter code, based on their presumed start codons; stop codon is indicated by "stop." Numbers on the left represent nucleotides; those on the right represent amino acids. Some restriction sites are indicated. The 5' end of *glnKamtB* RNA is indicated by +1. Promoter sequences similar to the -24/-12 consensus, a putative integration host factor (IHF) binding site and a putative NtrC binding site, are indicated. A putative ribosome-binding site (*rbs*) and a putative Rho-independent transcriptional terminator (---> <---) are indicated. Oligonucleotide synthesized for primer extension analysis is indicated as *olig1*. Putative membrane spanning helices are underlined. Data will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession number AJ002489.

Identification of the *glnKamtB* promoter region.

Defined fragments of the regions upstream of both *glnK* and *amtB* were cloned into pMP220, a low-copy number, promoter-probing vector carrying the *E. coli lacZ* reporter gene (Spaink et al. 1987). The resulting plasmids were conjugated into the wild-type (CE3) and *ntrC*⁻ mutant (CFN2012) strains of *R. etli* and tested for β-galactosidase activity. As indicated in Figure 3A, only background activity was detected in either strain containing plasmid pAR135 carrying 294 bp upstream of the putative start codon of *amtB*. By contrast, plasmid pAR136, carrying the DNA region upstream of *glnK* gene, promotes transcription of *lacZ*. The β-galactosidase activity was twelvefold higher when the wild-type strain was grown on glutamate (520 ± 45 units), as compared with NH₄Cl (42 ± 6 units) as a sole nitrogen source. Only background levels of β-galactosidase activity were detected in the *ntrC*⁻ mutant of *R. etli* (strain CFN2012) containing plasmid pAR136. These

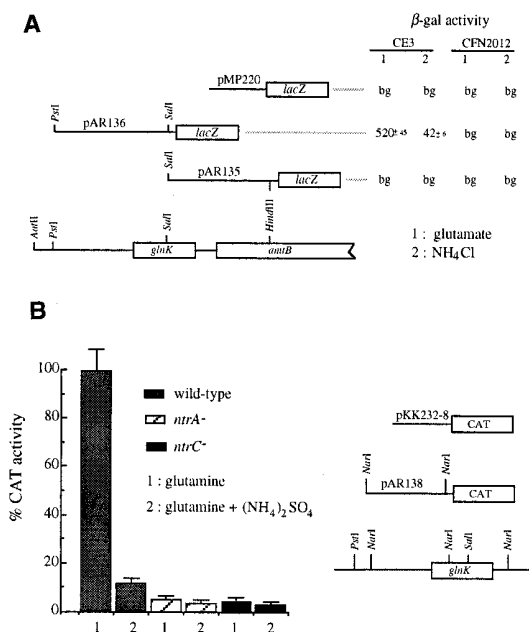


Fig. 3. A, Identification of the *glnKamtB* operon promoter region. DNA fragments from both the *glnK* and *amtB* upstream regions were cloned into the promoter-probing vector pMP220 (Spaink et al. 1987) carrying the *Escherichia coli lacZ* reporter gene. Resulting plasmids were conjugated into *Rhizobium etli* strain wild-type (CE3) or *ntrC*⁻ mutant (CFN2012). β-Galactosidase activities (Miller 1972) of bacteria grown in RMM minimal medium with as sole nitrogen source either 10 mM glutamate (1) or 10 mM NH₄Cl (2), are indicated. Values are average of three different experiments and standard errors (±) are indicated. Background activity (bg) was 48 to 54 units. **B**, Functional analysis of the *glnK* promoter in different strains of *Klebsiella pneumoniae*. Right part of the panel shows a partial restriction map of the *R. etli glnK* genomic region and plasmid pAR138, a derivative of pKK232-8, used in these assays. These plasmids were transformed in *K. pneumoniae* UNF122 (wild-type), UNF2792 (*ntrA*⁻), and UNF1828 (*ntrC*⁻). Bacteria were grown in NFD minimal medium with, as nitrogen source, either 200 μg ml⁻¹ glutamine (1) or 200 μg ml⁻¹ glutamine plus 1 mg ml⁻¹ ammonium sulfate (2). Numbers indicate relative specific activity of chloramphenicol acetyltransferase (CAT), with [¹⁴C]-chloramphenicol as substrate. Radioactive signals were quantified with a PhosphorImager and values were normalized to signal obtained with extracts from wild-type strain carrying plasmid pAR138 when grown with glutamine as a sole nitrogen source. Values are average of three different experiments and standard errors (vertical bar) are indicated.

results show that, while no promoter activity is detected with a fusion carrying the DNA region upstream of *amtB*, the *glnK* upstream region harbors a nitrogen-regulated promoter that requires NtrC for expression.

In order to confirm the absence of weak promoter activity in the *amtB* upstream region, a more sensitive analysis by RNase protection was performed. With the ³²P-labeled antisense RNA generated from clone pAR132 (a 534-bp *SalI/HindIII* DNA fragment from plasmid pAR131; see Figure 1) as a probe, only a fully protected fragment was observed (data not shown). This result demonstrates that *glnK* and *amtB* are co-transcribed and that if there is a promoter located in the *amtB* upstream DNA region it has very low transcriptional activity, undetectable by RNase protection experiments.

To test the influence of P_{II} on the regulation of transcription of the *glnKamtB* operon, plasmid pAR136 carrying the *glnK* promoter (Fig. 3A) was conjugated into a *glnB*⁻ derivative of *Rhizobium leguminosarum* bv. *viciae* (strain BS11; Amar et al. 1994). Constitutive expression of *lacZ* gene from the *glnKamtB* promoter in the presence of NH₄Cl was found (data not shown), indicating that this promoter responds to a signal transduction pathway that includes the P_{II} protein.

Functional analysis of the *R. etli glnKamtB* promoter in *Klebsiella pneumoniae*.

To characterize the promoter of the *glnKamtB* operon in more detail we performed a transcriptional analysis of the *glnKamtB* promoter in a heterologous background, using *K. pneumoniae*, which is a recognized model system for the study of *ntr*-regulated promoters. We made use of different *K. pneumoniae* strains into which we introduced a derivative of plasmid pKK232-8 (Brosius 1984) carrying the promoterless chloramphenicol acetyltransferase (CAT) reporter gene. Since the mutant strains of *K. pneumoniae* analyzed are tetracycline resistant we could not use the *lacZ* transcriptional fusions previously described (Fig. 3A). A 433-bp *NarI* DNA fragment containing 313 bp upstream of the putative start codon of *glnK* was cloned into pKK232-8. The resulting plasmid (pAR138) was transformed into *K. pneumoniae* wild type (strain UNF122), *ntrC*⁻ mutant (UNF1828), and *rpoN*⁻ mutant (UNF2792) (Fig. 3B).

The *glnK* promoter showed tenfold higher activity when the wild-type strain of *K. pneumoniae* was grown in minimal medium (NFD) using only glutamine as opposed to glutamine plus (NH₄)₂SO₄ as nitrogen source (Fig. 3B). Glutamine or glutamine plus ammonium, used as nitrogen sources, represent nitrogen-limiting and nitrogen-sufficient conditions, respectively. Furthermore, only background activity (less than 5% of that in the wild-type strain) was observed when protein extracts were prepared from either the *K. pneumoniae ntrC*⁻ or *rpoN*⁻ strains carrying plasmid pAR138. These results show that in *K. pneumoniae* this promoter is subject to nitrogen control and that it requires both the *ntrC* and *rpoN* gene products to initiate transcription.

Determination of the transcription initiation site.

To determine the 5' end of the *glnKamtB* mRNA an RNase mapping experiment was performed with RNA purified from the wild-type strain of *R. etli* grown either on glutamate or ammonium as sole nitrogen source and the ³²P-labeled antisense RNA generated from clone pAR137 as a probe. This clone covers the *glnKamtB* promoter region determined as

described above with the transcriptional fusion. Only a protected fragment of 189 nucleotides was observed (data not shown), indicating that *glnKamtB* transcription initiates within the region protected by this fragment and that only one transcription initiation site is present upstream of *glnK*.

To map the transcription start site more precisely, a ³²P end-labeled synthetic oligonucleotide (5'-CCTCATCGAGCTTG AACGGC-3') complementary to the sequence indicated as *olig1* in Figure 2 was used in primer extension assays. The size of the elongated products was determined with, as a marker, a sequencing ladder obtained with the same oligonucleotide. As shown in Figure 4A, we detected one 5' end, located at position -62 with respect to the putative start codon of *glnK* and corresponding to the protected fragment obtained by RNase mapping. Upstream of the *glnKamtB* transcription start site a 16-bp sequence (TGGCACGTCCTTTGAA), located between positions -26 and -11 (underlined in Figure 2), shows significant homology to the -24 (GG)/-12 (GC) promoter consensus sequence for σ^N(σ⁵⁴)-dependent promoters (Merrick 1993). In the -12 region, a single substitution is observed with A replacing the more normal C at -11. The same substitution was revealed in the -24/-12 promoter sequence of *R. etli* *nifHa*, *nifHb*, and *nifHc* (Quinto et al. 1985). Moreover, by a sequence analysis, a putative IHF (integration host factor) binding site (between positions -37 and -72) and a putative NtrC binding site (between positions -76 and -91) were identified upstream of the -24/-12 promoter sequence (see Figure 2).

The *R. etli* NtrC protein is essential for expression of *glnKamtB* operon.

Only a background level of β-galactosidase activity was detected in the *ntrC*⁻ mutant of *R. etli* carrying a *glnKamtB*-

lacZ transcriptional fusion (see Figure 3A). In order to confirm the absence of any weak NtrC-independent promoter activity, a double RNase protection analysis was performed (Fig. 4B). RNA was purified from the wild-type (CE3) and the *ntrC*⁻ (CFN2012) strains of *R. etli*, grown on either glutamate or NH₄Cl as sole nitrogen source, and hybridized to an excess of a mixture of two different ³²P-labeled antisense RNA probes generated from clones pAR137 (*glnKamtB*; this work) and pEP24a (*ntrBC*; Patriarca et al. 1993), respectively. We chose to use as a probe an antisense RNA generated from the *ntrBC* operon since its expression was previously found to be negatively autoregulated by NtrC (Martino et al. 1996). As expected, the intracellular level of *ntrBC* mRNA was two- to threefold higher when the wild-type strain was grown on NH₄Cl, relative to glutamate. In the *ntrC*⁻ strain the level of *ntrBC* mRNA was 20-fold higher than in the wild-type and showed the same twofold increase when bacteria were grown on NH₄Cl, relative to glutamate. By contrast, the intracellular level of *glnKamtB* mRNA was 10-fold higher when the wild-type strain was grown with glutamate as opposed to NH₄Cl as sole nitrogen source. In the *ntrC*⁻ strain transcripts corresponding to the *glnKamtB* operon were not detected. These data confirm that in *R. etli* the transcriptional activator NtrC is essential for *glnKamtB* expression.

Methylammonium uptake activity in different strains of *R. etli*.

Transport activity was determined with radioactive [¹⁴C]-methylammonium ([¹⁴C]-MA) as an NH₄⁺ analogue (Kleiner 1985 and references therein). Under these conditions the [¹⁴C]-MA uptake activity was optimal at pH 6.5 (Fig. 5A) and was found to be linear for at least 3 min (Fig. 5B). The [¹⁴C]-

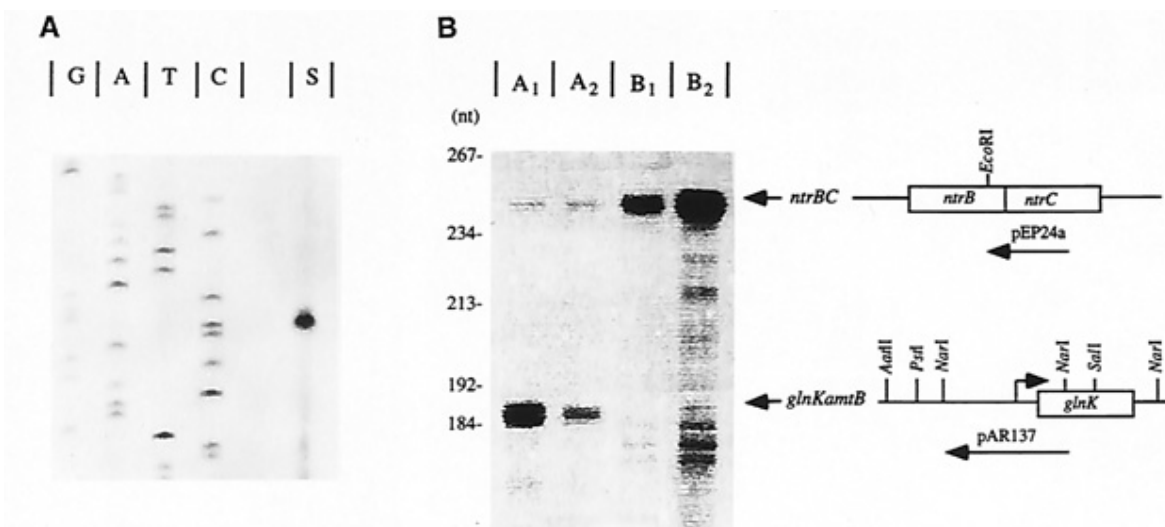


Fig. 4. **A**, Identification of the 5' end of the *glnKamtB* RNA. Primer extension analysis into the *glnKamtB* promoter region. Primer extension (lane S) was carried out extending a ³²P end-labeled synthetic oligonucleotide (5'-CCTCATCGAGCTTG AACGGC-3') complementary to the sequence (*olig1*) shown in Figure 2. Annealing was performed with 10 μg of RNA extracted from *Rhizobium etli* wild-type strain CE3 grown on minimal medium containing glutamate as a sole nitrogen source. Size markers were dideoxy sequencing reactions specific for the G, A, T, C residues, respectively, primed with the same ³²P end-labeled oligonucleotide used for the RNA analysis. **B**, RNase protection experiment showing that the *glnKamtB* transcript is absent in the *ntrC*⁻ strain (CFN2012) of *R. etli*. Right part of the figure shows a partial restriction map of both the *ntrBC* and *glnKamtB* region and the plasmids used, pEP24a (a 245-bp DNA fragment covering the *ntrBC* intergenic region; Patriarca et al. 1993) and pAR137 (a 433-bp *NarI* DNA fragment carrying the *glnK* upstream region), to prepare the ³²P-labeled antisense RNA. Both probes were mixed with RNA extracted from *R. etli* wild-type strain CE3 (**A**) and *ntrC*⁻ strain CFN2012 (**B**) grown on minimal medium containing glutamate (1) or ammonium chloride (2) as a nitrogen source. Arrows indicate the transcript, 245 nucleotides corresponding to the *ntrBC* RNA and 189 nucleotides corresponding to the *glnKamtB* RNA, identified on the basis of their mobility relative to that of the ³²P-labeled DNA molecular weight markers indicated on the left.

MA is taken up as rapidly when KNO_3 constitutes the only available nitrogen source. Uptake activity was reduced to less than 10% when the wild-type strain was grown on 10 mM NH_4Cl as the nitrogen source (data not shown) or in the *ntrC*⁻ strain (CFN2012) grown on glutamate (Fig. 5B). This residual [¹⁴C]-MA uptake activity was also observed in the absence of a carbon source and was found to increase as a function of pH (data not shown), suggesting that it may be the consequence of passive diffusion of methylamine.

To test the correlation between the lack of [¹⁴C]-MA uptake activity and the absence of *amtB* expression, a transcriptional fusion (plasmid pAR144) carrying the *R. etli* *amtB* gene under the control of the *R. meliloti* *dctA* promoter (*dctAp*) was constructed. In *R. etli*, *dctAp* was found to be inducible when dicarboxylic acids, such as succinate, were used as sole carbon source in minimal medium (R. Taté and E. J. Patriarca, unpublished results). As a control, plasmids pAR140, carrying *amtB* without a promoter, and pAR147, carrying an internal deletion of the *amtB* gene (deletion *SalI/SalI* between positions 1857 and 2118 of the nucleotide sequence shown in Figure 2), were used (Fig. 5C). Plasmids pAR140, pAR144, and pAR147 were conjugated in the *ntrC*⁻ strain of *R. etli* and 3, 5, and 12 h after induction of *dctAp* with 20 mM succinate, samples were harvested and the specific [¹⁴C]-MA uptake activity was assayed (Fig. 6C). The *ntrC*⁻ strain was found to be able to transport [¹⁴C]-MA only when containing plasmid pAR144 (Fig. 5C) and after induction of the heterologous promoter (*dctAp*) with succinate. The *amtB*-mediated [¹⁴C]-MA uptake activity was also tested in the presence of a fivefold molar excess of competitors. Uptake was inhibited 79 to 87% by ammonium and 32 to 34% by methylammonium; no inhibition was observed with K^+ as a competitor (data not shown).

Expression of the *glnKamtB* promoter during nodule development.

During the symbiotic interaction, *glnKamtB* promoter activity was followed by histochemical localization of β -galactosidase activity expressed from a *glnKamtB-lacZ* fusion (Fig. 6). Roots of *Phaseolus vulgaris* plantlets were inoculated with *R. etli* (strain CE3) transconjugants containing plasmid pRT124 or pAR136 carrying the *nifH* or the *glnKamtB* promoter fused to the *lacZ* gene, respectively. At different times after inoculation, roots were harvested and in situ expression of β -galactosidase was observed after staining with X-Gal. In an 8-day-old developing nodule, the *nifH* promoter is inactive in growing bacteria inside the infection threads and in bacteroids located in the first invaded cells of the nodule primordium (data not shown). Later on, in a 12-day-old nodule, the *nifH* promoter (pRT124) is active in bacteroids located in all invaded cells (Fig. 6B). In contrast, no β -galactosidase activity (or a very weak staining) was observed in a 12-day-old developing nodule induced by strain CE3(pAR136) (Fig. 6A), thus indicating that the *glnKamtB* promoter is inactive (or very weakly active) in bacteroids.

R. leguminosarum bv. *viciae* infecting *Vicia hirsuta* gives indeterminate nodules. We analyzed expression of the *glnKamtB* promoter in this heterologous background because in a longitudinal section of a mature indeterminate nodule it is possible to recognize all the developmental zones (Vasse et al. 1990; Yang et al. 1991). Roots of *V. hirsuta* plantlets were inoculated with *R. leguminosarum* bv. *viciae* (strain 1004) transconjugants containing either pAR66, pRT124, or pAR136 carrying the ORF1-*ntrBC*, the *nifH*, or the *glnKamtB* promoter fused to the *lacZ* gene, respectively. In an 8-day-old nodule the ORF1-*ntrBC* promoter (900 to 1,000 units of β -

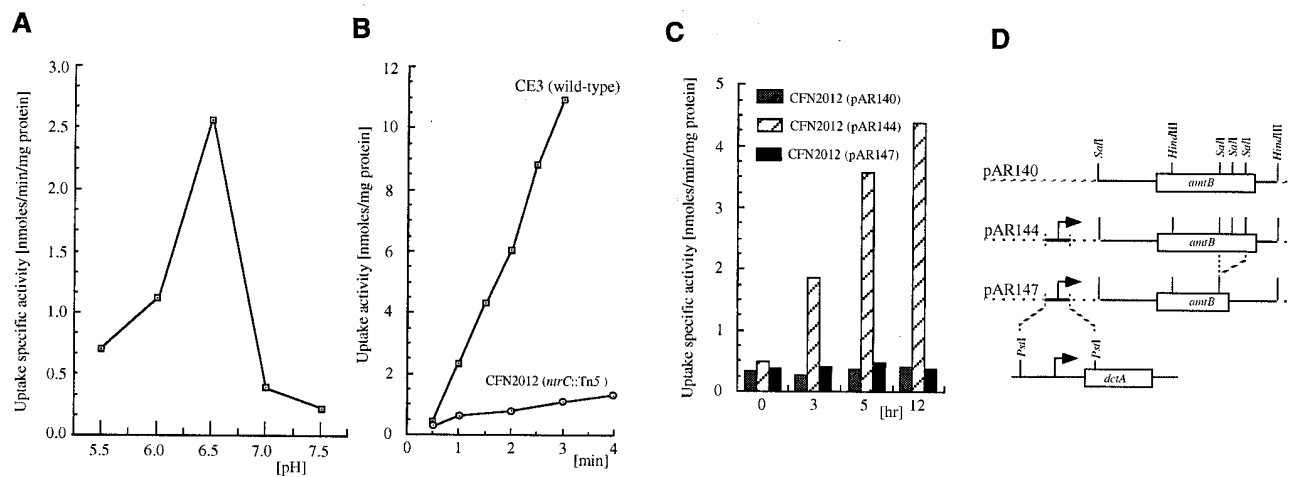


Fig. 5. The [¹⁴C]-methylammonium ([¹⁴C]-MA) uptake by *Rhizobium etli*. Bacteria were grown to mid-exponential phase in minimal medium (RMM) with glutamate as the sole nitrogen source. Cells were washed twice and resuspended in RMM without glutamate. **A**, pH dependence of [¹⁴C]-MA uptake by *R. etli* bacteria. Uptake activity was calculated from measurements of accumulated radioactive label in cells incubated at 30°C with 100 μM [¹⁴C]-MA and at the pH indicated. **B**, Time course of [¹⁴C]-MA uptake by different strains of *R. etli*. Uptake activity was determined as in **A** but at pH 6.5. **C**, Ability of the *amtB* gene to restore high [¹⁴C]-MA uptake activity in the *ntrC* mutant strain of *R. etli*. Bacteria were grown to mid-exponential phase in minimal medium (RMM) with mannitol as sole carbon source. Cells were washed and resuspended in the same medium but with 20 mM of succinate as carbon source. At the times indicated the [¹⁴C]-MA uptake was determined. Strains CFN2012(pAR140) (solid bar) and CFN2012(pAR144) (hatched bar). **D**, Plasmid pAR140 carrying the *R. etli* *amtB* gene without promoter, plasmid pAR144 carrying the *amtB* gene under the control of the *R. meliloti* *dctA* promoter and plasmid pAR147 carrying a deletion of the *amtB* gene under the control of the *R. meliloti* *dctA* promoter are shown. Both plasmids are derivatives of the low copy number promoter-probing vector pMP220 (Spaink et al. 1987).

galactosidase activity) is active in growing bacteria inside the infection threads and in bacteroids released in the first invaded cells of the nodule primordium (Fig. 6C). In a more mature nodule (12-day-old) the activity is restricted to bacteroids present in the younger invaded cells of zone II (Fig. 6F). In

contrast, the *nifH* promoter is inactive in either bacteria or bacteroids of a 8-day-old nodule (Fig. 6E). Later on, in a 12-day-old nodule, the *nifH* promoter is switched on sharply in bacteroids located in a single cell layer, the interzone II-III, and it is expressed throughout zone III (Fig. 6H). Finally, the

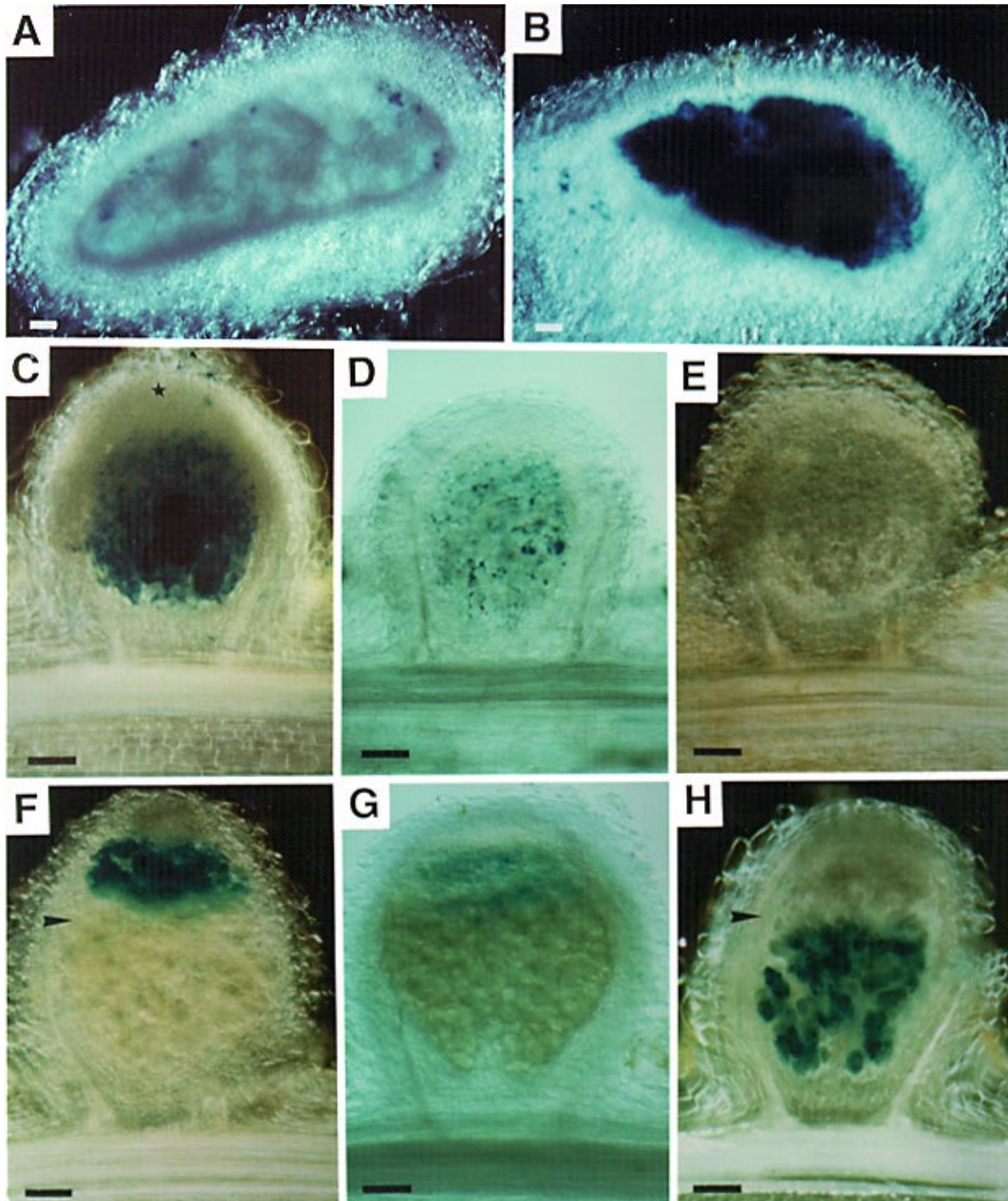


Fig. 6. Activity of the *glnKamtB* promoter observed in bacteroids of determinate-type of nodules induced by *Rhizobium etli* on roots of *Phaseolus vulgaris*. **A**, Section of a 12-day-old nodule induced by *R. etli* strain CE3(pAR136) carrying the *glnKamtB* promoter. **B**, Section of a 12-day-old nodule induced by strain CE3(pRT124) carrying the *nifHp-lacZ* fusion. Activity of the *glnKamtB* promoter observed during bacteroid differentiation in indeterminate-type of nodules induced by *R. leguminosarum* bv. *viciae* on the root of *Vicia hirsuta*. Longitudinal sections of 8- and 12-day-old nodules, respectively, induced by (**C** and **F**) *R. leguminosarum* bv. *viciae* strain 1004(pAR66) carrying the *ntrBCp-lacZ* fusion, (**D** and **G**) *R. leguminosarum* bv. *viciae* strain 1004(pAR136) carrying the *glnKamtBp-lacZ* fusion, and (**E** and **H**) *R. leguminosarum* bv. *viciae* strain 1004(pRT124) carrying the *nifHp-lacZ* fusion.

glnKamtB promoter is active in growing bacteria inside the infection threads in an 8-day-old nodule (Fig. 6D), but inactive or weakly active only in the invasion zone of a 12-day-old nodule (Fig. 6G).

DISCUSSION

We have cloned and characterized an *R. etli* operon that we designate *glnKamtB*, in which *glnK* encodes a protein belonging to the P_{II} family of signal transduction proteins and *amtB* encodes an ammonium transporter. The identification of *R. etli glnK* indicates that, as in *E. coli*, two genes encoding P_{II}-like proteins are present in *Rhizobiaceae*. The first such gene is the *glnB* gene located upstream of and cotranscribed with the *glnA* gene coding for GSI (Colonna-Romano et al. 1987; Martin et al. 1989; Arcondéguy et al. 1996).

It has previously been shown that NtrC-dependent promoters such as *glnIIp* (Patriarca et al. 1994) and *glnBAP* (Chiurazzi and Iaccarino 1990) are constitutively expressed in an *R. leguminosarum glnB* mutant that lacks P_{II}, suggesting that the *glnB* product in *Rhizobium* acts, as in enteric bacteria, to transmit a nitrogen status signal via the histidine protein kinase NtrB, thereby regulating NtrC phosphorylation (Amar et al. 1994). The protein encoded by *glnK* shares extensive sequence similarity to the *glnB* product but we found that in a *glnB* mutant strain of *R. leguminosarum* a plasmid carrying the *R. etli glnK* gene, under the control of its own promoter, could not restore repression of *glnIIp* under nitrogen-excess conditions (data not shown). This suggests that GlnK is unable to modulate the phosphorylation status of NtrC.

Why *glnK* and *amtB* genes form an operon in *R. etli* is not evident, but recent data from a number of laboratories indicate that this genomic organization is not unusual in bacteria since these genes are contiguous in *B. subtilis (nrgA,nrgB*; Wray et al. 1994), in *Azotobacter vinelandii (nrgB,nrgA*; Meletzus et al. 1995), and in *E. coli (glnK,amtB*; van Heeswijk et al. 1996). On the other hand, a *glnK*-like gene is not located upstream of the *amt* gene of *Corynebacterium glutamicum* (Siewe et al. 1996). The role of *R. etli* GlnK remains to be established, but it is possible that coordinate transcription of a gene encoding an ammonium transporter and a nitrogen sensory protein has functional implications. The *glnB* gene product (P_{II}) of *R. meliloti* is known to modulate, in response to the nitrogen status, the activity of the adenylyltransferase enzyme responsible for regulating GSI activity (Arcondéguy et al. 1997). It is therefore possible that the P_{II}-like protein encoded by *glnK* regulates, directly or indirectly, the activity of the AmtB protein in response to the cellular nitrogen status.

In agreement with the transcriptional analysis (Figs. 3 and 4), the [¹⁴C]-MA uptake activity in *R. etli* appeared to be strongly regulated by the nitrogen status. We cannot exclude the presence in *R. etli* bacteria of other (methyl)ammonium transporter(s), but the results shown in Figure 5 indicate that in strain CFN2012 (*ntrC*⁻) of *R. etli* such putative transport systems are (i) expressed at a very low level; (ii) inactive; or (iii) have a very low affinity for [¹⁴C]-MA. Furthermore, as shown in Figure 5, a deleted version of the *R. etli amtB* gene (*SalI/SalI* in frame deletion; Fig. 2) cannot rescue the ability of strain CFN2012 to transport [¹⁴C]-MA, thus indicating that the deleted amino acid sequence (including two putative trans-

membrane domains) is essential for the stability and/or the activity of AmtB.

The *glnK* and *amtB* genes are cotranscribed from a single σ^{54} -dependent promoter, which is activated, directly or indirectly, by NtrC (Fig. 3). Furthermore, transcription of the *glnKamtB* operon is activated under nitrogen-limiting conditions (Figs. 3 and 4). In *R. etli* the DNA binding activity of NtrC, but not its intracellular level, was found to be strongly regulated by the nitrogen availability (Patriarca et al. 1993, 1994; Martino et al. 1996). These data suggest that in *R. etli*, as in enteric bacteria, the activity of NtrC may be regulated by phosphorylation in response to the intracellular ratio of α -ketoglutarate to glutamine and not directly by the intracellular ammonium concentration (see Merrick and Edwards 1995). Hence, as *glnKamtB* transcription is activated by NtrC, its expression should also be dependent on the intracellular glutamine levels rather than on ammonium levels. Such regulation would facilitate recovery from the periplasmic space of ammonia lost by diffusion when cells are growing on poor nitrogen sources, such as nitrate or glutamate.

A sequence analysis revealed the presence of an IHF-binding site in the *glnKamtB* promoter region. It has previously been found that the IHF influence the expression of the *glnHPQ* operon (coding for the components of the high affinity glutamine transport system) of *E. coli*. It has been proposed that the IHF-induced DNA bend can facilitate or obstruct the initiation of transcription of this operon that is induced by the phosphorylated forms of NtrC, from a σ^{54} -dependent promoter, under nitrogen-limiting conditions (Claverie-Martin and Magasanik 1991). The role of IHF in the regulation of transcription of the *glnKamtB* operon of *R. etli* is currently unknown but worth investigation.

We have previously shown that transcription of the *R. etli* ORF1-*ntrBC* operon, coding for the NtrB/NtrC two component system, is down-regulated during bacteroid differentiation (Patriarca et al. 1996). Since the NtrC protein is essential for the transcription of the *glnKamtB* operon under free-living conditions (Figs. 3 and 4) and it is absent in terminally differentiated N₂-fixing bacteroids (Patriarca et al. 1996), we speculate that transcription of this operon should be either down-regulated during bacteroid differentiation or activated by an alternative transcription factor(s), such as NifA, which is specifically induced in bacteroids. The experiments reported here (Fig. 6) demonstrate that transcription of the *glnKamtB* operon in *R. etli* is switched off during nodule development (Fig. 6). β -Galactosidase activity expressed from the *glnKamtB* promoter is detectable in bacteria growing inside infection threads, but not in bacteroids within younger invaded cells. This result was unexpected since NtrC is expressed in bacteroids located in younger invaded cells (Patriarca et al. 1996), but the same pattern of expression was observed when nodules were induced with the wild-type strain of *R. etli* carrying a *glnIIp-lacZ* transcriptional fusion (data not shown). It has been previously demonstrated that the *glnII* promoter absolutely requires NtrC for activity (Patriarca et al. 1992, 1994). Therefore, we suggest that the inactivation of NtrC (most probably by dephosphorylation) and not its transcriptional down-regulation, which occurs at a later stage of the bacteroid differentiation (Patriarca et al. 1996), may be the first step of the regulatory mechanism acting to uncouple nitrogen fixation and assimilation in differentiated bacteroids.

Furthermore, it is also evident that no other σ^{54} -dependent transcriptional activator that is present in bacteroids (such as NifA) is able to activate transcription from NtrC-dependent promoters, such as those controlling *glnII* and *glnKamtB*. This may be due to the absence of specific binding sites for other transcriptional activators in these promoters.

Based on experiments performed in vitro with isolated symbiosomes, it has previously been proposed that NH_3 produced by the bacteroidal nitrogenase passively diffuses across the bacteroid membrane into the relatively acidic peribacteroidal space (PBS) where it is converted to NH_4^+ , which then enters the plant cytosol through a channel located on the PBM (Tyerman et al. 1995). In agreement with this model the lack of *glnKamtB* expression (Fig. 6) could explain the absence of an active transport mechanism for the uptake of ammonium ions in differentiated nitrogen-fixing bacteroids (Brown and Dilworth 1975; Howitt et al. 1986). At present our data do not exclude the possibility that transfer of fixed nitrogen NH_4^+ from the bacteroid to the plant is an active (NH_4^+) rather than a passive (NH_3) process and that a transport system, like a channel located on the bacteroidal membrane, would facilitate NH_4^+ efflux to the PBS. We hypothesized that induction of the expression of an ammonium uptake system in N_2 -fixing bacteroids could help to address the question as to whether fixed nitrogen was indeed transferred from the bacteroid to the plant in the form of ammonium. The NH_4^+ carrier of the bacteroid should compete with the channel located on the PBM for the ammonium ions present in the PBS, thereby reducing the efficiency of the symbiotic interaction. In fact, we observed that during development of nodules induced by the wild-type strain of *R. etli* harboring a plasmid carrying the *amtB* gene under the control of the *nifH* promoter, the bacteroid differentiation process was strongly delayed or arrested (data not shown). Therefore, repression or inactivation in N_2 -fixing bacteroids of NH_4^+ transporter(s) (for uptake or influx), such as the *amtB* gene product, appears to be essential for an efficient symbiotic interaction. That genes coding for nitrogen assimilation activities of *R. etli* bacteria must be down-regulated during nodule invasion for an efficient symbiotic interaction is also supported by the following observations: (i) a *ntrC*⁻ mutant strain of *R. etli* forms efficient nitrogen-fixing nodules (Moreno et al. 1992), thus indicating that, unlike free-living diazotrophs, *nif* genes are activated independently of a centralized nitrogen control system; and (ii) the expression of *E. coli* glutamate dehydrogenase (*gdh* gene involved in NH_4^+ assimilation) in *R. etli* interferes with nodulation (Mendoza et al. 1995) and with nitrogen fixation (Bravo and Mora 1988).

MATERIALS AND METHODS

Bacterial strains and plasmids.

R. etli wild-type strain CE3 (Segovia et al. 1993) and its *ntrC*::Tn5 derivative strain CFN2012 (Moreno et al. 1992); *R. leguminosarum* bv. *viciae* wild-type strain LPR1105, a Rif derivative of RCR1001 and its *glnB-kan* derivative (Amar et al. 1994) were grown at 30°C in TYR medium containing (g l^{-1}): tryptone (5) and yeast extract (3). RMM was used as a chemically defined medium containing (g l^{-1}): $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.25), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.1), K_2HPO_4 (1), KH_2PO_4 (1); and ($\mu\text{g l}^{-1}$): $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (10), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (20), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (20), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (20), CoCl_2 (20), Na_2MoO_4 (20), biotin

(20), calcium pantothenate (20), and thiamin (20). *E. coli* strains were grown at 37°C in TY medium containing (g l^{-1}): tryptone (10), yeast extract (5), NaCl (10). Antibiotics otherwise indicated ($\mu\text{g ml}^{-1}$): tetracycline (5); nalidixic acid (20); kanamycin (50); rifampicin (100); ampicillin (100). These were added to the medium as needed. All media were solidified with 1.5% agar (Difco, Detroit, MI).

Conjugative crosses between *E. coli* and *Rhizobium* strains were performed as previously described (Amar et al. 1994). Plasmid pKK232-8 and its derivative pAR138 were transformed in *K. pneumoniae* wild-type strain UNF122 (Holtel and Merrick 1988), *ntrC*⁻ mutant strain UNF1828 (Espin et al. 1982) and *rpoN*⁻ mutant strain UNF2792 (Coppard and Merrick 1991). *K. pneumoniae* strains were grown in NFDM medium (Dixon et al. 1977) at 30°C with 200 μg of glutamine or 200 μg of glutamine plus 1 mg ml^{-1} ammonium sulfate as nitrogen sources, in the presence of 200 $\mu\text{g ml}^{-1}$ ampicillin and 100 $\mu\text{g ml}^{-1}$ carbenicillin.

DNA and RNA biochemistry.

DNA biochemistry and Southern blot experiments were carried out as described (Sambrook et al. 1989). DNA was sequenced on both strands with the Sequenase kit (USB, Cleveland, OH) and in many cases gels containing 40% formamide. For sequence analysis the software package of the University of Wisconsin Genetics Computer Group (Devereux et al. 1984) was used and several data bases were screened. Codon usage frequency analysis was performed with a codon preference table compiled from all published sequences of *R. leguminosarum* and *R. etli*. RNA was extracted and purified as described elsewhere (Patriarca et al. 1993). Before each experiment the integrity of the RNA was checked on 1.5% agarose gels. Appropriate DNA fragments were cloned into pGEM vectors (Promega, Madison, WI) and ^{32}P -labeled antisense RNA were synthesized with either SP6 or T7 RNA polymerase (Promega) and [α - ^{32}P]UTP (Amersham, Milan, Italy) after linearization of the template. RNase protection experiments were performed as previously described (Patriarca et al. 1993). The protected RNA fragments were dissolved in 80% formamide, denatured for 5 min at 90°C, electrophoresed on a 6% polyacrylamide gel containing 7 M urea, dried, and autoradiographed. Protected fragments were quantitatively analyzed with a PhosphorImager gel scanner (Molecular Dynamics, Sunnyvale, CA). End-labeling of oligonucleotides and primer extension reactions were performed as described elsewhere (Patriarca et al. 1992).

CAT and β -galactosidase assays.

Chloramphenicol acetyltransferase (CAT; acetyl-CoA:chloramphenicol 3-*O*-acetyltransferase EC 2.3.1.28) activity was measured as described (Gorman et al. 1982) in crude extracts from logarithmically growing bacteria. Protein concentration in crude extracts was determined with a protein assay kit (BioRad, Milan, Italy) with bovine serum albumin as a standard. β -Galactosidase activity was assayed according to the method of Miller (1972).

Methylammonium uptake assays.

Assuming that the ammonium transport system is activated under nitrogen-limiting conditions of growth, assay conditions were initially optimized for the wild-type (CE3) strain of *R.*

etli grown in RMM minimal medium with glutamate as the sole nitrogen source. Bacteria were grown to mid-exponential phase (OD₅₉₀ of 0.6) and cells were harvested by centrifugation (6,000 × g, 5 min, 4°C), washed, and resuspended in minimal medium without any nitrogen source. The experiments were started by the addition of [¹⁴C]-methylamine hydrochloride (Amersham) to a final concentration of 100 μM. Samples (200 μl) were taken at timed intervals during 5 min, immediately vacuum filtered through a 0.45-μm membrane (Millipore, Bedford, MA), and washed twice with 5 ml of buffer containing 50 μM methylammonium. Radioactivity of samples was detected by placing the filter in 5 ml of scintillation (Insta-gel; Packard, Downers Grove, IL) liquid and measuring in a Beckman counter. Protein concentration of each cell suspension was determined as described above.

Plant growth and histochemical localization.

Seeds were germinated and inoculated as previously described (Amar et al. 1994). Roots were collected at various times after inoculation and fixed, under a brief and gentle vacuum, with 2% glutaraldehyde, 0.3 M mannitol, and 10 mM 2-[N-morpholino]ethanesulfonic acid, pH 5.6 for 1 h at room temperature. Hand-cut (made with a razor blade) nodule sections were washed three times with 50 mM phosphate buffer pH 7.2 and immersed in a staining solution containing 10 mM phosphate buffer, pH 7.2, 150 mM NaCl, 1 mM MgCl₂, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.03% Triton-X100 and 1 mM 5-bromo-4-chloro-3-indolyl β-galactopyranoside (X-Gal, Inalco SPA, Milan, Italy). The samples were incubated in the dark at 37°C for the required time, washed in phosphate buffer, rinsed in 70% ethanol, 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 stain. The stained sections were photographed with a Nikon microscope in bright-field and epipolarization optics.

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