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Identification and Characterization of the *Rhizobium meliloti ntrC* Gene: *R. meliloti* Has Separate Regulatory Pathways for Activation of Nitrogen Fixation Genes in Free-Living and Symbiotic Cells

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We show here that *Rhizobium meliloti*, the nitrogen-fixing endosymbiont of alfalfa (*Medicago sativa*), has a regulatory gene that is structurally homologous to previously characterized *ntrC* genes in enteric bacteria. DNA sequence analysis showed that *R. meliloti ntrC* is homologous to previously sequenced *ntrC* genes from *Klebsiella pneumoniae* and *Bradyrhizobium* sp. (*Parasponia*) and that an *ntrB*-like gene is situated directly upstream from *R. meliloti ntrC*. Similar to its counterparts in *K. pneumoniae* and *Escherichia coli*, *R. meliloti ntrC* is expressed when the cells are grown in nitrogen-limiting media. In addition, *R. meliloti ntrC* is required for growth on media containing nitrate as the sole nitrogen source and for the ex planta transcription of several *R. meliloti nif* genes. On the other hand, root nodules elicited by *R. meliloti ntrC* mutants fix nitrogen as well as nodules elicited by wild-type *R. meliloti*. These latter results indicate that *R. meliloti* has separate regulatory pathways for activating *nif* gene expression ex planta and during symbiotic nitrogen fixation.

The soil bacterium *Rhizobium meliloti* fixes dinitrogen (N_2) during symbiosis with specific host legumes such as *Medicago sativa* (alfalfa). The establishment of the symbiosis is a multistepped, interactive process that requires the expression of specific plant and bacterial genes. At least two *R. meliloti* operons, *nifHDK* and *fixABCX*, which encode the nitrogenase polypeptides (2) and other functions required for nitrogen fixation (2, 23), respectively, require the product of the *nif*-specific regulatory gene *nifA* for transcription under symbiotic conditions (1, 24a).

In this study, we have sought to determine whether *nifA*-mediated activation of *R. meliloti nif* genes is a direct response to nitrogen limitation as is the case for *nif* regulation in the enteric bacterium *Klebsiella pneumoniae*. In *K. pneumoniae* in response to nitrogen limitation the products of the general nitrogen assimilatory regulatory genes *ntrC* and *ntrA* activate transcription of the regulatory *nifLA* operon (see references 1 and 24a for reviews). The *K. pneumoniae nifA* product in turn activates the transcription of six additional *nif* operons (1, 24a).

Although free-living *R. meliloti* organisms have not been shown to fix nitrogen, previously published data from our laboratory have shown that transcription of an *R. meliloti nifH-lacZ* fusion is activated in nitrogen-starved, free-living *R. meliloti* cells (48). These data suggest that *R. meliloti nifA* transcription may be activated by the product of an *ntrC*-like gene. However, S1 nuclease mapping studies with RNA isolated from alfalfa root nodules have shown that the *R. meliloti nifA* promoter is not similar to other promoters activated by *ntrC-ntrA* or *nifA-ntrA* products (13). These latter promoters are characterized by two highly conserved consensus sequences, 5′-TTGCA-3′ at −15 to −10 and 5′-CTGG-3′ at −26 to −23 (9, 36). The regulation of *nifA* expression in *R. meliloti* is further complicated by the fact that the *nifA* gene is directly downstream of the *fixABCX* operon and at least 50% of *nifA* mRNA in nodules is a result of readthrough transcription from the *nifA*-activated *fixA* promoter (13, 27). Because the *K. pneumoniae ntrC* product can activate the *R. meliloti nifH* promoter (49), it is conceivable that the *nifH* and *fixA* promoters are activated directly by an *R. meliloti ntrC*-like gene in response to nitrogen limitation and that *nifA* transcription is subsequently activated by its own product via the *fixA* promoter.

To determine whether centralized nitrogen regulation plays a role in *nif* expression in *R. meliloti*, we have isolated and characterized an *R. meliloti ntrC*-like gene. Our results show that the *R. meliloti ntrC* product is required for *nif* gene activation when the bacteria are in the free-living state, but that the *ntrC* product is not required during symbiotic nitrogen fixation.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. The bacterial strains and recombinant plasmids used are listed in Table 1.

The rich media were TY (0.5% tryptone [Difco Laboratories], 0.3% yeast extract, and 0.05% calcium chloride dihydrate) and LB (31). Two defined media were used. NFDM minimal medium contained 1× Nif salts (0.02% $MgSO_4 \cdot 7H_2O$, 0.00001% $CaCl_2 \cdot 2H_2O$, 0.001% NaCl, 0.2% sequestrene, and 0.0005% $NaMoO_4 \cdot 2H_2O$), 0.1 M potassium phosphate buffer pH 7.4, 2% glucose, 20 μg of vitamin B1 per ml, 0.2 μg of biotin per ml, and a nitrogen source as specified. Glucose defined medium (GDM) contained RDM salts and vitamins (40), 0.4% glucose, and a nitrogen source as specified and was solidified with 1.2% Difco Noble agar as required. When appropriate, the media were supplemented with antibiotics as described previously (50).

DNA biochemistry. Plasmid DNA was prepared by the boiling method of Holmes and Quigley (26) or the cleared

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
R. meliloti strains		
Rm1021	Wild-type, Sm^r	31
Rm1352	*nifA*::Tn*5* Sm^r Nm^r	50
Rm1354	*nifA*::Tn*5* Sm^r Nm^r	50
Rm5001	*ntrC*::Tn*5* Sm^r Nm^r	This study
Rm5002	*ntrC*::Tn*5* Sm^r Nm^r	This study
Rm5003	*ntrC*::Tn*5* Sm^r Nm^r	This study
Rm3600	Deletion of the *nif-nod* region of the *R. meliloti* megaplasmid	S. Gibbons
Rm4400	Deletion of the *nif-nod* region of the *R. meliloti* megaplasmid	S. Gibbons
Plasmids (phages)		
M13mp18, M13mp19		57
pUC13		53
pRMCjd12	Ap^r Tc^r; cosmid clone (in pHC79 [25]) containing *Rhizobium ntrC* DNA	This study
pRmC3.8R	Tc^r; 3.8-kb *R. meliloti ntrC* fragment cloned into the *Eco*RI site of pACYC184 (16)	This study
pGln53Y	Ap^r;*E. coli ntrC* cloned in pBR322 (10)	3
pFB514	Cm^r; *K. pneumoniae glnA-ntrBC* DNA inserted into the *Hin*dIII site of pACYC184	19
pGS9	Cm^r Nm^r; Source of Tn*5* DNA	44
pALbI	Tc^r; alfalfa leghemoglobin cDNA cloned into the *Pst*I site of pBR322	K. Dunn
pRKM1a, pRKM1b	Ap^r Tc^r; pMC1403 (15) inserted into the *Eco*RI site of pRK290 in both orientations	This study
pRKP9a, pRKP9b	Ap^r Tc^r; pVSP9 (*nifH-lacZ* [49]) cloned in the *Eco*RI site of pRK290 in both possible orientations	This study
pCE201	Ap^r; *R. meliloti fixA* promoter fused to pMC1403 (15)	C. Earl
pRKP2a, pRKP2b	Ap^r Tc^r; pCE201 inserted into the *Eco*RI site of pRK290 in both orientations	This study
pRmR2	Tc^r; *R. meliloti nifHD* DNA (3.8 kb) cloned into the *Eco*RI site of pACYC184	43
pNRA2	Tc^r; 1.6-kb *Bam*HI fragment containing *R. meliloti nifA* inserted into pNS184[a]	This study
pCE116	Tc^r; 3.4-kb fragment containing *R. meliloti fixABCX* cloned into the *Eco*RI-*Hin*dIII site of pBR325 (38)	23
pWB50	Ap^r; 1.9-kb *R. meliloti nifB* DNA cloned into pUC13 (53)	11

[a] PNS184 is PACYC184 with the *Eco*RI site converted into a *Bam*HI site (N. Schultes, unpublished data).

lysate method of Clewell and Helinski (17). Genomic *R. meliloti* DNA was prepared as described previously (31), and DNA restriction fragments were purified from low-melting-point agarose (Bio-Rad Laboratories) by the method of Weislander (56). DNA labeling and filter hybridizations were carried out as described previously (50).

Isolation of the *R. meliloti ntrC* gene. Nine hundred and sixty members of an *R. meliloti* genomic library in pHC79 (12) were replicated from 96-well microtiter plates onto sterilized GeneScreen filters (New England Nuclear Corp.) placed on LB plates. The plates were incubated at 37°C overnight, and rows of 12 colonies were pooled. Plasmid DNA was purified from these bacterial mixtures, digested with *Eco*RI, transferred to GeneScreen filters, and hybridized with ^{32}P-labeled *Escherichia coli ntrC* DNA (the 2-kilobase [kb] *Eco*RI-*Hin*dIII fragment purified from pGln53Y [3]). Plasmid DNA was purified from the individual colonies contained in one particular row that showed a positive hybridization signal. These DNAs were again digested with *Eco*RI, transferred to GeneScreen filters, and hybridized with radioactive *ntrC* DNA. Of 960 clones screened in this manner, 1 contained *R. meliloti* sequences homologous to *E. coli ntrC*.

Transposon Tn*5* mutagenesis. Plasmid pRm3.8R DNA was mutagenized in *E. coli* with Tn*5* as described by de Bruijn and Lupski (20). The positions of Tn*5* insertions were mapped by digestions with the enzymes *Eco*RI, *Bam*HI, *Hin*dIII, and *Pst*I. The *Eco*RI fragments from pRm3.8R containing the desired insertions were recloned into pRK290 at the *Eco*RI site, conjugated into Rm1021, and recombined into the genome of Rm1021 by the method of Ruvkun and Ausubel (42). The location of each Tn*5* insertion in the resulting strains was verified by digesting their genomic DNA with *Bam*HI and *Hin*dIII, and then probing the digested DNA with ^{32}P-labeled pRm3.8R and pGln53Y DNA (data not shown).

Lysis of bacteria in gels. A modification of the methods of Eckhardt (24) and Buikema et al. (12) was used to permit the use of horizontal gels. Log-phase cells (1.5 ml) were washed once with 0.1% Sarkosyl in TE8 (10 mM Tris, 1 mM EDTA, pH 8.0) and suspended in 50 μl of 20% Ficoll 400 in TBE (12). Cells (20 μl) were mixed with 10 μl of lysozyme mix (7,500 U of lysozyme per ml, 0.3 U of RNase I, 0.05% bromphenol blue in TBE containing 20% Ficoll 400) and added to the wells of a previously poured horizontal 0.7% agarose gel containing 0.2% sodium dodecyl sulfate. After 5 min at room temperature, electrophoresis was carried out in the cold (0.4 V/cm for 1 h, 5 V/cm for several hours). During electrophoresis, the gel was placed in the tank in such a way that only the two ends were in contact with the buffer (sterile TBE).

DNA sequencing. The 2.3-kb *Eco*RI-*Bgl*II fragment from pRm3.8B was subcloned into pUC13, and the resultant plasmid was used to generate deletion derivatives as described by Poncz et al. (37). A series of deleted fragments was subcloned into M13mp18 and M13mp19. In addition to this nested set of deletions, several random clones obtained by the shotgun cloning strategy of Bankier and Barrell (5) were used. Sequencing reactions with single-stranded M13 DNA templates were carried out essentially as described previously (5), except that [^{35}S]dATP was used instead of [^{32}P]dATP. The 15-base primer 5′-TGTAAAACGACGGCC-3′, synthesized in the laboratory of J. Smith, Department of Molecular Biology, Massachusetts General Hospital, was

used in the sequencing reactions. Compressions were resolved by running selected clones on sequencing gels in the presence of 25% formamide.

Sequence data were entered into a PDP11/780 VAX/VMS computer by using a GTCO (Rockville, Md.) digitizing tablet and the GELREAD program of W. Buikema (this laboratory). The sequence data were arranged in coherent form by using the DBAUTO, DBUTIL, and DBCOMP programs of Staden (46). The sequence was confirmed in its entirety with data from both strands.

RNA biochemistry. One liter of *R. meliloti* cells grown to the midlog phase (about 5×10^8 cells per ml) in nitrogen-excess (NFDM–0.2% glutamine) or nitrogen-limiting (NFDM–0.01% glutamine) media were washed once with TE8–0.1 M NaCl and suspended in 5 ml of 0.2 M Tris (pH 9.0)–0.4 M KCl–35 mM $MgCl_2$–25 mM EGTA. After the cell pellet was dispersed evenly, the mixture was made up to 5% Triton X-100, 1 mg of lysozyme per ml, and 10 mM vanadyl nucleoside (47) and placed in a boiling water bath. After lysis had occurred (about 2 min), the mixture was placed on ice. Chromosomal DNA and cell debris were removed by centrifugation at 8,000 rpm for 15 min. The supernatant solution obtained was extracted four times with phenol (or until the interphase no longer decreased), extracted once with chloroform, made up to 0.3 M sodium acetate, and precipitated with 2 volumes of ethanol. The RNA preparation was treated with RNase-free DNase (Promega Biotec RQ1 DNase) by incubation at 37°C for 15 min under conditions specified by the manufacturer. The DNase-treated RNA was extracted once with phenol and once with chloroform and precipitated with ethanol. The RNA was stored in ethanol at −80°C until ready for use. Before each experiment, the integrity of the RNA was checked on a 1% agarose gel. All the solutions used for RNA purification were treated with 0.1% diethylpyrocarbonate for at least 2 h and autoclaved.

For dot blots, RNA was suspended in a solution containing 6% formaldehyde, 1.0 M NaCl, and 30 mM NaH_2PO_4, incubated at 70°C for 15 min, and blotted onto nitrocellulose filters with a Schleicher & Schuell Minifold SRC-96-0. Hybridizations were carried out at 37°C for 24 h as described by Wahl et al. (55) (50% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5× Denhardt solution, 10% dextran sulfate, and 20 mM sodium phosphate buffer, pH 6.5). The filters were washed (55), air dried and exposed for autoradiography.

Ex planta activation of *nifH* and *fixA* promoters. Log-phase cultures of *R. meliloti* strains carrying fusion plasmids were washed with sterile water and suspended in nitrogen-limiting medium (NFDM–0.01% glutamine, 1 to 3 dilution) and nitrogen-excess medium (NFDM–0.2% glutamine, 1 to 10 dilution). After incubation for 36 h at 32°C, the cells were washed with Z buffer (33), suspended in Z buffer, and sonicated for 15 s with the microtip of a Heat Systems Ultrasonics model W-375 sonicator. Cell debris was removed by centrifugation, and the protein concentration of the supernatant solution was assayed by using the Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories). A volume of supernatant solution corresponding to 10 μg of protein was assayed for β-galactosidase activity as described by Miller (33). The time course of each reaction was monitored on a Beckman DU 7-Spectrophotometer. One enzyme unit was calculated as 1 nmol of orthonitrophenol produced per mg of soluble protein per min.

Plant assays. Alfalfa seeds were surface sterilized by immersion in concentrated H_2SO_4 for 12 min, washed thoroughly with sterile distilled water, germinated overnight on 0.8% water agar, and transferred aseptically to sterilized 18- by 150-mm test tubes capped with plastic sponges and containing 8 ml of Jensen seedling agar (54) solidified at a slant. The seedlings were grown overnight at room temperature and then inoculated with 0.1 ml of a saturated *R. meliloti* culture resuspended in sterile water. Plants were grown under controlled environmental conditions (16-h day; 22°C day, 18°C night), and nitrogen fixation was assayed by acetylene reduction (31) at periods from 9 to 42 days after inoculation. Nodules were assayed for β-galactosidase activity as described previously (7), except that individual nodules were used. Bacteria were isolated from surface-sterilized nodules as described previously (50).

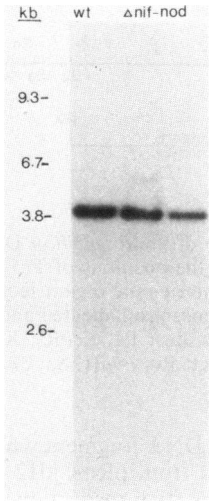

FIG. 1. Hybridization of *R. meliloti* genomic DNA to *E. coli ntrC* DNA. pGln53Y DNA was digested with *Eco*RI and *Hin*dIII, and the 2-kb fragment corresponding to *ntrC* (3) was purified, labeled, and hybridized to a GeneScreen filter containing *Eco*RI digested total DNA from Rm1021 (wild type; left lane), Rm3600 (Δ*nif-nod*; middle lane), and Rm4400 (Δ*nif-nod*; right lane).

RESULTS

Cloning and physical characterization of the *R. meliloti ntrC* gene. To determine whether *R. meliloti* contains an *ntrC*-like gene, wild-type *R. meliloti* (Rm1021) DNA was digested with *Eco*RI and hybridized with ^{32}P-labeled pGln53Y DNA. (pGln53Y is a plasmid containing the *E. coli ntrC* gene [3].) Because cloned *R. meliloti nifA* DNA cross-hybridizes with *E. coli ntrC* DNA (pGln53Y) (50), *Eco*RI-digested total DNA from two *R. meliloti* strains which contain *nifA* deletions (Rm3600 and Rm4400; S. Gibbons, unpublished) was also included in the hybridization studies to avoid possible confusion between hybridization of the *E. coli ntrC* probe to *R. meliloti ntrC* and *nifA*. In all three strains, only a 3.8-kb *Eco*RI restriction fragment hybridized to pGln53Y (*E. coli ntrC*) DNA (Fig. 1). Because the *R. meliloti nifA* gene is located on a 5.0-kb *Eco*RI fragment (50), these results suggest that an *ntrC* homolog is located on the 3.8-kb *Eco*RI fragment.

To isolate the presumptive *R. meliloti ntrC* gene, a ^{32}P-labeled *E. coli ntrC* DNA fragment (the 2-kb *Eco*RI-*Hin*dIII fragment purified from pGln53Y [50]) was used as a probe to screen an *R. meliloti* genomic library (12), and a cosmid clone containing *ntrC*-hybridizing sequences (pRmCjd12) was obtained (for details, see Materials and Methods). Subsequently, the 3.8-kb *Eco*RI fragment that corresponded

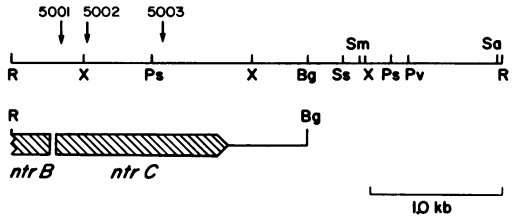

FIG. 2. Physical map of the *R. meliloti* DNA in pRm3.8R. The vertical arrows indicate the positions of Tn*5* insertions. The lower portion of the figure illustrates the region sequenced. The locations of *ntrB*- and *ntrC*-like genes are indicated and transcription is from left to right. Restriction sites: RI, *Eco*RI; X, *Xho*I; Ps, *Pst*I; Bg, *Bgl*II; Ss, *Sst*I; Sm, *Sma*I; Pv, *Pvu*II; Sa, *Sal*I.

in size to the genomic DNA fragment which hybridized to *E. coli ntrC* was purified from pRmCjd12 and subcloned into pACYC184 (16). A partial restriction map of this clone (pRmC3.8R) is shown in Fig. 2.

DNA sequence analysis. To confirm that the 3.8-kb *Eco*RI fragment cloned in pRmC3.8R contains an *ntrC*-like gene, we obtained the complete DNA sequence of the 2.3-kb *Eco*RI-*Bgl*II fragment in pRmC3.8R which contains the presumptive *R. meliloti ntrC* gene (see Materials and Methods for details). The results (Fig. 3; summarized schematically in Fig. 2) indicated that the 2.3-kb *Eco*RI-*Bgl*II fragment does indeed carry an *ntrC*-like gene. Comparison of the predicted amino acid sequence of the *R. meliloti ntrC* gene product with the published amino acid sequences of the products of the *ntrC* genes from *K. pneumoniae* (13) and *Bradyrhizobium* sp. (*Parasponia*) (34) (Fig. 3) showed that the *R. meliloti ntrC* and *Bradyrhizobium* sp. *ntrC* products are 74.5% homologous, the *R. meliloti ntrC* and *K. pneumoniae ntrC* products are 44.4% homologous, and the *Bradyrhizobium* sp. *ntrC* and *K. pneumoniae ntrC* products are 45.3% homologous, respectively. When all three *ntrC* products are compared, there is 37% amino acid identity.

In *K. pneumoniae* and *Bradyrhizobium* sp. (*Parasponia*), the nitrogen regulatory gene *ntrB* is situated directly upstream of the *ntrC* gene (28, 34). Examination of the DNA sequence of the 2.3-kb *Eco*RI-*Bgl*II fragment revealed the presence of an *ntrB*-like gene upstream of *R. meliloti ntrC*. The partial *R. meliloti ntrB*-sequence includes 84 carboxy-terminal amino acids, 50 and 26 of which are identical to the carboxy-terminal amino acids of the *Bradyrhizobium* sp. (*Parasponia*) and *K. pneumoniae ntrB* genes, respectively (Fig. 3).

Lack of linkage between *R. meliloti ntrC* and *glnA*. In enteric bacteria, *ntrC* is closely linked to *glnA*, the structural gene for glutamine synthetase, as part of the complex *glnA-ntrBC* operon (30). Several studies have shown that *E. coli glnA* is an analog of the gene which encodes glutamine synthetase I in *Rhizobium* species (14, 45). DNA hybridization experiments showed that *glnA* DNA (the *K. pneumoniae* 1.0-kb *Eco*RI fragment containing *glnA* purified from plasmid pFB514; Table 1) did not hybridize to the *R. meliloti* DNA contained in pRMCjd12, the cosmid clone containing *R. meliloti ntrC* (data not shown). Because the 3.8-kb *Eco*RI fragment containing *R. meliloti ntrC* is not located at either end of the *R. meliloti* DNA insert in pRMCjd12 (data not shown) and because *R. meliloti ntrC* DNA hybridized to a 12-kb genomic *Bam*HI restriction fragment and *K. pneumoniae glnA* DNA hybridized to two different *Bam*HI restriction fragments (18 and 13 kb) (data not shown), it is unlikely that *R. meliloti ntrC* is closely linked to *glnA*.

Generation of *R. meliloti ntrC* mutants. Plasmid pRmC3.8R (containing *R. meliloti ntrC*) was mutagenized with transposon Tn*5* (6, 20) to obtain Tn*5* insertions (Fig. 2). Three mutated DNA fragments carrying different *R. meliloti ntrC*::Tn*5* insertions were recloned into the broad-host-range plasmid pRK290 (21), conjugated into wild-type *R. meliloti* (Rm1021) and recombined into the Rm1021 genome (42), generating three independent *R. meliloti ntrC*::Tn*5* mutants (Rm5001, Rm5002, and Rm5003) (Fig. 2).

Chromosome location of the *R. meliloti ntrC* gene. Most, if not all, *R. meliloti* symbiosis specific genes, including *nifA*, are located on a large indigenous plasmid (megaplasmid) (4, 12, 41). To determine whether the *Rhizobium ntrC* gene is on the bacterial chromosome or on the megaplasmid, the megaplasmids contained in wild-type *R. meliloti* (Rm1021) as well as in strains carrying Tn*5* insertions in *nifA* and *ntrC* were fractionated from chromosomal DNA (Fig. 4A) in a horizontal agarose gel (see Materials and Methods for details). When the DNA contained in the gel was transferred to a GeneScreen filter and hybridized with ^{32}P-labeled *R. meliloti nifA* DNA as a positive control, hybridization to the megaplasmid was detected in all strains (Fig. 4B). In contrast, when the same filter was hybridized with ^{32}P-labeled Tn*5* DNA, only the megaplasmid bands contained in *nifA*::Tn*5* strains, but not those in *ntrC*::Tn*5* strains, showed positive hybridization (Fig. 4C). These results demonstrate that the *R. meliloti ntrC* gene is located on the bacterial chromosome.

Growth of *R. meliloti ntrC*::Tn*5* mutants on various nitrogen sources. In contrast to wild-type cells, *R. meliloti ntrC*::Tn*5* strains failed to grow on 0.5 mM potassium nitrate as the sole nitrogen source in either NFDM or GDM minimal medium. However, no growth deficit of the *ntrC* mutants was observed in GDM medium when arginine, proline, or aspartate was added as sole nitrogen source; the mutant cultures attained final cell densities similar to those of the wild type.

In contrast to the results obtained in GDM minimal medium, the *ntrC* mutants grew poorly compared with the wild type in NFDM medium when 0.01% arginine, proline, or aspartate was added as the sole nitrogen source. However, the *ntrC* mutants grew as well as the wild type in NFDM minimal medium with 0.01% histidine, urea, ammonium sulfate, or glutamine as the only nitrogen source.

Although we cannot explain the discrepancy between the results obtained in GDM and NFDM media, these results demonstrated that *R. meliloti ntrC*, unlike its counterpart in enteric bacteria (30, 52), is not required for the utilization of several amino acids as sole nitrogen sources.

Transcription of the *R. meliloti ntrC* and *glnA* genes. RNA dot-blot analysis was carried out to determine whether the transcription of *ntrC* itself is induced under nitrogen starvation conditions as is the case in enteric bacteria (39). RNA was purified from wild-type (Rm1021) and *ntrC*::Tn*5* (Rm5001; Fig. 2) strains grown under nitrogen excess (NFDM–0.2% glutamine) or nitrogen-limiting (NFDM–0.01% glutamine) conditions. Samples (10 μg) of each RNA preparation were blotted onto nitrocellulose filters and hybridized with ^{32}P-labeled *ntrC* DNA (Fig. 5). ^{32}P-labeled total *R. meliloti* DNA and alfalfa leghemoglobin DNA were used as positive and negative controls, respectively. Total *Rhizobium* DNA hybridized evenly to all the RNA preparations (Fig. 5), indicating that the amount of RNA contained per dot was similar. As expected, alfalfa leghemoglobin DNA (negative control) did not hybridize to any of the RNA dots. *ntrC* DNA hybridized significantly only to RNA from nitrogen-starved wild-type cells, indicat-

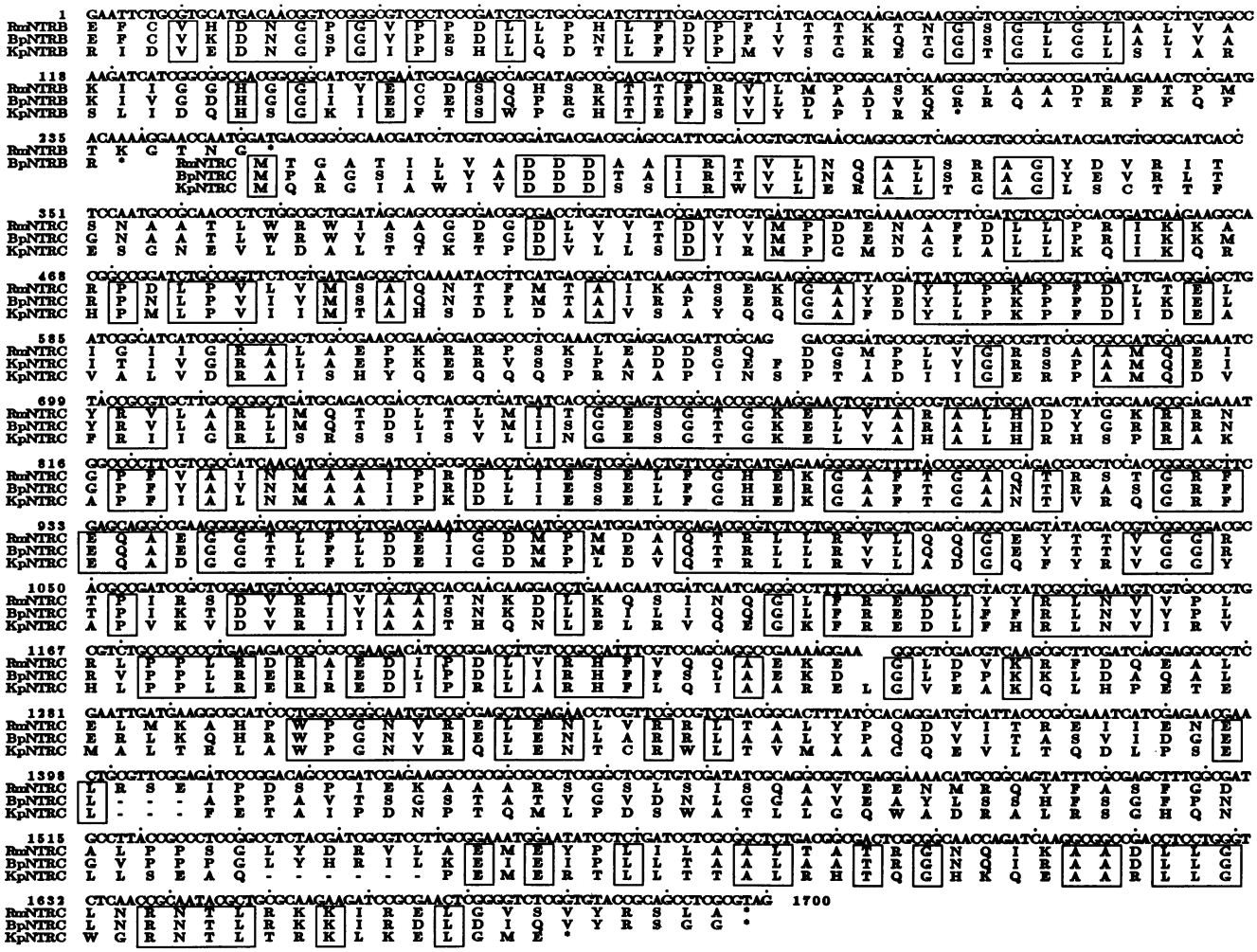

FIG. 3. DNA and derived amino acid sequences of the *R. meliloti* (Rm) *ntrC* gene and the 3′ end of the *ntrB* gene compared with the amino acid sequences of the *K. pneumoniae* (Kp) (13) and *Bradyrhizobium* sp. (*Parasponia*) (Bp) (34) *ntrC* products and the C-terminal portions of the *K. pneumoniae* (22) and *Bradyrhizobium* sp. (*Parasponia*) (34) *ntrB* products.

ing that transcription of the *R. meliloti ntrC* gene is induced under nitrogen-limiting conditions. In contrast, ^{32}P-labeled *glnA* DNA hybridized at the same level to all of the RNA preparations (Fig. 5). Therefore, it appears that the transcription of the *R. meliloti* glutamine synthetase I gene, coded by *glnA*, does not require *ntrC* for transcription and does not change in response to the nitrogen levels of the medium.

The reason for the lack of detectable *ntrC* RNA induction in Rm5001(*ntrC*::Tn*5*) cells is not clear. It is possible that the truncated *ntrC* RNA in Rm5001 is unstable. It is also possible that *ntrC* is required for its own activation, as is the case in enteric bacteria (30).

***R. meliloti ntrC* activates the *nifH* and *fixA* promoters ex planta.** Earlier studies have shown that an *R. meliloti nifH*::*lacZ* fusion carried on plasmid pVSP9 can be activated in *E. coli* by the *K. pneumoniae nifA* or *E. coli ntrC* products (47, 49). Furthermore, the *R. meliloti nifH* promoter can also be activated in nitrogen-starved, free-living *R. meliloti* wild-type or *nifA* mutant cells (48).

To determine whether the ex planta activation of *nifH* is mediated by *ntrC*, pVSP9, containing the *R. meliloti nifH*::*lacZ* fusion and linearized with *Eco*RI, was inserted in both orientations into the broad-host-range plasmid pRKP290 (21) at the *Eco*RI site. The cointegrate plasmids pRKP9a (transcription of the *nifH* promoter toward the *Sal*I site of pRK290) and pRKP9b (the opposite orientation) were conjugated into wild-type, *ntrC*::Tn*5*, and *nifA*::Tn*5* *R. meliloti* strains. As controls, the plasmids pRKM1a and pRKM1b (pRKM1a and pRKM1b are pRKP9a and pRKP9b, respectively, without the *R. meliloti nifH* promoter) were also conjugated into the same strains. These strains were grown to the log phase, washed, suspended in nitrogen-limiting or nitrogen-excess medium, incubated at 32°C for 36 h, and then assayed for β-galactosidase activities.

Wild-type (pRKP9a) and *nifA* mutant (pRKP9a) cells grown in nitrogen-limiting medium contained significantly more β-galactosidase than the same cells grown in nitrogen-excess medium (Table 2). In contrast, *ntrC* mutant (pRKP9a) cells contained low amounts of β-galactosidase whether grown in nitrogen-limiting or nitrogen-excess medium. Because similar results were obtained when the same strains were carrying pRKP9b (data not shown), it is likely that the observed induction of *lacZ* was via the *R. meliloti nifH* promoter rather than a plasmid promoter.

The *R. meliloti fixA* promoter is structurally homologous

TABLE 2. Ex planta expression of *R. meliloti nifH-lacZ* and *fixA-lacZ* fusions in various *R. meliloti* strains

Host	Plasmid	β-Galactosidase activity (U)	
		Nitrogen-limited medium	Nitrogen-excess medium
Wild type	pRKM1a (no promoter)	30	32
NifA⁻	pRKM1a (no promoter)	28	29
NtrC⁻	pRKM1a (no promoter)	29	27
Wild type	pRKP9a (*nifH*::*lacZ*)	1,238	98
NifA⁻	pRKP9a (*nifH*::*lacZ*)	725	52
NtrC⁻	pRKP9a (*nifH*::*lacZ*)	98	55
Wild type	pRKP2a (*fixA*::*lacZ*)	505	41
NifA⁻	pRKP2a (*fixA*::*lacZ*)	368	39
NtrC⁻	pRKP2a (*fixA*::*lacZ*)	48	46

to the *nifH* promoter (8) and during symbiosis also requires *nifA* for transcription (50). To investigate whether the *fixA* promoter can also be activated by *ntrC* ex planta, additional *lacZ* fusion experiments were carried out. Plasmid pCE201 (carrying the *R. meliloti fixA* promoter fused to *E. coli lacZ* [23]) was inserted into the *Eco*RI site of pRK290 in both orientations. The recombinant plasmids pRKP2a (*fixA* transcription toward the *Sal*I site of pRK290) and pRKP2b

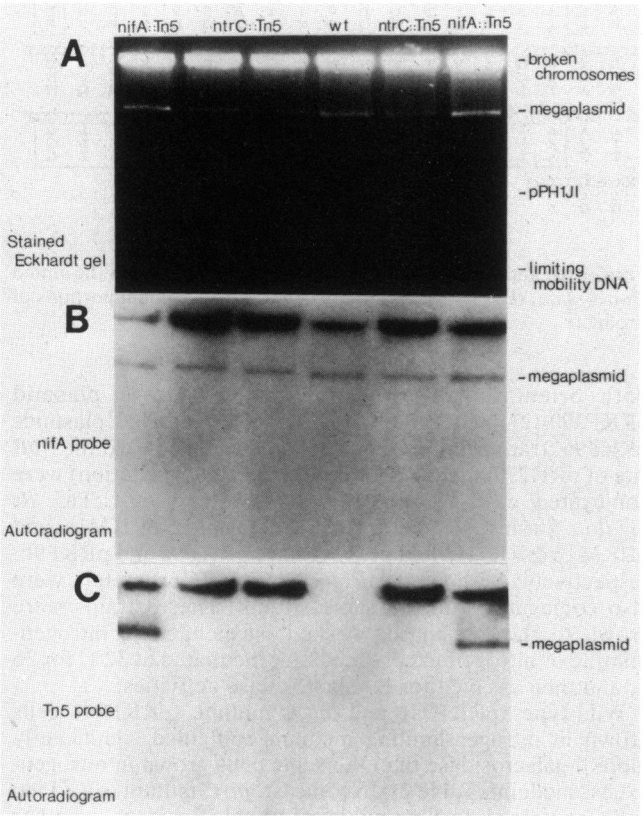

FIG. 4. Chromosomal location of the *R. meliloti ntrC* gene. The megaplasmid DNA from various *R. meliloti* strains was fractionated away from chromosomal DNA (see Materials and Methods) (A), transferred to a GeneScreen filter, and hybridized with ^{32}P-labeled pNRA2 (*nifA*, Table 1) (B). After autoradiography, the filter was washed overnight with 0.5 M KOH and rehybridized with ^{32}P-labeled pGS9 (Tn*5*, Table 1) (C).

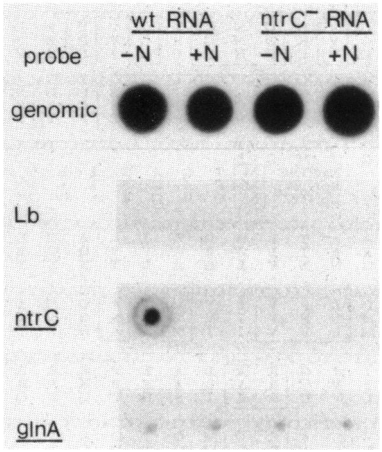

FIG. 5. Transcription of the *R. meliloti ntrC* and *glnA* genes. Samples of 10 μg of RNA purified from Rm1021 (wild type) and Rm5001 (*ntrC*) grown under nitrogen-excess or nitrogen-limited conditions (see Materials and Methods) were spotted onto nitrocellulose and hybridized with ^{32}P-labeled *ntrC* (the 2.3-kb *Eco*RI-*Bgl*II fragment of pRmC3.8R, the 1.1-kb *Eco*RI fragment that cross-hybridizes with *E. coli ntrC*, or the 1.2-kb *Pst*I-*Bgl*II fragment that contains the presumptive 3′ end of *R. meliloti ntrC*; Fig. 2), *glnA* (the 1-kb *glnA* fragment from pFB514; Table 1), total *R. meliloti* (positive control), and alfalfa leghemoglobin (Lb) (pALbI; see Table 1 negative control) DNA.

(opposite orientation) were conjugated into various *R. meliloti* strains, and studies similar to those described above for pRKP9a and pRKP9b were undertaken (Table 2). The activation of *fixA* in nitrogen-starved, free-living *R. meliloti* cultures, like the activation of *nifH*, was dependent on *ntrC* but not on *nifA*.

The experiments described in this section were repeated in GDM medium, and similar results were obtained (data not shown).

***R. meliloti ntrC* induces the transcription of several *nif* genes.** RNA dot-blot analyses were also carried out to corroborate and extend the fusion studies described above. RNA purified from induced (grown in nitrogen-limiting medium) and uninduced (grown in nitrogen-excess medium) wild-type and *ntrC* cells was blotted onto nitrocellulose filters as described in the legend to Fig. 5 and hybridized with ^{32}P-labeled *nifHD*, *fixABCX*, *nifB*, and *nifA* probes. Figure 6A shows the physical organization of these genes on the *R. meliloti* megaplasmid.

As expected from the results described above, high levels of *nifHD* and *fixABCX* RNA were detected in induced wild-type cells but not in uninduced wild-type or *ntrC* (induced or uninduced) cells (Fig. 6B). This corroborates the results presented in Table 2. Interestingly, wild-type cells also contained significantly higher levels of *nifA* and *nifB* RNA compared with uninduced or *ntrC* (induced or uninduced) cells. It is likely that *R. meliloti ntrC* directly activates the *nifB* gene, because the *nifB* promoter region contains the consensus sequence characteristic of *ntrA* activated promoters (13). On the other hand, because the *R. meliloti nifA* promoter does not contain the *ntrA* consensus sequences, the observed accumulation of *nifA* mRNA under nitrogen limitation conditions most likely results from readthrough from the *fixA* promoter (13, 27).

Although the transcription of several *nif* genes could be detected when the cells were starved for fixed nitrogen,

acetylene reduction assays indicated that nitrogen fixation did not occur at a detectable level in these cells.

Symbiotic phenotype of *R. meliloti ntrC* mutants. Strains carrying *ntrC*::Tn*5* mutations nodulated alfalfa plants at the same time and formed approximately the same number of nodules per plant as the wild-type strain. Moreover, nodules elicited by the *ntrC* mutants showed wild-type levels of acetylene reduction (nitrogenase activity), which was first detectable 9 days after inoculation and which reached a maximum about 12 days after inoculation. In addition, nodules elicited by Rm5002(pRKP9a) (*ntrC*::Tn*5*, *nifH-lacZ*) exhibited a similar amount of β-galactosidase activity (1,553 Miller units) as nodules elicited by strain Rm1021(pRKP9a) (wild type, *nifH-lacZ*) (1,402 Miller units) when assayed 12 days after inoculation.

DNA hybridization analyses showed that bacteria recovered from nodules elicited by *ntrC* mutants still contained Tn*5* insertions in *ntrC* (data not shown). Furthermore, the *ntrC* mutants recovered from nodules had the same symbiotic properties when inoculated onto alfalfa as the original inoculating strains.

The results presented in this section show that the *ntrC* product is not required for the activation of *nif* genes during symbiosis.

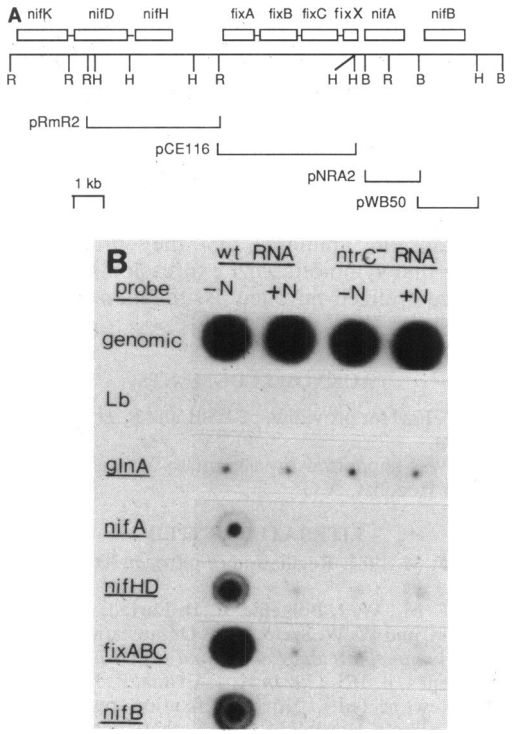

FIG. 6. Transcription of *R. meliloti nif* genes ex planta. (A) The organization of selected *nif* and *fix* genes on the *R. meliloti* megaplasmid and various cloned DNA fragments used as hybridization probes in the experiment shown in Fig. 6B. (B) Samples of 10 μg of RNA purified from Rm1021 (wild type) and Rm5001 (*ntrC*) grown under nitrogen-excess or nitrogen-limited conditions (see Materials and Methods) were spotted onto nitrocellulose and hybridized with ^{32}P-labeled *R. meliloti* genomic (positive control), alfalfa leghemoglobin (negative control), *glnA* (uninduced control), *nifHD* (pRmR2), *fixABCX* (pCE116), *nifA* (pNRA2 or the 0.7-kb *Eco*RI-*Bam*HI fragment in pNRA2 containing the 5′ end of *nifA* that does not cross-hybridize with *ntrC* [50]), and *nifB* (pWB50) DNA.

DISCUSSION

The data presented in this paper demonstrate that *R. meliloti* has a regulatory gene that is structurally and functionally similar to the *ntrC* genes of *K. pneumoniae* and *Bradyrhizobium* sp. (*Parasponia*). Moreover, as in several enteric species and in *Bradyrhizobium* sp. (*Parasponia*), *R. meliloti ntrC* is situated directly downstream of an *ntrB*-like gene (Fig. 3).

Structure of the *R. meliloti ntrC* product. The *R. meliloti*, *K. pneumoniae*, and *Bradyrhizobium* sp. (*Parasponia*) *ntrC* gene products share three major blocks of homology (Fig. 3), supporting the proposal that the *ntrC* product consists of three functional domains (22, 34). The N-terminal region (nucleotide positions 252 through 605, Fig. 3) of the *R. meliloti ntrC* product is also homologous to the N-terminal regions of a diverse set of other regulatory proteins and may function in the reception of information about the nitrogen status of the cell through interaction with *ntrB* product (22, 29, 34).

The central conserved portion of the *R. meliloti ntrC* product (nucleotides 650 through 1350, Fig. 3) shows a high degree of homology to *nifA* products from several species (13, 34) and to the *Rhizobium leguminosarum dctD* product (C. Ronson, unpublished data). Because all of these proteins activate transcription in conjunction with the *ntrA* sigma factor (C. Ronson, unpublished data), it is possible that this region is required for interaction with *ntrA* product. Finally, the conserved C-terminal region of the *R. meliloti ntrC* product (nucleotides 1605 through 1670) displays the characteristic helix-turn-helix motif of DNA binding proteins and likely represents a DNA binding domain (22).

Structure of the *R. meliloti ntrB* product. The *R. meliloti ntrB*-like gene product contains a region of C-terminal homology common to several regulatory proteins (34). We have previously proposed that this region is important for transducing information about environmental conditions to the regulatory proteins that share N-terminal homology to the *ntrC* product (34).

Role of *R. meliloti ntrC* in nitrogen assimilation. In enteric bacteria, the transcription of *ntrC* is autoactivated in response to nitrogen limitation. The observation that *R. meliloti ntrC* is transcribed under nitrogen-limiting conditions in wild-type but not in *ntrC* mutant bacteria (Fig. 5) suggests that *ntrC* may be similarly regulated in *R. meliloti*. However, despite the similarities in structure and regulation, there are also significant differences between the roles of *R. meliloti ntrC* and enteric *ntrC* products in nitrogen assimilation in their respective hosts.

In contrast to enteric *ntrC* mutants, *R. meliloti ntrC* mutants exhibited wild-type growth in the presence of low levels of ammonia or glutamine or when a variety of other amino acids were provided as the sole nitrogen source. The reason for the failure of the *ntrC*::Tn*5* mutants to grow in NFDM medium containing arginine, aspartate, or proline as the nitrogen source is unclear; the *ntrC* mutants grew as well as the wild type and reached similar final cell densities when cultured in another minimal medium (GDM) containing these nitrogen sources. The only nitrogen source that the *R. meliloti ntrC* mutants were clearly unable to utilize was nitrate, suggesting that *ntrC* product may be required for the induction of assimilatory nitrate reductase. Such a role has been demonstrated for the *ntrC* product in *Azotobacter vinelandii* (51).

In enteric bacteria, growth on limiting amounts of ammonia or glutamine leads to *ntrC*-mediated activation of *glnA*

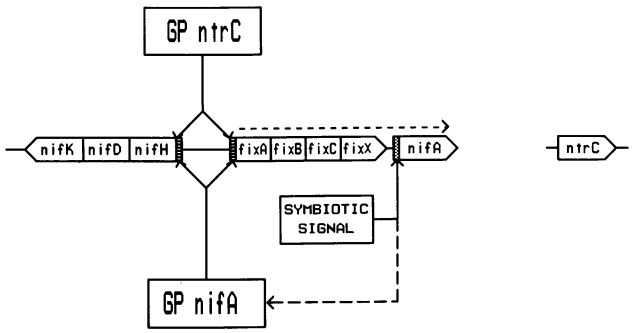

FIG. 7. A model of *nif* regulation in *R. meliloti*. Ex planta (top), the *nifH* and *fixA* promoters are activated by the *ntrC* gene product under ammonia limitation conditions. In the symbiotic state, the *fixA* and *nifH* promoters are activated by the *nifA* gene product. In the bacteroid (bottom), it appears that a symbiotic specific signal regulates either *nifA* transcription or the activity of the *nifA* gene product or both.

transcription (39) because glutamine synthetase (the *glnA* product) is required for ammonia assimilation under these conditions. However, *R. meliloti glnA* mRNA levels were independent of ammonia supply (Fig. 6B). One feature of *R. meliloti* which may account for the difference in *glnA* regulation when compared with enteric bacteria is the fact that several *Rhizobium* species, including *R. meliloti*, have two glutamine synthetases, I and II (18), encoded by different genes. The *R. meliloti* glutamine synthetase structural gene (*glnA*) is analogous to the *glnA* gene of enteric bacteria (45).

Role of *R. meliloti ntrC* in *nif* regulation. The most interesting difference between *K. pneumoniae* and *R. meliloti* with respect to the data presented here concerns the regulation of the nitrogen fixation genes. Although *K. pneumoniae ntrC* and *nifA* products activate promoters which share common structural features and in many cases both *ntrC* and *nifA* products appear to be able to activate the same promoters (32, 35), *ntrC* does not activate any of the *K. pneumoniae nif* promoters except the *nifLA* promoter (1). In *K. pneumoniae*, therefore, *nifA* plays the role of a specific activator of *nif* gene expression. In contrast, as illustrated in the model of *R. meliloti nif* regulation depicted in Fig. 7, both *R. meliloti ntrC* and *nifA* products appear to be able to activate the *nif* and *fix* structural gene promoters; which regulatory gene product is operative (*ntrC* or *nifA*) depends on whether the *R. meliloti* organisms are free living or have entered into the symbiotic state.

In free-living *R. meliloti* organisms under conditions of nitrogen limitation, the transcription of the *nifH* and *fixA* promoters is absolutely dependent on *ntrC* (Table 2). Although *R. meliloti nifA* mRNA can also accumulate as a result of readthrough transcription from the *fixA* promoter in free-living cultures under the nitrogen-limiting conditions used here (Fig. 5) (13, 27), the *nifA* product apparently has little, if any, role in *nif* expression ex planta (Table 2). In nodules, on the other hand, *R. meliloti nifA* is absolutely required for *nif* gene activation, whereas *ntrC* appears to play no significant role. Therefore, although part of the circuitry of *nif* regulation, namely, *nifA* activation of *nif* operon transcription, has been conserved in evolution between *R. meliloti* and *K. pneumoniae*, the mechanism(s) which regulates *nifA* transcription or *nifA* protein activity differs between the two species.

In alfalfa nodules, *R. meliloti nifA* RNA is transcribed from an *nifA*-specific promoter which does not contain the *ntrA* promoter consensus sequences as well as from the *fixA* promoter (13, 27). Because *R. meliloti ntrC* is not required for symbiotic nitrogen fixation, *nifA* transcription probably initially originates from the *nifA* promoter. This suggests that *nifA* transcription is either constitutive or that it is regulated by a mechanism unrelated to *ntrA* activated transcription. One possibility (Fig. 7) is that a symbiosis-specific signal either activates *nifA* transcription at the *nifA* promoter or activates a constitutively produced *nifA* product at the posttranscriptional level. It is interesting to note that the *ntrC* gene of *A. vinelandii*, an obligate aerobe that fixes nitrogen in free-living culture, is also not required for nigrogen fixation (51).

The stringent coregulation of *K. pneumoniae nif* genes by *ntrC* and *nifA* in a hierarchical fashion makes sense physiologically because nitrogen fixation is energy intensive and because *Klebsiella* species can occupy both nitrogen-rich and nitrogen-poor habitats. The two-tiered system is most likely designed to prevent expression of *K. pneumoniae nif* genes until all other nitrogen sources have been depleted.

In contrast to *K. pneumoniae*, the physiological significance of *ntrC* control of *R. meliloti nif* genes in the free-living state is not clear. Under conditions of free-living growth in the laboratory, it has not been possible to demonstrate that fast-growing *Rhizobium* species are capable of nitrogen fixation as measured by the acetylene reduction assay. It is possible, therefore, that the *ntrC*-mediated activation of the *nifH* and *fixA* promoters under nitrogen-limiting conditions is a laboratory artifact. On the other hand, it is also possible that *Rhizobium* species are capable of low levels of nitrogen fixation in their normal habitat. If this were the case, then stringent control of *R. meliloti nif* genes may not be as important as it appears to be in *K. pneumoniae* because the obligatory aerobic lifestyle of *R. meliloti* might preclude high levels of nitrogen fixation ex planta. In contrast, in the symbiotic state, *Rhizobium* species export rather than assimilate the ammonia that they produce; thus, it makes sense that symbiotic *nifA* expression should be independent of centralized nitrogen assimilation control and of *ntrC* expression.

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