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Rhizobium meliloti ntrA (*rpoN*) Gene Is Required for Diverse Metabolic Functions

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We report the identification and cloning of an *ntrA*-like (*glnF rpoN*) gene of *Rhizobium meliloti* and show that the *R. meliloti ntrA* product (NtrA) is required for C_4-dicarboxylate transport as well as for nitrate assimilation and symbiotic nitrogen fixation. DNA sequence analysis showed that *R. meliloti* NtrA is 38% homologous with *Klebsiella pneumoniae* NtrA. Subcloning and complementation analysis suggested that the *R. meliloti ntrA* promoter lies within 125 base pairs of the initiation codon and may be constitutively expressed.

In the enteric bacteria *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella typhimurium*, the products of the three genes *ntrA* (*glnF rpoN*), *ntrB* (*glnL*), and *ntrC* (*glnG*) regulate the transcriptional activation of several operons involved in the assimilation of poor nitrogen sources (22, 24). The products of *ntrB* (NtrB) and *ntrC* (NtrC) are bifunctional regulatory proteins that act in concert to effect transcriptional repression or activation (22, 23, 24). The functional state of NtrB is modulated by the products of the *glnB* and *glnD* genes in response to the intracellular glutamine to 2-ketoglutarate ratio (3). Depending on its state, NtrB is able to switch NtrC between repressor and activator forms by phosphorylating or dephosphorylating it (31). NtrB, in its C-terminal domain, and NtrC, in its N-terminal domain, share homology with a variety of two-component bacterial regulatory systems that may therefore share the same mode of signal transduction (11, 32).

The requirement for the *ntrA* product (NtrA) for transcriptional activation by NtrC has recently been explained by the demonstration that NtrA is a sigma factor which is required to confer specificity on core RNA polymerase for NtrA-specific promoter sequences (17, 19). Promoters activated by NtrC-NtrA do not contain canonical −35 and −10 sequences but instead have the consensus −26 CTGGYAYR-N_4-TTGCA-10 (16). NtrA is able to bind to core RNA polymerase, and transcription from the NtrC-regulated promoter *glnAp2* can be activated in vitro by highly purified core enzyme together with NtrA and NtrC (17, 19). Based on these observations, it has been proposed that *ntrA* (*glnF*) be renamed *rpoN* (19).

The regulation of the *ntrA* gene has been studied in *K. pneumoniae* (7, 28) and *E. coli* (5). In each case constitutive expression of the *ntrA* promoter that was independent of the nitrogen source was observed, suggesting that the cells always contain a subpopulation of core RNA polymerase bound with NtrA. The *ntrA* gene from *K. pneumoniae* has been sequenced, and a potential constitutive promoter was identified (27) on the basis of homology with the canonical *E. coli* promoter (−35 TTGACA-N_{17}-TATAAT-10).

In *K. pneumoniae* the nitrogen fixation regulatory operon *nifLA* is one of the operons that is activated by NtrC-NtrA (for a review, see reference 16). Like NtrC, the *nifA* product (NifA) is a transcriptional activator which requires the concerted action of NtrA for the expression of the other six *nif* operons (16). NifA and NtrC share strong homology in their central and C-terminal domains (4, 11), and NifA-activated promoters have a similar consensus with NtrC-activated promoters (16).

Homologs of the enteric *ntrC* and *nifA* genes have been identified in the symbiotic diazotroph *Rhizobium meliloti* (44, 45), and homologs of *nifA* have been identified in several other *Rhizobium* and *Bradyrhizobium* species as well (for a review see reference 16). In *R. meliloti* NtrC is required for the utilization of nitrate as a nitrogen source and the activation of the *nifH* gene in free-living culture in response to nitrogen limitation (44), while NifA is required for the activation of *nif* genes, including *nifH*, in the symbiotic state (45). In contrast to *K. pneumoniae*, *R. meliloti* NtrC is not required for the activation of the *nifA* gene (44). The *R. meliloti* NtrC and NifA proteins (4, 44) and the promoters that they activate (2, 43) show strong homology with their enteric counterparts, suggesting that *R. meliloti* also contains an *ntrA* gene. Such a gene has not been identified, however, despite extensive searches based on hybridization to the *K. pneumoniae ntrA* gene and complementation of *E. coli ntrA* mutants (32a). Recently, homologs of *ntrC* and *nifA* have also been found in *Rhizobium* sp. strain ORS571, the stem-nodulating symbiont of *Sesbania rostrata* (32a).

In a recent study of the genetic regulation of C_4-dicarboxylate transport in *Rhizobium leguminosarum* (36; C. W. Ronson, P. M. Astwood, B. T. Nixon, and F. M. Ausubel, manuscript in preparation), we observed that *dctA*, a structural gene required for the transport of succinate, fumarate, and malate, contains an NtrA-like promoter sequence. We also identified two positive regulatory genes, *dctB* and *dctD*, that are required for activation of *dctA* transcription. Interestingly, the C terminus of the *dctB* gene product (DctB) is homologous with the C terminus of NtrB, and the N terminus of the *dctD* product (DctD) is homologous with the N terminus of NtrC. Furthermore, DctD also

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Relevant characteristics	Source or reference
E. coli		
HB101	*ara xyl mtl pro leu thi lacY supE rpsL endA recA hsdR hsdM*	H. Boyer
JM101	Δ(*lac-pro*) *supE thi* (F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)	J. Messing
MC1061	*ara leu lac gal hsdR rpsL*	M. Casadaban
MM294	*pro thr endA hsdR supE*	M. Meselson
R. meliloti		
1021	Wild-type Str^r	26
1354	*nifA*::Tn*5* Str^r Nm^r	45
5002	*ntrC*::Tn*5* Str^r Nm^r	44
1680	*ntrA1*::Tn*5* Str^r Nm^r	This study
1681	*ntrA2*::Tn*5* Str^r Nm^r	This study
1682	*ntrA5*::Tn*5* Str^r Nm^r	This study
R. leguminosarum R1538	*dctD* Str^r	C. Ronson
Plasmids		
pRK290	IncP Tc^r	9
pWB5A	Derivative of pRK290 with polylinker containing *Eco*RI, *Cla*I, *Hin*dIII, *Xba*I, *Bgl*II, *Pst*I, and *Bam*HI sites	W. Buikema
pLAFR1	Derivative of pRK290 with λ *cos* site	14
pSUP106	IncQ Tc^r Cm^r	35
pCR63	pLAFR1 with (*dctA-lacZ*) *dctB*$^+$ *dctD*$^+$ insert	C. Ronson
pRKP9a	pRK290 with *nifH-lacZ* fusion	44
pRK2013	*rep* (ColE1) Mob$^+$ Tra$^+$ Km^r	9
pRK602	pRK2013 *npt*::Tn*9* Ω::Tn*5*	T. Finan and E. Signer
pUC8	Ap^r	46
pNtr3.5BE	pUC8 with 3.5-kb *Bam*HI-*Eco*RI insert containing *ntrA*	This study
pNtrA10	pLAFR1 cosmid clone containing *ntrA*	This study
pNtrTn1	pUC8 with 23-kb *ntrA1*::Tn*5* *Eco*RI fragment from Rm1680	This study
pNtrTn4	pUC8 with 23-kb *ntrA2*::Tn*5* *Eco*RI fragment from strain 1681	This study
pHP45Ω	Ap^r Sp^r; source of omega fragment	13, 34
M13mp18		47

shares strong homology with NtrC and NifA in the central domain of these proteins that is postulated to interact with NtrA. These observations suggest that NtrA might be required for the expression of *dctA*; therefore, we formulated a selection scheme for mutations in a putative *R. meliloti ntrA* gene based on the supposition that NtrA is required for *dctA* expression. We report here the isolation and characterization of *R. meliloti ntrA* mutants, the cloning of the *R. meliloti ntrA* gene, and the *R. meliloti ntrA* DNA sequence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are described in Table 1. Construction of the gene bank of *R. meliloti* 1021 DNA in the cosmid vector pLAFR1 has been described previously (14). Complex media were LB for *E. coli* and LB or TY for *R. meliloti*. Defined medium contained RDM salts and vitamins (39), 0.4% glucose as the carbon source unless otherwise specified, and 5 mM ammonium chloride as the nitrogen source unless otherwise specified. When 10 mM sodium succinate was used as the carbon source, the medium was buffered with 50 mM MES (morpholineethanesulfonic acid; pH 6.1) (38).

R. meliloti 1021 was mutagenized with transposon Tn*5* (by Q. Gu and M. Honma, Department of Molecular Biology, Massachusetts General Hospital) by mating *R. meliloti* 1021 with strain MM294 (pRK602) followed by selection for streptomycin- and neomycin-resistant transconjugants on LB medium. Approximately 10,000 neomycin-resistant *R. meliloti* 1021 transconjugants were pooled by suspending the colonies in LB; the pooled colonies were stored in 20% glycerol at −70°C.

Bacterial crosses. All bacterial crosses were triparental spot matings with MM294 donor strains and the helper strain MM294(pRK2013) (9, 37), and streptomycin was used to counterselect the *E. coli* strains. For identification of complementing cosmids from the gene bank, the mating mixture was streaked for single colonies directly on defined medium containing succinate, tetracycline, and streptomycin. For complementation studies with pWB5A derivatives, the *R. meliloti* transconjugants were first selected on LB medium containing streptomycin and tetracycline and then screened on appropriate defined media for phenotypic characterization. For complementation studies with pSUP106 derivatives, the mating mixture was streaked directly onto defined medium containing succinate, potassium nitrate, and streptomycin but without drugs to select for plasmid transfer because the chloramphenicol resistance gene of pSUP106 is expressed poorly in *R. meliloti*.

Growth and β-galactosidase assays. Strains were grown to late log phase in TY medium, spun down, suspended in an equal volume of RDM salts, and inoculated 1 in 50 into defined medium containing either no nitrogen source (control culture) or the specified nitrogen source. Growth was monitored by measuring the A_{600} of the cultures and by plating for single colonies on TY. To assay induction of the *nifH*::*lacZ* fusion, cultures were grown in defined medium containing glutamine at either 0.005% (nitrogen limited) or 0.2% (nitrogen excess). Samples were taken at 20 h (mid-log phase) and 44 h (stationary phase) and assayed for β-galactosidase activity as described previously (29). Similar values were obtained at each time point. Nodules were assayed for β-galactosidase activity as described previously (44).

Plant assays. Plant assays and isolation of bacteria from nodules were done as described previously (44).

DNA manipulations. Large- and small-scale plasmid preparations were made by the boiling method (18). Methods for the isolation of genomic DNA (41), labeling of probes by random primer extensions (12), and Southern hybridizations (32) have been described previously. Standard procedures (25) were used for restriction enzyme digestions, ligations, gel electrophoresis, and transformations.

DNA sequencing. To prepare fragments for sequencing, 20 μg of pNtr3.5BE DNA was linearized by digestion with either *Eco*RI or *Bam*HI and then digested in a final volume of 500 μl with *Bal* 31 nuclease at a concentration (usually a 1 in 200 dilution of enzyme obtained from New England BioLabs, Inc., Beverly, Mass.) that was predetermined to remove about 200 base pairs (bp) per minute from each terminus. Fractions of 50 μl were taken at 1-min intervals and made 20 mM with EGTA [ethylene glycol-bis(β-

aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], ethanol precipitated, suspended, and then digested with either *Bam*HI or *Eco*RI. The *Bal* 31-digested fragments were resolved on a 1% low-melting-point agarose gel, extracted from the agarose, and ligated into M13 mp18 cut with *Sma*I and either *Bam*HI or *Eco*RI. The ligation mixture was transformed into strain MC1061 and plated on a lawn of JM101, and the resultant plaques were used to prepare template DNAs. Sequencing was done by the dideoxy method with a commercial 17-mer as the primer and [^{35}S]dATP as the label (1). This procedure yielded about 1.5 kilobases (kb) of sequence from the *Eco*RI and *Bam*HI ends. To sequence the remainder of the 3.5-kb *Bam*HI-*Eco*RI fragment, the replicative forms of the M13 phage containing the largest deletion in each direction were purified, and the *Bal* 31 procedure was repeated (33). The final sequence was completely double stranded.

To determine the sequence from the site of the Tn*5* insertion in *R. meliloti* 1681, genomic DNA from strain 1681 was digested with *Eco*RI, ligated to *Eco*RI-cleaved pUC8, and transformed into MC1061 with selection for ampicillin plus kanamycin resistance. The resultant plasmid containing Tn*5* and flanking genomic DNA was cleaved with *Bam*HI or *Eco*RI-*Bam*HI, and the fragments containing the arms of Tn*5* and flanking *Rhizobium* DNA were cloned into M13 mp18 in the appropriate orientation for sequence determination from the ends of Tn*5* by using a Tn*5*-specific primer (40). The Tn*5*-specific primer 3′-GTTCATCGCAGGACTTG-5′ was synthesized in the laboratory of J. Smith, Department of Molecular Biology, Massachusetts General Hospital.

Computer analysis. DNA sequences were compiled by using the DBAUTO and DBUTIL programs (42) and were analyzed by using programs from the University of Wisconsin, Madison (8). Pair-wise comparisons between proteins were made with the National Biomedical Research Foundation ALIGN or RELATE programs (6) by using the mutation data matrix and a gap penalty of 16. The (standard deviation) scores given represent the number of standard deviations separating the maximum score of the real sequences from the mean of the maximum scores of 100 random permutations of the two sequences (6).

RESULTS

Selection of *R. meliloti ntrA* mutants. We designed a selective scheme to isolate *R. meliloti ntrA* mutants based on the hypothesis that *ntrA* mutants would be unable to activate *dctA* expression. To monitor *dctA* expression in *R. meliloti*, we used an in-frame *dctA-lacZ* fusion that was present on pCR63, a broad-host-range plasmid that contains the entire *dct* regulon from *R. leguminosarum*. The *leguminosarum dctA-lacZ* fusion was activated to approximately the same extent in response to succinate in both *R. leguminosarum* and *R. meliloti* (data not shown). Furthermore, *R. meliloti* 1021 containing pCR63 formed white colonies on defined medium containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) with glucose as the sole carbon source and blue colonies on defined medium containing X-gal with glucose plus succinate as carbon sources.

Plasmid pCR63 was conjugated from *E. coli* MM294 into a culture of *R. meliloti* 1021 which was mutagenized earlier with transposon Tn*5* (see above), and transconjugants were plated on defined medium containing X-gal, glucose, succinate, streptomycin, and neomycin. Because the *dct* regulatory genes *dctB* and *dctD* are present on pCR63, we reasoned that any mutant of *R. meliloti* 1021 that contained pCR63 and formed white colonies when plated on medium containing X-gal, glucose, and succinate should be mutated in the *R. meliloti ntrA* gene. Three white colonies were observed among approximately 5,000 colonies that were screened; these colonies were purified on the same medium.

TABLE 2. Phenotypic properties of *R. meliloti dctD*, *ntrC*, *nifA*, and *ntrA* mutant strains

Phenotype	Strains			
	5002 (*ntrC*)	1354 (*nifA*)	R1538 (*dctD*)	1681 (*ntrA2*)
Growth on succinate	+	+	−	−
Induce *dctA* fusion in response to succinate[a]	+ (280)[b]	+ (260)	− (7)	− (9)
Growth on 0.5 mM KNO_3 as the sole nitrogen source	−	+	+	−
Induce *nifH* fusion in nitrogen-limited free-living culture[a]	− (8)	+ (210)	NT[c]	− (10)
Induce *nifH* fusion in nodules	+ (1,490)	− (18)	NT	− (12)
Form Fix$^+$ nodules	+	−	+	−

[a] Induction assays were carried out as described in the text. The *dctA-lacZ* fusion was carried on pCR63, and the *nifH-lacZ* fusion was carried on pRKP9a.

[b] Numbers in parentheses denote units of β-galactosidase calculated by the method described by Miller (29).

[c] NT, Not tested.

Before further physiological studies were done, the three mutant strains were cured of pCR63. Because we observed that plasmids which are stable in free-living cells are frequently lost from strains which are recovered from root nodules, the mutant strains carrying pCR63 were inoculated onto alfalfa plants, and the bacteria isolated from the resultant nodules were screened for tetracycline-sensitive colonies. Tetracycline-sensitive derivatives were obtained at a frequency of 40% and one tetracycline-sensitive derivative of each of the three mutants *R. meliloti* 1680 (*ntrA1*::Tn*5*), *R. meliloti* 1681 (*ntrA2*::Tn*5*), and *R. meliloti* 1682 (*ntrA5*::Tn*5*) was saved. In subsequent studies the three mutants were found to display identical phenotypes; and hence, in some cases results from only one, *R. meliloti* 1681 (*ntrA2*::Tn*5*), are reported here.

Phenotypic properties of putative *ntrA* mutant and revertant strains. Because *K. pneumoniae* NtrA works in concert with NtrC and NifA to activate genes involved in nitrogen assimilation and nitrogen fixation, respectively, we assayed the presumptive *ntrA* mutant strains 1680, 1681, and 1682 for NtrC$^-$ and NifA$^-$ phenotypes. We also assayed the mutants for DctD$^-$ phenotypes (Table 2).

As expected from the results obtained on succinate–glucose–X-gal plates, the *dctA-lacZ* fusion carried on plasmid pCR63 was not activated in strain 1681 when the strain was unable to grow on succinate as the sole carbon source (Table 2).

R. meliloti ntrC::Tn*5* mutants are unable to grow on 0.5 mM potassium nitrate as the nitrogen source (44). When *R. meliloti* 1681 (*ntrA2*::Tn*5*) and *R. meliloti* 5002 (*ntrC*::Tn*5*) were cultured with potassium nitrate as the sole nitrogen source, strain 1681 was completely unable to grow on concentrations from 0.5 to 5.0 mM, whereas strain 5002 was unable to grow on 0.5 mM KNO_3 but grew slowly on 2 and 5 mM KNO_3.

R. meliloti ntrC::Tn*5* mutants elicit Fix$^+$ nodules which reduce acetylene at wild-type levels, whereas *nifA*::Tn*5*

mutants elicit Fix⁻ nodules (44, 45). Similarly, a *nifH-lacZ* fusion is activated to wild-type levels in alfalfa root nodules in an *ntrC*::Tn*5* host but is not activated at all in a *nifA*::Tn*5* host (44). In contrast, a *nifH-lacZ* fusion is not activated during free-living growth on limiting nitrogen in an *ntrC*::Tn*5* host but is partially activated in a *nifA*::Tn*5* host (44). *R. meliloti* 1681 (*ntrA2*::Tn*5*) elicited Fix⁻ alfalfa root nodules, and a *nifH-lacZ* fusion in 1681 was not activated in root nodules or during free-living nitrogen-limited growth (Table 2). The results presented above indicate that strain 1681 has the phenotypic properties of *Rhizobium ntrC*, *nifA*, and *dctD* mutants, as would be expected for an *ntrA* mutant.

Because *E. coli*, *K. pneumoniae*, and *S. typhimurium ntrC* and *ntrA* mutants are characterized by glutamine auxotrophy and the inability to grow on several amino acids as the sole nitrogen source, we tested the ability of *R. meliloti* 1681 (*ntrA2*::Tn*5*) to grow in defined liquid medium containing glucose as the carbon source and glutamine, histidine, aspartate, arginine, proline, alanine, or ammonium chloride at 50 μg/ml as the nitrogen source. Both strains 1681 and 5002 (*ntrC*::Tn*5*) grew at a rate similar to that of the wild type and reached final cell densities similar to that of the wild type in all of these media.

Three revertants of *R. meliloti* 1681 (*ntrA2*::Tn*5*) that were able to grow on succinate were isolated after a culture was plated on defined medium containing succinate as the carbon source and ammonium chloride as the nitrogen source. Two of the revertants were able to utilize nitrate and were neomycin sensitive, suggesting that the succinate-negative and nitrate-negative phenotypes of strain 1681 are both caused by the Tn*5* insertion. The third revertant was unable to utilize nitrate and was neomycin resistant, suggesting that it is a Dct-specific second-site revertant.

Molecular characterization of the putative *ntrA* mutants. When ^{32}P-labeled pUC8::Tn*5* DNA was used to probe genomic DNA from strains 1680 (*ntrA1*::Tn*5*), 1681 (*ntrA2*::Tn*5*), or 1682 (*ntrA5*::Tn*5*) that was digested with *Eco*RI, a single band of about 23 kb was detected in each case. When the genomic DNA was digested with *Eco*RI-*Bam*HI, two bands of 5.0 and 4.2 kb were detected in strain 1680 DNA, while two bands of 4.9 and 4.3 kb were detected in 1681 and 1682 DNA. Because Tn*5* is 5.7 kb long and contains a single *Bam*HI site, these data indicate that each mutant contains a single Tn*5* insertion located within an 18-kb *Eco*RI fragment and a 3.5-kb *Bam*HI-*Eco*RI or *Bam*HI fragment and suggests that strains 1681 (*ntrA2*::Tn*5*) and 1682 (*ntrA5*::Tn*5*) are isogenic.

Total DNA from strains 1680 (*ntrA1*::Tn*5*) and 1681 (*ntrA2*::Tn*5*) was digested with *Eco*RI, ligated to *Eco*RI-cut pUC8 DNA, and transformed into *E. coli* MC1061. Plasmid DNA was isolated from several of the resulting ampicillin- and kanamycin-resistant transformants; most of these contained pUC8 with the 23-kb *Eco*RI insert, and representative plasmids derived from 1680 (pNtrTn1) and 1681 (pNtrTn4) were chosen for futher study. Restriction enzyme mapping of pNtrTn1 and pNtrTn4 showed that Tn*5* was inserted within a 3.5-kb *Bam*HI-*Eco*RI fragment in each case (see below).

To determine whether Tn*5* in strain 1681 (*ntrA2*::Tn*5*) was inserted into a gene that was homologous with *K. pneumoniae ntrA*, the 4.3-kb *Bam*HI and 4.9-kb *Eco*RI-*Bam*HI fragments from pNtrTn4 that contained Tn*5* and flanking genomic sequences were cloned into M13 mp18. An oligonucleotide primer homologous to the ends of Tn*5* was used to determine the DNA sequence directly adjacent to the insertion site of Tn*5*. Significant homology at the amino acid level was detected to the N-terminal region of the *ntrA* gene from *K. pneumoniae* (see below), confirming that the Tn*5* in 1681 is inserted into an *ntrA*-like gene.

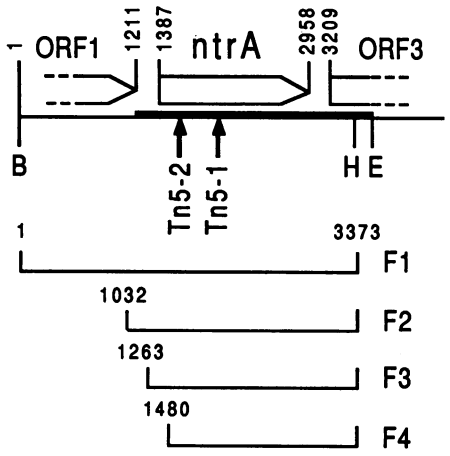

FIG. 1. Genetic organization of the 3.5-kb *Bam*HI-*Eco*RI fragment containing *ntrA* and map of fragments used for complementation tests. The numerals refer to nucleotides from the *Bam*HI site and correspond to those in Fig. 2. The precise start site of ORF1 is unknown but is between 300 and 500 bp from the *Bam*HI site (unpublished data). The sites of the Tn*5* insertions in strains 1680 (*ntrA1*::Tn*5*), Tn5-1, and 1681 (*ntrA2*::Tn*5*), Tn5-2, are marked. The heavy line indicates the region the sequence of which is presented in Fig. 2. Restriction site abbreviations: B, *Bam*HI, E, *Eco*RI, H, *Hin*dIII.

Molecular cloning of the *R. meliloti ntrA* gene. To clone the *R. meliloti ntrA*-like gene, a pLAFR1 gene library of strain 1021 DNA was mated into strains 1680, 1681, and 1682; and four Dct⁺ transconjugants from each strain were isolated onto defined medium containing succinate as the sole carbon source. These Dct⁺ colonies also grew on nitrate as the sole nitrogen source. The complementing pLAFR1 cosmids from the 12 Dct⁺ colonies were conjugated into *E. coli* HB101 in a triparental mating, using MM294(pRK2013) as the helper. Plasmid DNA was isolated from the 12 *E. coli* strains containing the pLAFR1 cosmids. All 12 plasmid DNAs gave identical *Eco*RI restriction patterns and contained an 18-kb *Eco*RI fragment.

The 4.3-kb *Bam*HI fragment from pNtrTn4 (see above) that contained part of Tn*5* and flanking genomic sequences from strain 1681 was gel purified, labeled with ^{32}P, and used to probe the *Eco*RI-digested DNAs from the 12 complementing cosmids by using the Southern blotting and hybridization procedure. In each case, hybridization to the 18-kb *Eco*RI fragment was detected. These results confirm that the mutant phenotypes of strain 1681 were caused by the Tn*5* insertion and not an endogenous insertion sequence or some other mutation. One of the cosmids that complemented 1681, pNtrA10, was chosen for further study and was found to contain the expected 3.5-kb *Bam*HI-*Eco*RI fragment.

Nucleotide sequence of the *R. meliloti ntrA* gene and flanking DNA. To characterize further the *R. meliloti ntrA* gene, we determined the DNA sequence of the 3.5-kb *Bam*HI-*Eco*RI fragment containing the gene. The fragment was subcloned from the pLAFR1 cosmid pNtrA10 into pUC8, and the resultant plasmid pNtr3.5BE was used as the source of DNA for isolation of *Bal* 31-deleted fragments for sequencing. The sequence data revealed three open reading frames (ORFs) (Fig. 1), including a central one of 1,602 bp (Fig. 2), the product of which had extensive homology with the *K.*

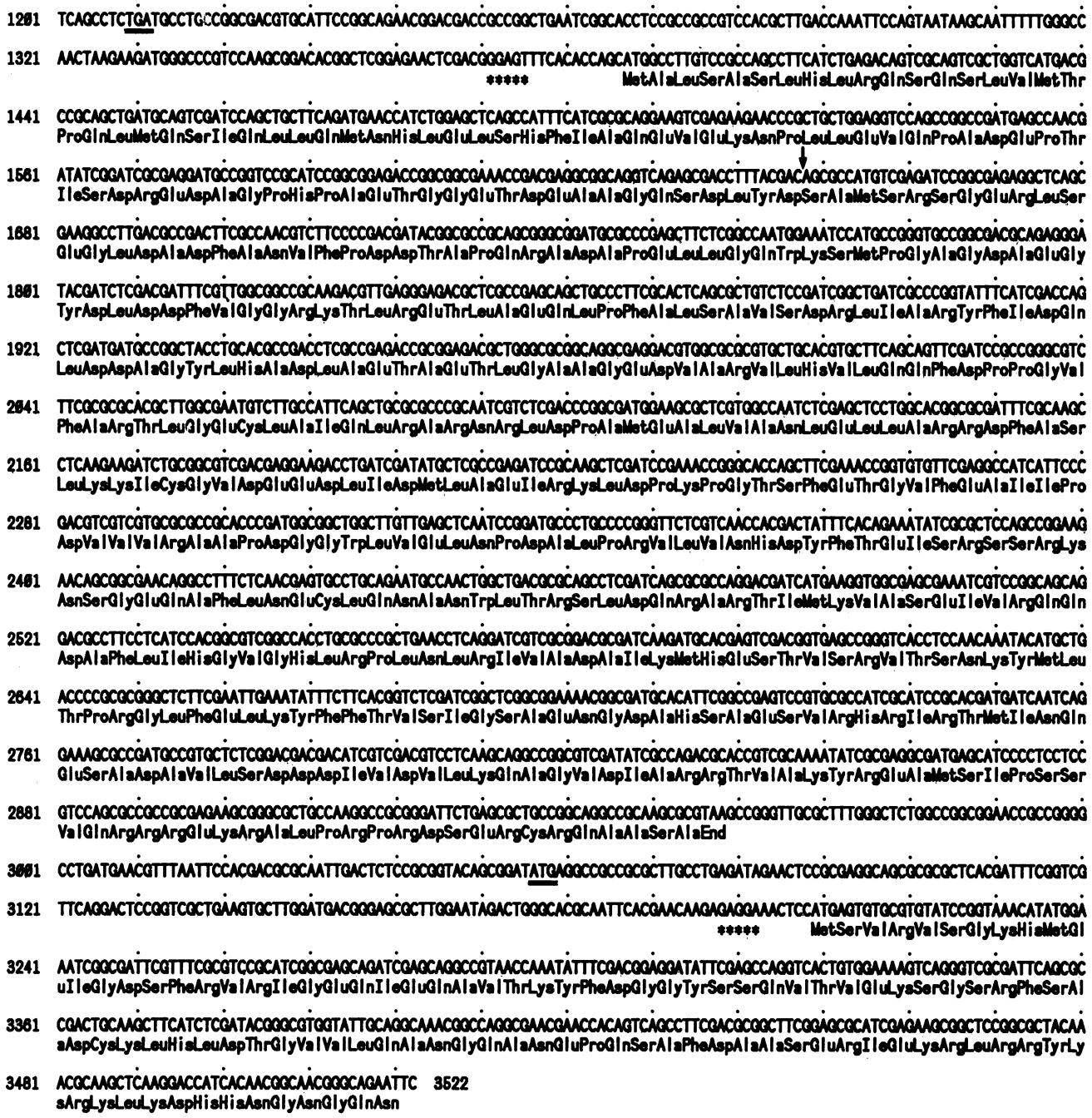

FIG. 2. Nucleotide sequence of a 2.3-kb region of the 3.5-kb *Bam*HI-*Eco*RI fragment containing the *R. meliloti ntrA* gene and 5′ region of ORF3 (see Fig. 1). Nucleotides are numbered from the first base of the *Bam*HI site (data not shown). Possible ribosome-binding sites are indicated with asterisks. The termination codon of ORF1 (see Fig. 1) and an in-frame ATG codon upstream of ORF3 are underlined. The position of the Tn*5* insertion in strain 1681 (*ntrA2*::Tn*5*) is indicated by a vertical arrow.

pneumoniae ntrA gene product (51.24 SD units by ALIGN analysis; Fig. 3), including 38% amino acid identity.

The start codon of the *R. meliloti ntrA* gene was provisionally assigned by comparison with the *K. pneumoniae ntrA* gene. The Tn*5* insertions in *R. meliloti* 1680 (*ntrA1*::Tn*5*; position determined by restriction site mapping) and *R. meliloti* 1681 (*ntrA2*::Tn*5*; position determined by sequence analysis) mapped within the gene (Fig. 1 and 2). The *R. meliloti ntrA* product was slightly larger than the *K. pneumoniae ntrA* product (524 versus 478 amino acids), but the products shared homology throughout their length and had similar amino acid compositions.

The *R. meliloti ntrA* gene was preceded by an ORF that terminated 176 bp before the initiation codon of *ntrA* (Fig. 1; data not shown). Although the 176-bp region between ORF1 and *ntrA* (Fig. 2) did not contain strong stem-loop structures

characteristic of [*rho*]-independent transcriptional terminators, complementation data (see below) suggest that ORF1 is probably not part of an *ntrA* operon. Nevertheless, we have observed a similar ORF upstream of the *K. pneumoniae ntrA* gene (unpublished data). The sequence of ORF1 and the phenotypes of strains containing ORF1 mutations will be published elsewhere.

The *R. meliloti* 3.5-kb *Bam*HI-*Eco*RI fragment also contained a third ORF (ORF3; Fig. 1) that was initiated downstream of *ntrA* and that continued through the *Eco*RI site. Two possible initiator ATG codons, one 98 bp and the other 251 bp downstream of the *R. meliloti ntrA* termination codon, could be identified; but only the latter was preceded by a potential ribosome-binding site (GAGGA) (Fig. 2). Merrick and Gibbins (27) also have identified a potential ORF that is initiated 23 bp downstream of *ntrA* in *K. pneumoniae*, and the available 75 amino acids of this ORF share weak but possibly significant homology (3.95 SD units by ALIGN analysis; data not shown) with the N-terminal 77 amino acids of ORF3, translated from the initiation codon 251 bp downstream of *R. meliloti ntrA*. Similarly to ORF1, complementation data (see below) indicated that ORF3 does not code for either an essential *R. meliloti* product or a product that is required for an $NtrA^+$ phenotype, assuming that Tn*5* causes polar mutations in *R. meliloti*.

Complementation analysis of the *ntrA* mutants. To determine whether the *R. meliloti ntrA* gene is part of an operon containing ORF1, various DNA fragments that contained *ntrA* with or without ORF1 were tested for their ability to complement strain 1681 (*ntrA2*::Tn*5*). To isolate such fragments, we took advantage of the nested deletions that were constructed for sequence determination. The replicative forms of M13 phage containing the deleted fragments were purified, and the fragments were excised with *Hin*dIII. (Each replicative form plasmid contained an *Hin*dIII site from the M13 mp18 polylinker 5′ of the fragment, and the *Hin*dIII site within the fragment 3′ of the *ntrA* gene.) The *Hin*dIII fragments were then cloned in each orientation into pWB5A, a low-copy-number plasmid derived from pRK290 (Table 1), and pSUP106, a moderate-copy-number plasmid derived from RSF1010 (35), and the ability of the resultant plasmids to complement the succinate-negative and nitrate-negative phenotypes of strain 1681 was determined.

The four fragments (F1 to F4) that were used are shown in Fig. 1; only the smallest fragment, F4, which was deleted within the *ntrA* gene, was unable to complement strain 1681. The next smallest fragment, F3, which contained none of the upstream gene and only 125 bp preceding the *ntrA* gene, complemented strain 1681 to wild-type growth rates. To eliminate the possibility that the observed complementation was due to readthrough from vector promoters that have not been described previously, the omega fragment which carries strong transcriptional stop signals at each end (13, 34) was inserted as a *Bam*HI fragment at the vector-insert junction upstream of *ntrA* in a pWB5A-based plasmid carrying F3 and in pSUP106-based plasmids carrying F3 in each orientation. The ability of the plasmids to complement strain 1681 was unaffected in each case.

***R. meliloti ntrA* is constitutively expressed.** The fragments in pSUP106 were cloned into the *Hin*dIII site within the *tet* gene, which is derived from pACYC184 (35). Insertion of a DNA fragment in this site disrupts the promoter of the *tet* gene but leaves the coding sequence intact (25). Therefore, a promoter within the inserted fragment that reads through into the *tet* gene can cause expression of tetracycline resistance. *E. coli* MM294 derivatives containing pSUP106 with F1, F2, F3, or F4 in either orientation were found to be tetracycline sensitive; however, *R. meliloti* 1681 derivatives containing pSUP106 with F1, F2, or F3 in the orientation such that *ntrA* was transcribed in the same direction as *tet* were tetracycline resistant, even when cultured on LB medium. The pSUP106 derivative containing the omega fragment upstream of F3 also conferred tetracycline resistance. Examination of the nucleotide sequences of the *tet* gene (25) indicates that ORF3 is terminated immediately after the *Hin*dIII site in these constructions (sequence was in frame with AAG CTT TAA). *R. meliloti* strains containing pSUP106 with F4 in either orientation or F1, F2, or F3 in the orientation such that *ntrA* was transcribed in the opposite direction to that of *tet* were tetracycline sensitive. These results suggest that the *R. meliloti ntrA* promoter is not expressed in *E. coli* but is constitutively expressed in *R. meliloti* and reads through ORF3.

```
RMNTRA   1 MALSASLHLRQSQSLVMTPQLMQSIQLLQMNHLELSHFIAQEVEKNPLLEVQPAD
KPNTRA   1   MKQGLQLRLSQQLAMTPQLQQAIRLLQLSTLELQQELQQALDSNPLLE-QTDL
Common         L LR SQ L MTPQL Q I LLQ  LEL    Q   NPLLE Q

RMNTRA  56 EPTISDREDAGPHPAETGGETDEAAGQQSDLYDSAMSRSGERLSEGLDADFANVFP
KPNTRA  53 HDEVETKEAEDRESLDTVDALEQKEMPEEL----------PLDASMDEIYT
Common        E       T          L             LDA

RMNTRA 111 DDTAPQRADAPELLGQMKSMPGAGDAEGYDLDDFV----GGRKTLRETLAEQLPF
KPNTRA  94 AGTPS----------------GNGVDYQDDELPVYQGETTQSLQDYLMMQVEL
Common      T                  G   Y D          L  L Q

RMNTRA 162 ALSAVSDRLIARYFIDQLDDAGYLHADLAETAETLGAAG---EDVARVLHVLQQF
KPNTRA 131 TPFTDTDRAIATSIVDAVDDTGYLTISVEDIVESIQDDEIGLEEVEAVLKRIQRF
Common        DR IA   D DD GYL      E G     E V VL  Q F

RMNTRA 214 DPPGVFARTLGECLAIQL----RARNRLDPAMEALVANLELLARRDFASLKKICG
KPNTRA 186 DPVGVAAKDLRDCLLVQLSQFAKETPMIEEARLIISDHLDLLANHDFRSLMRVTR
Common    DP GV A  L  CL  QL          A      L LLA  DF SL

RMNTRA 265 VDEEDLIDMLAEIRKLDPKPGTSFETGVFEAIIPDVVVRAAPDGGMFLVELNPDAL
KPNTRA 241 LKEEVLKEAVNLIQSLDPRPGQSIQTGEPEYVIPDVLVRKVNDR-MVVELNSDSL
Common      EE L     I  LDP PG S  TG  E  IPDV VR   D  V VELN D L

RMNTRA 320 PRVLVNHDYFTEISRSSRKNSGEQAFLNECLQNANMLTRSLDQRARTIMKVASEI
KPNTRA 295 PRLKINQQY-AAMGNSTRNDADGQ-FIRSNLQEARMLIKSLESRNDTLLRVSRCI
Common    PR  N Y     S R    Q F   LQ A VL  SL  R  T   V  I

RMNTRA 375 VRQQDAFLIHGVGHLRPLNLRIVADAIKMHESTVSRVTSNKYMLTPRGLFELKYF
KPNTRA 348 VEQQQAFFEQGEEFMKPMVLADIAQAVEMHESTISRVTTQKYLHSPRGIFELKYF
Common    V QQ AF  G    P  L  A A  MHEST SRVT  KY  PRG FELKYF

RMNTRA 430 FTVSIGSAENGDAHSAESVRHRIRTMINQESADAVLSDDDIVDVLKQAGVDIARR
KPNTRA 403 FSSHVNTEGGGEA-SSTAIRALVKKLIAAENPAKPLSDSKLTTMLSDQGIMVARR
Common    F        G A S   R     I E    LSD     L  Q  ARR

RMNTRA 485 TVAKYREAMSIPSSVQRRREKRALPRPRDSERCRQAASA*
KPNTRA 457 TVAKYRESLSIPPSNQRKQLV*
Common    TVAKYRE  SIP S QR
```

FIG. 3. Optimum alignment of *ntrA* gene products from *R. meliloti* (RMNTRA) and *K. pneumoniae* (KPNTRA) (27). The alignment was determined by using the program ALIGN, as described in the text.

DISCUSSION

We demonstrated that *R. meliloti* contains an *ntrA*-like gene, the product of which is required for at least three physiological processes: (i) growth on potassium nitrate as the sole nitrogen source, (ii) the activation of *nif* operons in the symbiotic state, and (iii) the activation of the C_4-dicarboxylate transport gene *dctA* in response to C_4-

dicarboxylates. In each case, *R. meliloti* NtrA acts in conjunction with another transcriptional activator: NtrC, NifA, and DctD, respectively (36, 44, 45; Ronson et al., in preparation). All three of these activators share strong homology in their central regions (4, 44; Ronson et al., in preparation), suggesting that this region is involved in the interaction with the *ntrA* product. In addition, the operons that they activate contain the NtrA-consensus promoter sequence, which is consistent with the finding that NtrA is a sigma factor (17, 19). These observations suggest that the presence of the NtrA-consensus promoter sequence is diagnostic of an operon that requires NtrA for activation and that the presence of the conserved central domain in a regulatory protein is predictive of a system that requires NtrA as a coactivator.

The *ntrA* gene was initially identified on the basis of its role in the activation of nitrogen-regulated operons (15). However, only DctA and not DctD is required for symbiotic nitrogen fixation (Ronson et al., in preparation), suggesting that *dctD* has not evolved specifically to take advantage of the availability of NtrA in the nodule. Instead, it seems likely that *ntrA* has a broader physiological role than originally envisaged. Consistent with this idea, NtrA-like promoter sequences have been observed in the *xylA* (20), carboxypeptidase G2 (30), and pilin (21) genes from *Pseudomonas* species; and *ntrA* is required for the *xylR*-mediated activation of *xylA* in *E. coli* (10). Furthermore, the conserved portion of *nifA* from *K. pneumoniae* hybridizes to multiple restriction fragments of *Rhizobium* sp. strain ORS571 (32a) and to multiple restriction fragments of *Bradyrhizobium japonicum*, *R. meliloti*, and *E. coli* DNA (T. Adams, Ph.D. thesis, Michigan State Unversity, East Lansing, 1986). Finally, we observed six strongly hybridizing bands and four weakly hybridizing bands in *Bradyrhizobium* sp. (*Parasponia*) DNA that were probed with a 17-bp oligonucleotide designed from a conserved portion of the central region of *nifA*, *ntrC*, and *dctD* genes (unpublished data). Taken together, these results suggest that there may be several additional regulatory genes that require NtrA as a coactivator.

It is not clear why genes such as *ntrC*, *nifA*, and *dctD* have evolved to use NtrA rather than the *rpoD* product σ^{70} for transcriptional initiation; however, the metabolic pathways that these genes regulate are not essential for growth or viability. *ntrA* mutants of *R. meliloti* grow as well as the wild type in defined medium with glucose as the carbon source and ammonium chloride as the nitrogen source.

Results of comparisons of nucleotide sequences surrounding the *ntrA* gene in *R. meliloti* (Fig. 2) and *K. pneumoniae* (27) suggest that genes that are transcribed in the same direction and possibly in the same operon as *ntrA* are present upstream (ORF1) and downstream (ORF3) of the *ntrA* gene in both species (Fig. 1 and 2; unpublished data). DNA fragments containing only the *R. meliloti ntrA* gene, however, were able to complement the *R. meliloti ntrA* mutants, suggesting that the *ntrA* promoter lies within 125 bp of the initiation codon of the *ntrA* gene. Nevertheless, our data do not exclude the possibility that transcription through ORF1 reads through *ntrA*, and the strong conservation in sequence and location of ORF1 between *R. meliloti* and *K. pneumoniae* suggests that ORF1 may play a role in *ntrA* product function. Furthermore, our data suggest that transcription from the *ntrA* promoter reads through ORF3. We are currently mapping the transcriptional initiation and termination sites in the *ntrA* region and isolating mutants in ORF1 and ORF3 in an attempt to determine their function.

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