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Title: Purification and Characterization of R. meliloti RNA Polymerase from Wild Type and Mutant Strains, and the Role of the Enzyme in the Specificity of *in vitro* Transcription of Nitrogen Fixation Genes

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Lyle R. Brown

The RNA polymerase from free-living cells of Rhizobium meliloti was purified, and the subunit structure of the enzyme was found to be similar, but not identical, to the RNA polymerase from other gram-negative bacteria. The molecular weights of the enzyme subunits are: β', 155,000; β, 151,000; σ, 93,000; and α, 43,000. In addition, a polypeptide with a molecular weight of 29,000, which was designated τ, was found associated with both core and holoenzyme. The stoichiometry of the purified holoenzyme was found to be $2\alpha:1\beta:1\beta':0.7\sigma:1\tau$. The 93,000 molecular weight subunit was positively identified as the σ subunit by its ability to stimulate transcription when added to purified core enzyme, which is characteristic of σ subunit function.

The identification of the 151,000 molecular weight protein as the β subunit, which is defined as the subunit that binds rifampicin, was determined by reconstitution assays using different combinations of subunits purified from wild type and rifampicin resistant RNA polymerases. The purified HYP9 rifampicin resistant RNA polymerase used for this analysis showed enhanced activity on poly(dAdT) and plasmid pRK290 templates in the presence of rifampicin, while the addition of rifampicin to partially purified RNA polymerase from this strain showed a reduction in activity to 70% of the level when no drug was present. A 65,000 molecular weight protein isolated from the partially purified enzyme was found to be responsible for this lower level of rifampicin resistance.

The HYP9 RNA polymerase was found to stimulate transcription from the nifH region of the nifHDK operon, as analyzed by hybridization of in vitro transcripts prepared from a supercoiled plasmid template to Southern blots. The addition of a partially purified bacteroid protein extract containing the R. meliloti nifA-like regulatory protein stimulated incorporation of activity by the purified RNA polymerase on linear DNA templates containing the nifP1 or nifP3 promoters. The increased incorporation of activity on the nifP3 promoter template was identified as a specific transcript of expected length by analysis of the run-off transcript generated from this linear template. This specific nifA-activated expression was only observed when the template contained the entire 160 base pair conserved nif gene promoter region upstream of the transcription start site.

Purification and Characterization of *R. meliloti* RNA Polymerase from Wild Type and Mutant Strains, and the Role of the Enzyme in the Specificity of *in vitro* Transcription of Nitrogen Fixation Genes

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for Marty,
Peter, and Daniel

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INTRODUCTION

The symbiotic relationship between bacteria of the genus Rhizobium and leguminous plants leads to nitrogen fixation, and is of great agricultural significance. The bacteria carry the genetic information for nitrogen fixation (nif genes) on very large plasmids, but the majority of species are unable to transcribe the genes for the nitrogenase enzyme under normal free-living conditions. Expression requires infection of the host plant roots by the bacteria, and the development of nodules on the plant roots within which differentiated forms of the bacteria, termed bacteroids, are produced. It is within these nodules that bacteroids express the nitrogen fixation genes, leading to the reduction of atmospheric nitrogen to ammonia, which can then be utilized by the bacteroids or exported to the plant (Verma and Long 1983). The mechanism by which transcription of the nif genes is activiated in Rhizobium bacteroids is not known, although many comparisons have been made between this system and the expression of nitrogen fixation in the free-living bacteria Klebsiella pneumoniae. K. pneumoniae fixes nitrogen anaerobically under nitrogen limiting conditions. Much of the understanding of the genetics and regulation of nitrogen fixation was determined in studies with this organism. In the model for control of nitrogen fixation in K. pneumoniae, the products of a nif regulatory gene (nifA) and a general nitrogen metabolism regulatory gene (glnF)

are both required for activation of transcription of the other 15 nif genes arranged in 6 or 7 transcription units (Ausubel 1984; Dixon 1984). Although there are similarities, and a nifA-like regulatory gene has been identified in R. meliloti, it is not certain whether the same control mechanism is involved in Rhizobium, where nitrogen fixation is expressed only during symbiosis, rather than being activated by nitrogen limitation as in K. pneumoniae.

The work in this thesis was initiated in order to gain a better understanding of the molecular basis of nitrogen fixation (nif) gene control in Rhizobium meliloti. To do this, the RNA polymerase from free-living R. meliloti cells was purified and its subunit composition characterized. The stoichiometry of the enzyme and the positive identification of the σ subunit were determined.

Purification of RNA polymerase from a rifampicin resistant R. meliloti strain, which gives a 20% higher plant yield on alfalfa plants inoculated with it, was also carried out. Reconstitution assays using individual RNA polymerase subunits from this strain and from the wild-type strain were performed to identify the subunit responsible for rifampicin resistance. Purified RNA polymerase from this strain actually showed a stimulation of polymerizing activity in the presence of rifampicin, while partially purified enzyme fractions showed partial sensitivity to the drug. Further characterization of this mutant RNA polymerase was carried out to identify a protein in the partially purified enzyme fraction which is responsible for this rifampicin sensitivity.

R. meliloti bacteroids were purified from alfalfa nodules, and bacteroid proteins were compared with RNA polymerase purified from

free-living cells on silver-stained polyacrylamide gels. Nodule proteins were also examined in this way.

Comparison of *in vitro* transcription by the purified mutant and wild type RNA polymerases was done by hybridization of mRNA probes prepared from a plasmid template containing *R. meliloti nif* genes to Southern blots of restriction-digested plasmid DNA. Analysis of specific transcription from *nif* gene promoters was carried out by measuring *in vitro* run-off transcripts on formaldehyde-denaturing agarose gels. The activation of *nif* gene transcription by the addition of a partially purified bacteroid protein extract containing the *R. meliloti nifA*-like regulatory protein was examined.

REVIEW OF LITERATURE

Rhizobium is a genus of aerobic, gram-negative bacteria which carries genetic information for the enzymatic reduction of atmospheric dinitrogen to ammonia (Corbin et al. 1982; 1983), a process termed nitrogen fixation. Although in some slow-growing species of Rhizobium nitrogen fixation can be induced in free-living cells under microaerophilic conditions (Keister 1975; Kurz et al. 1975; Pagan et al. 1975; Kaneshiro et al. 1978), the nitrogenase enzyme is normally only produced during symbiosis with a host legume plant (Verma and Long 1983). The bacteria interacts with the host plant via the root hairs, and induces the plant to form nodules on its roots. A differentiated form of the bacteria, termed bacteroids, develops within the nodules, and it is within the bacteroids that nitrogen fixation occurs (Burns and Hardy 1975; Verma and Long 1983). Each Rhizobium species is specific for a limited host range, and the species associated with many different legumes have been isolated and characterized (Beringer et al. 1979). The species associated with alfalfa is Rhizobium meliloti; with soybean, R. japonicum (recently assigned a new genus name, Bradyrhizobium (Jordan 1982)); and with peas, R. leguminosarum. Although other bacteria, such as certain species of Klebsiella (MacNeil et al. 1978), Enterobacter (Singh et al. 1983), and Rhodopseudomonas (Arp and Zumft 1983), and blue-green algae, such as Anabaena (Tumer et al. 1983), also carry genetic information for nitrogen fixation, Rhizobium is unique in its ability to establish an endosymbiotic relationship with legume plants.

The specificity of the Rhizobium-legume interaction suggests that a mechanism for recognition between the plant and the bacteria exists. A model has been proposed by several authors which involves recognition of carbohydrates on the bacterial surface by specific sugar-binding proteins, called lectins, which are located in the plant cell walls (see Vance 1983). Haptens of soybean and clover lectins blocked attachment of bacteria to specific host root hairs, presumably by competing for binding sites on the bacterial cell surface (Dazzo et al. 1975; Stacey et al. 1980). However, other evidence indicates that lectins may not be the determinants of specificity. Soybean lectin has been shown to bind heterologous strains of Rhizobium (Chen and Phillips 1976), and soybean lines which lack detectable soybean lectin still nodulate normally (Su et al. 1980). Although it is not clear how the plant specifically recognizes the proper Rhizobium strain, and if lectins are involved, additional work suggests that bacterial exopolysaccharides may be involved as elicitors of nodulation specificity (see Vance 1983; Sander et al. 1978; Dazzo 1981).

After recognition and binding of the bacteria to the root hairs, the infection process begins. This process involves several steps, including (1) root hair curling, (2) penetration of cortical cells by an infection thread of bacteria, (3) induction of meristematic activity resulting in nodule formation on the plant roots, (4) localization of bacteria within the nodules, and (5) differentiation of the bacteria into the enlarged and osmotically sensitive bacteroid

form (Verma and Long 1983; Sutton et al. 1984). Both plant and bacterial mutants have been isolated which block this process at various stages (Bergersen and Nutman 1957; Nutman 1968; Maier and Brill 1976; Vance et al. 1980; 1983; Kondorosi et al. 1984).

Several proteins unique to the nodules are coded for by the plant (Legocki and Verma 1979; 1980; Fuller et al. 1983; Halverson and Stacey 1984; Sutton et al. 1984). These proteins, termed nodulins, may include nodule structural proteins, enzymes required for assimilation into the plant of the fixed, reduced nitrogen, and proteins that support and facilitate bacteroid function and nitrogen reduction. Functions for the nodulins identified have not been established except for uricase (Legocki and Verma 1980), and for leghemoglobin, which comprises up to 40% of the total protein in effective soybean nodules (Verma et al. 1974; 1981). Leghemoglobin is an oxygen binding protein, and protects the oxygen-labile nitrogenase enzyme produced in the bacteroids, while allowing sufficient oxygen for oxidative phosphorylation to penetrate through the nodule into the bacteroid. Enzymes for producing the heme prosthetic group of leghemoglobin are produced by the bacteria (Leong et al. 1982), while the apoleghemoglobin is coded for by the plant (Verma et al. 1974).

The benefit of this infection of legume plants by specific Rhizobium species is the enzymatic reduction of atmospheric nitrogen to ammonia by the bacteroids within the root nodules, with the fixed ammonia then becoming available for use by the plant. The enzyme nitrogenase is responsible for the conversion of atmospheric nitrogen

to ammonia, according to the reaction:

$$N_2 + 8H^+ + 8e^- + nATP \rightleftarrows 2NH_3 + H_2 + nADP + nP_i$$

The level of nitrogen fixation is limited by the energy supply, and the number of moles of ATP required for reduction of one mole of N_2 is estimated to be between 22 and 70 (Pate et al. 1981). Along with reducing nitrogen, nitrogenase also causes the reduction of protons to hydrogen gas, which wastes electrons and adds to the ATP cost of nitrogen fixation (Schubert and Evans 1976). R. japonicum and some other species of Rhizobium, but not R. meliloti, have an uptake hydrogenase enzyme which recycles hydrogen gas to protons to regenerate energy and electrons (Carter et al. 1978). The presence of this enzyme system in Rhizobium has been found to increase nodule efficiency in symbiotic nitrogen fixation (Albrecht et al. 1979; Hanus et al. 1981).

Ammonia produced by nitrogen fixation can be utilized by the enzymes glutamine synthetase and glutamate synthase, which catalyze the respective reactions:

$$\text{glutamate} + NH_3 + ATP \rightarrow \text{glutamine} + ADP + P_i$$

$$\text{glutamine} + \alpha\text{ketoglutarate} \rightarrow 2\ \text{glutamate}$$

or by the enzyme glutamate dehydrogenase, which requires high ammonia levels and catalyzes the reaction:

$$\alpha\text{ketoglutarate} + NADPH + NH_3 \rightleftarrows \text{glutamate} + NADP^+$$

The glutamine synthetase/glutamate synthase pathway is the most likely mechanism by which ammonia produced by symbiotic nitrogen fixation is processed, as a ^{13}N label is initially taken up into glutamine in nodules, but is then quickly processed into glutamate (Meeks et al. 1978). In addition, levels of these two enzymes rise

during nodule development (Robertson et al. 1975; Groat and Vance 1981). The determination of the enzyme(s) involved is complicated by the presence of two forms of glutamine synthetase in Rhizobium (Ludwig 1980). The first is similar to the enteric form and is inactivated by adenylylation in the presence of ammonia, while the second form is heat-labile and does not undergo adenylylation (Magasanik 1982). It is not yet clear which form is involved in symbiotic nitrogen fixation.

Messenger RNA transcripts of the nitrogen fixation (nif) genes are first detected when rhizobia are within the infection thread (Paau and Brill 1982), and are most abundant in 6-8 week old root nodules. The synthesis of nitrogenase structural proteins also occurs early after infection (Van den Bos et al. 1983). The level of glutamine synthetase and glutamate dehydrogenase appears to be reduced in bacteroids (Brown and Dilworth 1975), and greater than 90% of the ammonia produced is excreted through the bacteroid membrane (Bergersen and Turner1967; 1978; Evan and Crist 1984). This NH_3 is then assimilated by enzymes of the plant system. Ureides are the primary nitrogen-containing compounds exported from soybean nodules (Matsumoto et al. 1977), while other legumes export fixed nitrogen as asparagine or glutamine (Pate 1976).

The nitrogenase enzyme is composed of two components; component I is a molybdenum-iron protein which binds the reducible substrate, and component II is an iron protein, which transfers electrons to component I. Component I is composed of two subunits, α and β, which are the products of the nifK and D genes, and component II is the product of the nifH gene. The nifHDK genes of several bacterial

nitrogen-fixing species have been found to be highly conserved at the DNA sequence level (Ruvkun and Ausubel 1981). The nifHDK structural genes of R. meliloti are expressed in a single transcriptional unit (Ruvkun and Ausubel 1980), while in R. japonicum these genes are transcribed in two separate units, nifH and nifDK, with the two operons being separated by DNA not required for nitrogen fixation (Fuhrman and Hennecke 1982; 1983; Kaluza et al. 1983). In R. meliloti and other Rhizobium species the genes involved in the establishment of symbiosis (sym or nod genes) and nitrogen fixation (nif genes) are closely linked and have been localized on very large plasmids (Rosenburg et al. 1981; Long et al. 1982). In R. meliloti, this 'mega'plasmid has a molecular weight of greater than 450 megadaltons, as estimated by gel electrophoresis (Rosenburg et al. 1981; 1982). Recently, electron microscopy was used to measure the length of this plasmid, which was determined to be 0.48 mm (Burkhardt and Burkhardt 1984). This corresponds to a molecular weight of approximately 1×10^9, which is about one-third the size of the E. coli chromosome. Other Rhizobium species also contain large plasmids, and strains cured of these plasmids lose the ablility to nodulate their hosts and to fix nitrogen (Zurkowski and Lorkiewicz 1978; Banfalvi et al. 1981; Morrison et al. 1983). Transposon mutagenesis into this large plasmid in R. meliloti has led to the localization of the nitrogenase structural gene operon (nifHDK), as well as two other nif gene operons that specify functions essential for nitrogen fixation but not nodule formation (Corbin et al. 1982; Zimmerman et al. 1983; Downie et al. 1983). Genes required for the establishment of symbiosis (nod genes) have also been localized on this

large plasmid (Banfalvi et al. 1983; Julliot et al. 1984; Buikema et al. 1983; Kondorosi et al. 1984). One gene cluster of 2.5-3.0 kilobases (Kb) located 20 Kb downstream from the nifHDK promoter carries genes involved in root hair curling, and another cluster contains 2 Kb and 1 Kb segments, separated by a 1 Kb region non-essential for nodulation (Kondorosi et al. 1984). The genes in this second cluster are possibly involved in the host specificity of nodulation.

Using transposon Tn5 mutagenesis in R. meliloti, a regulatory gene region was found in a 1.8 Kb region 5.5 Kb upstream from the nifH::lacZ fusion during symbiotic growth, and this region is also required for the expression of an additional nif operon located upstream of the nifHDK operon (Szeto et al. 1984). This regulatory gene region of R. meliloti was found to hybridize to the E. coli glnG gene, and to a lesser extent to the nifA regulatory gene of K. pneumoniae. Nuclease protection experiments suggested that this regulatory gene region has its own promoter, separate from the adjacent upstream nif operon. This regulatory region is sufficient to code for a protein with a molecular weight up to 60,000, which is similar to the size of the 57,000 dalton nifA protein of K. pneumoniae (Ausubel and Cannon 1981).

Transconjugation of one of the large plasmids from R. leguminosarum to R. phaseoli led to the ability of the R. phaseoli transconjugant to nodulate peas, the normal host of R. leguminosarum (Beynon et al. 1980), providing further evidence that plasmid-encoded genes are involved in host plant recognition and nodulation. This transfer of nodulating ability has also been shown to occur at high

frequency between other species of Rhizobium (Johnston et al. 1978; Beynon et al. 1980; Torok and Kondorosi 1981; Lamb et al. 1982). However, transfer of a large plasmid from R. loti to other Rhizobium species did not transfer the ability to nodulate the host Lotus plant to the recipient, suggesting that in this species the genes necessary for nodulation are present on a smaller plasmid or on the chromosome (Pankhurst et al. 1983). Recently, several laboratories have transferred the megaplasmid from R. meliloti into Agrobacterium tumefaciens and E. coli, resulting in the ability of these recipients to form pseudo-nodules on alfalfa but not other legumes (Hirsch et al. 1984; Banfalvi et al. 1983; Truchet et al. 1984). Nodules induced by the E. coli transconjugant were completely devoid of bacteria, and in A. tumefaciens-induced pseudonodules, some bacteria were seen but no bacteroid differentiation occurred. No nitrogen fixation occurred with any of these transconjugants.

The enteric bacteria Klebsiella pneumoniae is the best understood nitrogen-fixing organism because a genetic transfer system has been developed for genetic analysis. This organism fixes nitrogen at low oxygen tension in free-living cells when grown under fixed nitrogen-limiting conditions (Riedel et al. 1979). Seventeen closely linked genes located in 7 or 8 operons have been identified as being required for nitrogen fixation in K. pneumoniae (Beynon et al. 1983). Besides the nifHDK structural genes, the other genes are involved in cofactor synthesis and processing, and in electron transport from pyruvate to nitrogenase (Brill 1980; Ow et al. 1983). The regulation of nif gene expression in K. pneumoniae is controlled by the products of the nifLA operon. The nifL gene product causes repression of nif

gene expression in the presence of oxygen or ammonia (Merrick et al. 1982). The nifA gene product is a transcription activator required for expression of all the nif operons except its own (Buchanan-Wollaston et al. 1981; Ow et al. 1983). Constitutive expression of nifA in the absence of nifL results in the loss of nif gene expression sensitivity to oxygen and ammonia (Zhu et al. 1983).

In Klebsiella and other enteric bacteria, genes involved in nitrogen assimilation have been shown to be under the control of a central regulatory system, involving the products of 3 genes, glnF, glnG, and glnL (Magasanik 1982; Ausubel 1984). These genes have also been designated by some authors as ntrA, ntrC, and ntrB, respectively, because of their central role in nitrogen regulation (Merrick et al. 1982; Ow and Ausubel 1983), although this designation has not been totally accepted. In this thesis, the gln designation will be used. Under nitrogen limiting conditions, the glnF and glnG products act together to activate various nitrogen catabolism genes, such as the proline and histidine utilization genes. The glnL product acts along with the glnG product to repress the transcription of these same genes under conditions of nitrogen excess. This regulatory system also regulates expression of the nifLA operon of K. pneumoniae, as determined by introducing a plasmid carrying the nifLA operon promoter fused with the β-galactosidase (lacZ) gene into E. coli strains of various Gln phenotypes. Only when both glnG and glnF were present in the host was expression of β-galactosidase from the nifLA promoter observed (Drummond et al. 1983). The R. meliloti nifH::lacZ fusion and the K. pneumoniae nifH::lacZ fusion could both be activated by K. pneumoniae nifA in the presence of glnF (Ow and Ausubel 1983;

Buchanan-Wollaston et al. 1981; Sundaresan et al. 1983), suggesting that the nif genes are regulated similarly in both organisms. The K. pneumoniae nifA protein can replace glnG in activating genes normally requiring glnG (Ow and Ausubel 1983), so these genes may be evolutionarily related. However, the glnG product cannot replace nifA in activating the K. pneumoniae nifH::lacZ fusion (Ow et al. 1983).

Because of the possibility for similar control of nif gene expression, the promoters of the nif operons have been examined in both K. pneumoniae and R. meliloti. The promoters of 5 nif operons from K. pneumoniae have been sequenced, and conserved sequences at regions -26 to -23 (CTGG) and -14 to -10 (TTGCA) upstream of the transcription start site, with the first base incorporated being designated as +1, were found (Beynon et al. 1983; Ausubel 1984). These promoters also showed homology at the -70 region (Sundaresan et al. 1983), but not at the -10 and -35 regions that have been identified as contact points for RNA polymerase binding in promoters of gram-negative bacteria (Rosenberg and Court 1979). The -14 TTGCA sequence is also conserved among several other promoters regulated by glnG and nifA, such as glnA, argTr, and dhuA (Ow et al. 1983). Deletion of nucleotides greater than 150 bases upstream from the start site of nifLA transcription in K. pneumoniae resulted in a four-fold reduction in promoter activity, and this activity further decreased to 7% with deletions to -28. Promoter activity was totally abolished in deletions removing the -10 or -26 to -12 regions (Drummond et al. 1983). The same conserved sequences at -14 and -26 were also found in R. meliloti nif promoters (Brown and Ausubel 1984).

Three nif promoter regions have been identified in R. meliloti, and these promoters contain 75-85% sequence homology for 160 base pairs upstream from the transcription initiation site (Better et al. 1983). This entire 160 base region appears to be required for maximal activation of transcription (Better et al. 1984).

Despite these similarities between the structure and the regulation of the nif genes in K. pneumoniae and R. meliloti, there is some evidence that the regulation of nif gene expression is not identical in these two organisms. K. pneumoniae nif gene expression is inhibited by ammonia, and this control involved the gln nitrogen assimilation regulatory system as described earlier (Magasanik 1982; Ausubel 1984). The addition of 25 mM NH_4^+ to nitrogen-fixing R. leguminosarum bacteroids isolated from nodules did not repress nifHDK gene expression (Van den Bos et al. 1983), suggesting that the gln regulatory system may not be involved in Rhizobium nif gene expression, although this is not yet clear. The mechanism(s) by which the nifA regulatory protein of these two organisms enables RNA polymerase to recognize and initiate transcription from the unique nif gene promoters is not known.

The RNA polymerase from bacteria has the general form $\alpha_2\beta\beta'\sigma$ (Burgess 1969), and initiates transcription from specific promoter sites on the DNA. The promoters contain signals that direct the proper hydrogen bonding of RNA polymerase to the double-stranded DNA, via the major or minor grooves. This closed promoter-enzyme complex is then converted to the open form with local unwinding of the DNA, to enable the RNA polymerase to initiate synthesis of nascent RNA from the correct (sense) strand of the DNA. The sites for RNA

polymerase interaction with the promoters on the DNA have been localized to regions 10 and 35 bases upstream from the start site of transcription (Rosenberg and Court 1979). The DNA sequence of these regions in bacterial gene promoters have been highly conserved, and a consensus promoter sequence of E. coli genes has been compiled from a comparison of 112 promoters (Hawley and McClure 1983). The relative transcriptional activity of various promoters is referred to as promoter strength, and can be predicted by the degree of homology of any promoter with the consensus promoter sequence (Mulligan et al. 1984). The level of initiation from specific promoters can be altered by accessory proteins which may bind to the DNA adjacent to promoter sites, or directly to the RNA polymerase, to modify transcription initiation.

The RNA polymerase utilizes ribonucleoside triphosphates as substrate to transfer ribonucleoside monophosphates to the 3'-OH terminus of a growing RNA chain, incorporating bases complementary to the DNA template until a transcription termination site in the DNA sequence is reached, when the RNA polymerase, DNA, and nascent RNA dissociate (see von Hippel et al. 1984). The σ subunit is responsible for the specificity of the interaction of the enzyme with the DNA, and σ subunit mutants show distinct differences in transcriptional specificity (Nomura et al. 1984). The β' subunit is involved in binding the DNA, while the β subunit binds the nucleotide substrates and transcription inhibitors such as rifampicin. The α subunit appears to be involved in maintaining the correct association of the β and β' subunits (for reviews, see Yura and Ishihama 1979; von Hippel et al. 1984). Genetic studies indicate that β and β' subunit mutations alter the expression

of several genes, including those involved in metabolism (Uzan and Danchin 1978), DNA replication (Rasmussen et al. 1983), and phage growth (Snyder 1972). Kajitani and Ishihama (1984) have developed an *in vitro* mixed transcription system, in which mixtures of truncated DNA templates obtained by restriction endonuclease digestion, each carrying specific promoter(s), are transcribed simultaneously in the same reaction and the specific run-off transcripts are analyzed by gel electrophoresis and autoradiography. Using this system, they observed altered promoter specificity of selected *E. coli* ribosomal RNA and protein gene promoters by several RNA polymerase β and β′ subunit mutants, suggesting that not only the σ subunit, but also the β and β′ subunits are involved in promoter recognition (Nomura et al. 1984).

Several mechanisms of transcriptional control involving RNA polymerase modifications by accessory proteins have been described in *E. coli*, *Bacillus subtilis*, and other bacteria (Yura and Ishihama 1979). When *E. coli* is infected with T4 phage, the phage induces the synthesis of several proteins that bind specifically to the host RNA polymerase and alter its transcription specificity so that only phage genes are transcribed (Stevens 1972). Several *E. coli* metabolic genes such as *lac*, *gal*, and *ara* are positively regulated by the interaction of cAMP with the catabolite receptor protein at specific upstream sequences of the genes (Gilbert 1976; Rosenburg and Court 1979). This binding site involves bases from 54 to 71 bases before the start site of transcription, as determined by analyzing deletion mutants (Yu and Reznikoff 1984). Other genes are negatively controlled by repressors, such as the *lac* repressor, which bind to the gene to

prevent effective interaction of the RNA polymerase with the promoter until an inducer of the operon displaces the repressor (Rosenburg and Court 1979).

Nucleotide factors such as tRNA and ppGpp appear to be involved in altering the promoter specificity of the RNA polymerase (Travers 1981). Ammonia or carbon source limitation are causes of the stringent response in E. coli, during which cellular metabolism is altered, total RNA and protein synthesis decreases, and ppGpp is produced (Gallant 1979). In an in vitro mixed transcription system, the addition of ppGpp inhibited in vitro transcription of ribosomal RNA and ribosomal protein genes (Kajitani and Ishihama 1984). Other metabolic genes such as lac and trp were not affected, while transcription from the recA promoter was enhanced. This inhibition occurred even after initial RNA polymerase binding to the promoter, suggesting that ppGpp may destabilize RNA polymerase complexes at stringently controlled promoters.

Another method of transcriptional control used in the genus Bacillus involves multiple σ subunits, with at least 5 different σ subunits identified in B. subtilis (Doi 1982). These subunits are produced at various stages of growth, and while some catalyze recognition of the same consensus promoter sequence as E. coli RNA polymerase, others recognize unique promoters of genes that are expressed only at certain stages of growth, such as sporulation (Goldfarb et al. 1983). Each of these σ subunits has a different promoter recognition sequence, with the differences localized at the -35 and -10 regions (Doi 1982; Johnson et al. 1983).

The mechanism by which transcription of the nif genes is acti-

vated in K. pneumoniae and Rhizobium bacteroids is not yet known, and as stated previously appears to be different between these organisms. A nifA activator protein is clearly involved, and may bind to a subunit of the bacterial RNA polymerase to cause a structural change, resulting in the ability of the enzyme to recognize and bind the nif gene promoters. A second possibility is that the nifA protein may bind specifically to the 160 bp conserved promoter region of the nif operons to facilitate RNA polymerase binding. Another possibility in bacteroids is that a separate RNA polymerase specific for nif gene transcription is produced during symbiosis. The presence of just the promoter region of the R. meliloti or K. pneumoniae nifH gene on a high copy number plasmid inhibits expression from this promoter region, possibly due to titration of the nifA activator protein (Brown and Ausubel 1984). This suggests that the nifA protein binds to the nifH promoter, and that the level of nifA may be limiting. Other factors may also be involved in optimal expression of the nif genes in bacteroids, and it is possible that coordinate control of genes expressed during symbiosis may occur.

There is some evidence that modifications of the RNA polymerase in certain Rhizobium strains may result in a selective ability of these strains to express nitrogenase activity in either free-living culture or during symbiosis. First, a small proportion of rifampicin-resistant mutants were able to nodulate their proper host plants, but no nitrogen fixation occurred in the nodules (Pankhurst 1977; Pain 1979). Since rifampicin binds the β subunit of RNA polymerase, this suggests that the mutations may have altered the RNA polymerase structure, affecting the ability of the enzyme to transcribe the nif

genes. Second, the expression of nitrogen fixation was inhibited much more than cellular growth when rifampicin was added to free-living nitrogen-fixing cultures of slow-growing rhizobia (Werner 1978). Third, slow-growing Rhizobium strains able to express nitrogenase in free-living culture were found to be much more resistant to rifampicin than strains unable to express nitrogenase under the same conditions (Pankhurst et al. 1982). Furthermore, a rifampicin-resistant mutant of R. meliloti which confers a 20% higher plant yield on alfalfa seedlings inoculated with it has been isolated in this laboratory.

To identify the factors and understand the mechanisms involved in the regulation of transcription of the nitrogen fixation genes in Rhizobium, the RNA polymerase needs to be purified and characterized. The RNA polymerase has been purified from R. japonicum (Regensburger and Hennecke 1983) and R. leguminosarum (Lotz et al. 1981), and from another member of the Rhizobiaceae family, Agrobacterium tumefaciens (Knopf 1974; Cardarelli et al. 1981). The RNA polymerase from each of these species has the same general form $\alpha_2\beta\beta'\sigma$ as the enzyme from other bacteria, and the molecular weights of the subunits are similar, but not identical, to the corresponding subunits of E. coli RNA polymerase. Putative identification of the σ subunit has been made for each of these enzymes, but a stimulation of transcription upon addition of σ to core enzyme characteristic of σ function has not been demonstrated. The enzyme from R. japonicum and R. leguminosarum were found to specifically bind to E. coli T7 phage early gene promoters by electron microscopy (Lotz et al. 1981) and nitrocellulose filter binding assays (Regensburger and Hennecke

1983). However, *in vitro* transcription analysis of the *nifH* gene with *R. japonicum* RNA polymerase showed no specific transcription of this gene (Regensburger and Hennecke 1983). No difference in subunit structure or size could be seen in two-dimensional gels of proteins from microaerophilic nitrogen-fixing cells of *R. japonicum* (Regensburger and Hennecke 1983), so it appears that the same form of RNA polymerase which functions in free-living cells also functions in nitrogen-fixing cells. The product of the *nifA* gene and possibly other bacterial and/or plant proteins produced during the establishment of symbiosis apparently are required for transcription of the *nif* genes. *In vitro* transcription analysis with purified RNA polymerase will allow the identification of the protein factors in nitrogen-fixing bacteroids required for *nif* gene expression. DNA- and protein-binding experiments with these factors will allow the determination of the mechanism by which these factors activate *nif* gene expression. Analysis of deletion, insertion, and base change mutations in the conserved 160 base pair upstream region of the *nif* genes should yield an understanding of what bases in this region are involved in *nif* gene activation.

MATERIALS AND METHODS

Strains and plasmids. Bacterial strains and plasmids used in this work are listed in Table 1. The HYP9 rifampicin-resistant mutant strain of R. meliloti was selected for its ability to increase plant yield when used as inoculum to infect alfalfa seedlings.

Chemicals and reagents. Formamide, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), morpholinopropanesulfonic acid (MOPS), bovine serum albumin (BSA), unlabelled ribonucleoside triphosphates, medium EEO agarose, and electrophoresis buffer reagents were obtained from Sigma. Formaldehyde was from Mallinckrodt. Electrophoresis-grade sodium dodecyl sulfate (SDS), acrylamide, bisacrylamide, tetramethylethylenediamine (TEMED), and protein molecular weight markers were from BioRad. Poly(dAdT) was purchased from Miles Laboratories, and [5,6-^{3}H]-UTP (35 Ci/mmole) was obtained from ICN. Genescreen hybridization transfer membrane and [α-^{32}P]-UTP (760 Ci/mmole) were purchased from New England Nuclear. Glass fiber prefilters (25 mm, Type AP20) and nitrocellulose sheets (0.45 μm pore size) were from Millipore. NACS prepac columns, low melting point agarose, nuclease-free bovine serum albumin (BSA), and human placental ribonuclease inhibitor were obtained from BRL. Restriction endonucleases were from either New England Biolabs or BRL. T4 DNA ligase was from New England Biolabs. DEAE-cellulose and phosphocellulose were obtained from Whatman. Yeast extract, tryptone, and bacto-agar were purchased from Difco. E. coli ribosomal RNA was the gift of Henry Schaup, and ^{32}P-labelled molecular weight standards were kindly provided by Gary Merrill. Other chemicals were reagent grade or

Table 1. Sources of strains, plasmids, and phage

Medicago sativa cv. Anchor		North American Plant Breeders, Ames, Iowa
Rhizobium meliloti RM41		F. O'Gara
Rhizobium meliloti HYP9		Rifampicin-resistant derivative of *R. meliloti* 104A13 obtained in this laboratory
Escherichia coli	HB101pBR322	J. Beaty
	HB101pSP19	C. Mathews (Purohit and Mathews 1984)
	HB101pDC2	G. Ditta (Corbin et al. 1983)
	HB101pDC4	G. Ditta (Corbin et al. 1983)
	HB101pRM23-2	S. Long (Friedman et al. 1982)
	HB101pRK290	F. Ausubel (Ditta et al. 1980)
	JM103	B.R.L.
Bacteriophage M13mp8		B.R.L.
Bacteriophage T3 DNA		J. Hughes

better, and all solutions were prepared in deionized, distilled water.

RNA polymerase inhibitors. Rifampicin was purchased from Calbiochem. Lipiarmycin was obtained from John Trowsdale. Streptolydigin was the gift of G. B. Whitfield of the Upjohn Co. Heparin was purchased from Sigma.

Growth of Bacteria. Rhizobium meliloti cells were grown at 32°C with vigorous aeration and stirring to late log-phase in 10 l of LRB media, which contains per liter 3 g yeast extract, 0.5 g $K_2HPO_4 \cdot 3H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, and 0.1 g NaCl, adjusted to pH 6.8 and supplemented with 1% mannitol. Ten minutes before harvesting, 100 ml of 0.05 M PMSF in 95% ethanol was added to inhibit proteolysis. The culture was then placed on ice and 1 l of -70°C ice cubes of buffer containing 0.01 M Tris-HCl pH 7.9 and 0.01 M $MgCl_2$ was added. Cells were harvested using a water-cooled CEPA continuous centrifuge. The cells were washed 4 times with buffer containing 1.0 M KCl, 0.01 M Tris-HCl pH 7.9, and 0.01 M $MgCl_2$, and the pellet was stored at -70°C until used for RNA polymerase extraction. The yield from ten liters was 30 grams wet weight of cells.

RNA polymerase purification. All procedures were carried out at 5°C. To break the cells, ninety grams of cells were resuspended in 100 ml of breaking buffer (0.1 M Tris-HCl pH 7.9, 0.01 M $MgCl_2$, 1.0 mM EDTA, 0.3 mM DTT, 10% glycerol, 0.15 M KCl, and 2 mM PMSF), and the cells were passed three to four times through a French press at 15,000 psi. To this extract, 25 grams of hemoglobin-sepharose, prepared using cyanogen bromide-activated sepharose 4B according to the method of Chua and Bushuk (1969), was added to bind proteases,

and the mixture was stirred for 20 min. This was followed by low speed centrifugation at 30,000 x g for 30 min in a refrigerated Sorvall centrifuge to remove cell debris and the hemoglobin-sepharose. The supernatant was removed and diluted to 600 ml with buffer (0.02 M Tris-HCl pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 1.0 mM PMSF, and 10% glycerol) containing 50 mM ammonium sulfate. Solid ammonium sulfate was added to a concentration of 134 g/l, and the solution was stirred until the salt had dissolved. The mixture was centrifuged at 45,000 rpm (100,000 x g) in a Ti60 rotor (Beckman Instrument Co.) for 1.5 hr. The supernatant was collected, 280 g/l ammonium sulfate was added with gentle stirring, and the mixture was centrifuged for 45 min under the same conditions as above. Each of the resulting pellets was rinsed with buffer A (0.01 M Tris-HCl pH 7.9, 0.01 M $MgCl_2$, 0.1 mM DTT, 0.1 mM EDTA, 1 mM PMSF, and 10% glycerol), and then dissolved in 2.5 ml of this buffer. The fractions were pooled and dialyzed for 10 hr against 2 changes of buffer A. This protocol is a modified form of a procedure used successfully in this lab to purify the RNA polymerase from Bacillus subtilis.

DEAE-cellulose column chromatography. The dialysate from above was applied to a 32 x 2.5 cm DEAE-cellulose column which had been equilibrated with buffer A containing 0.1 M KCl. The column was washed with 100 ml of buffer A at 24 ml/hr, and 4 ml fractions were collected and monitored at $O.D._{280}$. Once the $O.D._{280}$ reached a base line level the column was sequentially eluted with buffer A containing 0.15 M KCl and then 0.25 M KCl. The peak $O.D._{280}$ fractions were assayed for polymerase activity and the active fractions were pooled

and dialyzed for 4 hr against 2 changes of buffer B (0.01 M Tris-HCl pH 7.9, 0.1 mM DTT, 0.1 mM EDTA, 1 mM PMSF, 20 % glycerol, and 0.2 M KCl).

DNA-agarose column chromatography. A 22.5 x 1.5 cm DNA-agarose column was prepared with salmon testes DNA according to the method of Schaller et al. (1972), and washed extensively with buffer B + 0.2 M KCl. The dialysate from above was then loaded and the column washed with additional buffer B containing 0.2 M KCl. Fractions were obtained by eluting the proteins first with buffer B + 0.45 M and then the same buffer with 1.0 M KCl. Fractions of 2.5 ml were collected at a flow rate of 15 ml/hr. Both protein and RNA polymerase activity were monitored.

Phosphocellulose column chromatography. For the preparation of core polymerase devoid of sigma subunit, fractions from the 1.0 M KCl wash of the DNA-agarose column were pooled and dialyzed against several changes of buffer B + 0.15 M KCl. The dialysate was loaded onto a 21 x 1.5 cm phosphocellulose column which had been equilibrated with buffer B + 1.0 M KCl and then washed with buffer B. After the material was loaded, the column was washed with 100 ml of buffer B + 0.15 M KCl at a flow rate of 20 ml/hr. The core enzyme was eluted with buffer B + 0.7 M KCl and the 2 ml fractions assayed with a poly(dAdT) template.

RNA polymerase assays. The assay conditions of Gross et al. (1976) were slightly modified to obtain maximum activity. The total assay volume was 100 µl and contained the following: 25 mM Tris-HCl pH 7.9, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM K_2HPO_4 pH 7, 150 mM NaCl, 0.2 mM CTP, 0.2 mM GTP, 0.2 mM ATP, 0.05 mM UTP, 1 µCi [5,6-^{3}H]-

UTP, 50 µg BSA, and 1.1 µg poly(dAdT). Assays were incubated for 10 min at 34°C and then 3 ml of cold 5% trichloroacetic acid (TCA) was added to precipitate the proteins and nucleic acid. The mixtures were filtered through a glass fiber prefilter which had been soaked in 0.2 mg/ml uridine to prevent non-specific binding of the radioactive substrate. The filters were rinsed 3 times with cold 5% TCA, once with cold 95% ethanol, dried, and placed in scintillation vials with 5 ml of a toluene-based scintillation mixture containing 0.1 g POPOP and 4 g PPO per liter. Samples were counted in a Beckman LS-8000 liquid scintillation counter.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). Preparative slab gels (160 x 100 x 1.5 mm) were stained with Coomassie blue R-250 for scanning densitometry, and mini-gels (100 x 70 x 0.8 mm) were stained with a highly sensitive silver stain procedure (Nielsen and Brown 1984). The Coomassie blue-stained gels were scanned with a Zeineh Soft Laser Scanning Densitometer and data analyzed with an Electrophoresis Reporting Integration Program (Biomed Instruments, Fullerton, CA) on an Apple IIe computer. This information was used to determine the stoichiometry of the RNA polymerase holoenzyme.

Protein determination. During the protein purification process the relative protein concentration of the fractions was determined by monitoring the $O.D._{280}$. The protein concentrations of the peak fractions were determined using the BioRad protein assay, with bovine serum albumin as the standard.

Elution of subunits from gels. RNA polymerase subunits were purified from preparative SDS-polyacrylamide slab gels according to the method of Hager and Burgess (1980), except that the elution buffer contained 50 µg/ml BSA to stabilize the RNA polymerase, and the dilution buffer contained 5 µg/ml BSA and 40% glycerol. Subunits were eluted from crushed gel pieces, and the eluted proteins were then denatured in the presence of 6 M guanidine-hydrochloride, and slowly renatured by a 1:50 dilution of the sample with dilution buffer +40% glycerol, incubating for 6-12 hours at room temperature. The renatured protein was stored at -20°C until use. Purity of the eluted proteins was established by SDS-polyacrylamide gel electrophoresis in a mini-gel system using the silver stain procedure mentioned above. With this procedure protein contamination can be detected at the nanogram level.

Reconstitution assays. To identify the β subunit, which is responsible for rifampicin sensitivity, various combinations of RNA polymerase subunits from rifampicin-sensitive and rifampicin-resistant strains were mixed together after elution from gels, and allowed to renature overnight at room temperature. The reconstituted enzyme was assayed for activity in the absence or presence of 260 µM rifampicin, which was added to the assay mixture before the addition of RNA polymerase. Other assays to identify the σ subunit, and to examine other proteins which may modify the RNA polymerase, were performed in a similar manner, adding interesting polypeptides eluted from preparative gels to purified core or holoenzyme.

Growth of alfalfa seedlings. Alfalfa seeds were surface-sterilized in a 20% solution of commercial bleach (1% hypochlorite) for

8-10 min with stirring, then washed several times with sterile deionized water to remove the bleach. The seeds were placed on the surface of a 1.5% agar plate, and the plate was inverted in a plastic bag containing a moistened filter disk. The seeds were allowed to sprout overnight at room temperature, and were then each transferred to a separate slant of modified Jensen's seedling agar (Jensen 1942), which contains per liter: 0.6 g $CaCl_2$, 0.2 g $MgSO_4 \cdot 3H_2O$, 0.1 g NaCl, 0.05 g Fe citrate, 0.5 g K_2HPO_4, 0.188 g KH_2PO_4, 15 g agar, and 10 ml of a trace element solution, with the pH adjusted to 7.0. The trace element solution contained per liter: 143 mg H_3BO_3, 102 mg $MnSO_4 \cdot 3H_2O$, 22 mg $ZnSO_4 \cdot 2H_2O$, 8 mg $CuSO_4 \cdot 5H_2O$, 10 mg $CoCl_2 \cdot 4H_2O$, and 5 mg $Na_2MoO_4 \cdot 2H_2O$. The bottom half of the tubes were wrapped with aluminum foil, and the seedlings were incubated under greenhouse conditions. After seven days, the seedlings were inoculated with 0.5 ml of an overnight culture of Rhizobium meliloti RM41 grown in LRB broth + 1% mannitol. Plants were watered every 7-10 days, and nodules were harvested 4-6 weeks after infection.

Bacteroid isolation from nodules. Four nodules were separated from the roots and placed into a sterile 1.5 ml tube, and washed 3 times with sterile deionized water to remove surface contamination. Bacteroids were then isolated from the nodules by the method of Zimmerman et al. (1983). The nodules were crushed in 1 ml nodule extraction buffer, which contained 50 mM Tris-HCl pH 7.4, 20 mM KCl, 10 mM $MgCl_2$, 0.5 M mannitol, 1 mM PMSF, and 0.1% polyvinylpyrrolidone. The crushed material was then centrifuged at 8000 rpm for 10 min, and the supernatant, which contains nodular proteins, was removed to

another tube. The pellet, containing the bacteroids and debris, was resuspended in 100 µl nodule extraction buffer, layered onto a 1.2 ml 5-20% sucrose gradient in a 1.5 ml microfuge tube, and centrifuged for 30 sec at 3000 rpm in a Sorvall HB-4 rotor. The bacteroids were found by phase microscopy to be in the top one-third of the gradient, while the debris went to the bottom. The bacteroid-containing fractions were then centrifuged at 8000 rpm for 10 min, and the resulting bacteroid pellet was resuspended in bacteroid protein extraction buffer (25 mM Tris-HCl pH 7.4, 1 mM DTT, 2% SDS, 1 mM PMSF, and 40% glycerol), and stored at -20°C. Samples of both the bacteroid and nodule protein fractions were analyzed on 10% SDS-polyacrylamide gels prepared by the method of Laemmli (1970), and stained with a color-forming silver stain (Nielsen and Brown 1984). Proteins in these fractions were compared to the subunits of purified RNA polymerase from free-living bacteria on the same gels to see if proteins of the same molecular weights as the purified enzyme subunits are present in the bacteroid protein fraction.

Isolation of 55,000-60,000 molecular weight proteins from bacteroids. The 55,000-60,000 molecular weight proteins of the bacteroid extract were eluted from preparative SDS-polyacrylamide gels and renatured according to the method of Hager and Burgess (1980) described earlier. This group of proteins likely contains the nifA-like regulatory protein required for activation of nif gene expression in R. meliloti (Szeto et al. 1984). This protein is limited to a maximum molecular weight of 60,000 by transposon mutagenesis into the nifA gene region. This gene shows considerable homology to the K. pneumoniae nifA gene, which codes for a protein with a molecular

weight of 57,000 (Ausubel and Cannon 1981). The purity of the renatured proteins was determined on SDS-polyacrylamide mini-gels.

Plasmid DNA and restriction fragment isolation. Plasmid DNA from E. coli strains in Table 1 was purified by chloramphenicol treatment of the cells to amplify the plasmid, followed by lysozyme-Triton X-100 lysis, CsCl density gradient centrifugation, phenol extraction, and ethanol precipitation. Restriction enzyme digests of plasmid DNA were separated on low melting point agarose gels. The desired fragments were excised, the agarose melted, and the DNA purified from the agarose by extraction with phenol saturated with 50 mM Tris-HCl pH 7.9, 10 mM EDTA, with 1:1 phenol:chloroform, and with 24:1 chloroform:isoamyl alcohol, followed by ethanol precipitation.

Subcloning of nif gene promoter fragments. Plasmids pDC2 and pDC4 were double digested with BglII and HindIII restriction endonucleases, and separated on 1% low melting point agarose gels in Tris-acetate buffer (pH 7.4). The fragments containing the 5' ends of the nif P1 and nif P3 gene regions (Fig. 1) were purified from the melted agarose pieces by phenol extraction and ethanol precipitation. Each of these fragments was then ligated with HindIII- and BamHI- digested M13mp8, as the BglII and BamHI ends have the same overlapping bases, and will anneal. Ligation mixtures contained, in 20 µl: 4 µl digested vector (20-40 ng DNA), 4 µl of the fragment to be cloned (12-120 ng DNA), 2 µl DNA ligase buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 100 mM DTT), 2 µl 1 mM ATP, and 0.0002 units T4 DNA ligase. Ligation reactions were incubated at 15°C for 4-18 hr.

Transfection of competent cells. Competent cells of E. coli host strain JM103 were prepared by calcium chloride treatment

(Mandel and Higa 1970). Each ligation mixture was added to 200 µl competent cells and placed on ice for 40 min, followed by a heat shock for 2 min at 42°C. To this mixture was added 10 µl 100 mM isopropylthio-β-galactoside (IPTG), 50 µl 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal, 2% in dimethyl formamide), 0.2 ml exponentially growing JM103 cells, and 3 ml YT soft agar (10 g tryptone, 5 g yeast extract, 5 g NaCl, 7 g agar per liter), and this mixture was poured onto a YT agar plate. The overlay was allowed to harden, and the plate was incubated overnight at 37°C. Blue plaques indicate there has probably been no insertion into the polylinker cloning region of the β-galactosidase gene in M13mp8, while clear plaques indicate an insertion has been made to inactivate the β-galactosidase gene and prevent hydrolysis of the X-Gal indicator.

Analysis of clear plaques. Infected cells from clear plaques were picked using sterile toothpicks into 2 ml YT broth in sterile tubes, and 20 µl of an overnight culture of JM103 was added to each. The tubes were incubated at 37°C with shaking for 6 hr, and then the culture was centrifuged for 1 min. The supernatant, which contains mature single stranded M13 recombinant phage, was removed to another tube and stored at 4°C. The pellet, which consists of infected cells containing the double-stranded replicative form of the M13 DNA, was treated with the alkaline-SDS lysis method (Birnboim and Doly 1979) to prepare enough DNA for restriction digestion analysis. After digestion with the desired restriction enzyme, DNA fragments were analyzed by electrophoresis on 1.2% agarose gels.

Southern blotting and hybridization. pDC4 plasmid DNA, which contains an insert of the R. meliloti nifHDK operon lacking 86 base

pairs at the 5' end of the 160 base pair conserved nif promoter region (Fig. 1), was double digested with BglII and EcoRI, or with BglII and SalI, and the fragments were separated on a 1.5% horizontal agarose gel in Tris-acetate buffer containing 0.5 µg/ml ethidium bromide. After electrophoresis the gel was photographed with a Polaroid MP-3 land camera, illuminating the gel with an ultraviolet transilluminator (Ultraviolet Products, Inc). The gel was then denatured in 0.2 N NaOH, 0.6 N NaCl for 30 min at room temperature with gentle agitation, followed by washing three times in 10XSSC (1.5 M NaCl, 0.15 M sodium citrate) for 1 hr. The DNA was transferred to Genescreen hybridization transfer membrane for at least 12 hrs by the capillary blot procedure, adding additional 10XSSC as needed. The membrane was then carefully rinsed with 10XSSC to remove agarose, air dried, and baked for 2-4 hrs at 80-100°C.

Labelled mRNA probes were prepared using the same conditions as for the RNA polymerase assays previously described, except the [5,6-^{3}H]-UTP was replaced by 10 µCi [α^{32}P]-UTP (760 Ci/mmole), and 1.1 µg pDC4 plasmid DNA or 1.1 µg pNB1 M13 phage DNA was used as template. Reactions were incubated for 30 min at 34°C, and then phenol extracted and ethanol precipitated. To remove unincorporated nucleotides, the pellet was resuspended in 10 mM Tris-HCl pH 7.9, 1 mM EDTA (TE buffer) + .5 M NaCl and loaded onto a NACS prepac column. The column was washed with additional buffer to remove the unincorporated [α^{32}P]-UTP, and then the mRNA was eluted with TE + 2 M NaCl. A 1/20 volume sample was taken, precipitated with ice cold 5% TCA after addition of 20 µg yeast tRNA to aid precipitation,

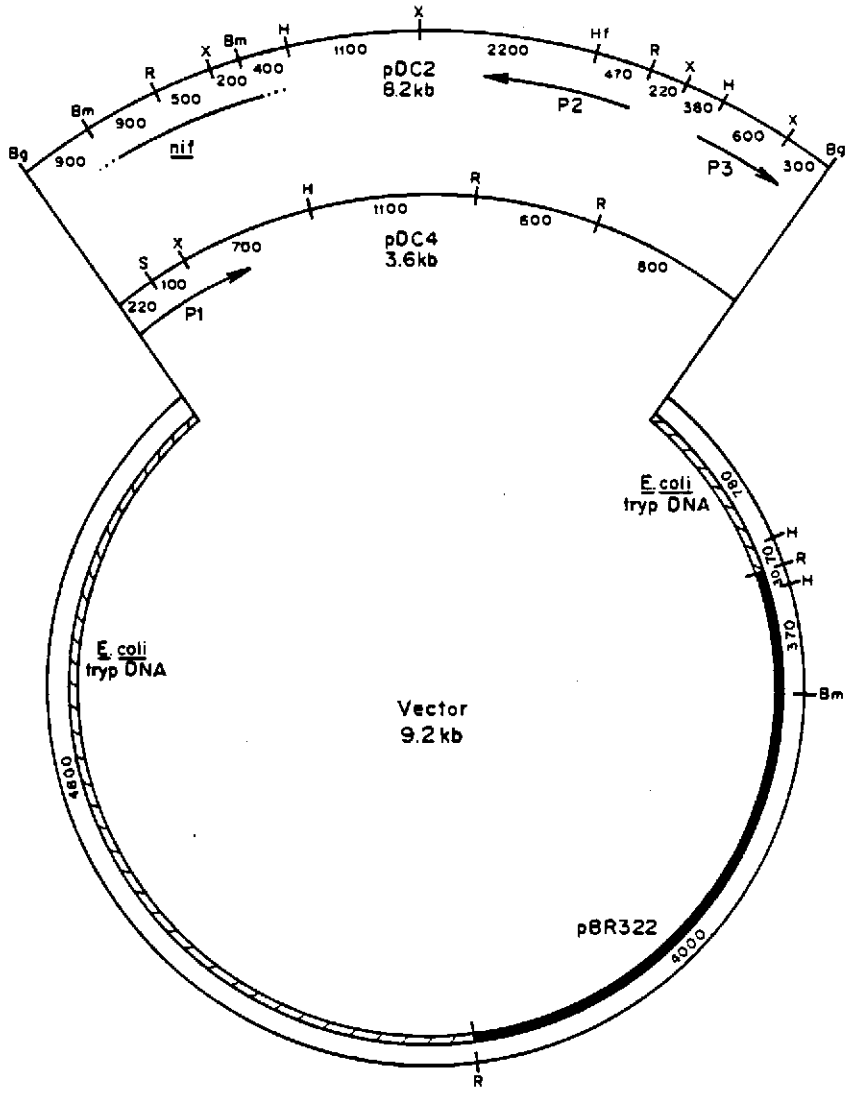

Fig. 1. Restriction endonuclease map of plasmids pDC2 and pDC4 (Corbin et al. 1983; Better et al. 1983; Szeto et al. 1984), obtained from G. Ditta. The vector consists of pBR322 fused to E. coli tryp DNA as shown, with corresponding fragments of R. meliloti DNA inserted into the BglII restriction site of the vector. The location of the transcription start sites and the direction of transcription for the nifP1 (nifHDK), nif P2, and nifP3 promoters are shown (Better et al. 1983), as well as the location of the nifA-like regulatory locus recently identified (Szeto et al. 1984). Restriction endonuclease sites for the following enzymes are shown: S, SalI; X, XhoI; H, HindIII; R, EcoRI; Bg, BglII; Bm, BamHI; and Hf, HinfI. The sizes of the fragments are listed as the number of base pairs.

filtered, and counted in a Beckman LS-8000 liquid scintillation counter to determine the total amount of activity incorporated.

Each blot was prehybridized with 10 ml of the following buffer: 50% deionized formamide, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.2% ficoll, 0.05 M Tris-HCl pH 7.5, 1.0 M NaCl, 0.1% sodium pyrophosphate, 1.0% SDS, 10% dextran sulfate, and 100 µg/ml denatured salmon sperm DNA. This solution was added to each blot in sealable plastic bags, and the bags were incubated at 42°C for at least 6 hrs. The radioactive probe from above was then added to 2-3 ml of the above buffer lacking NaCl and dextran sulfate, and this mixture was added to the bag. Hybridization was carried out for 16-24 hrs at 42°C with constant agitation. The hybridization solution was then removed, and the membrane was washed twice with 2XSSC for 5 min at room temperature, twice with 2XSSC + 1% SDS for 30 min at 65°C, and twice with 0.2XSSC for 30 min at room temperature. The membrane was wrapped, while slightly damp, in plastic wrap and placed against Kodak X-Omat AR film with an intensifying screen for 18-72 hrs. Blots could be reused by stripping the probe with 250 ml buffer containing 0.005 M Tris-HCl pH 8.0, 0.0002 M Na_2EDTA, 0.05% sodium pyrophosphate, 0.002% polyvinylpyrrolidone, 0.002% BSA, and 0.002% ficoll at 60-65°C for 3 hrs with constant agitation. The blots were then air dried and reprobed. Autoradiograms were scanned with a Zeineh Soft Laser Scanning Densitometer and data was analyzed with an Electrophoresis Reporting Integration Program on an Apple IIe computer.

Run-off transcription reactions. In vitro transcription reactions were carried out similar to the RNA polymerase assays described earlier, except that each assay contained 20 μCi [α^{32}P]-UTP (760 Ci/mmole) and 0.01 mM unlabelled UTP. Various plasmid DNA restriction fragments containing known promoters purified from 1% low melting point agarose gels were used as templates, and 3 units human placental ribonuclease inhibitor was added to each reaction to inhibit any ribonuclease which may be present. In addition, ribonuclease-free BSA was used in the reactions. Reactions were incubated in 1.5 ml microfuge tubes for 20 min at 34°C and then heparin was added to 50 μg/ml to block reinitiation. Incubation was continued for 10 min, and 20 μg tRNA was added to serve as a carrier for precipitation. A 1/20 volume sample from each reaction was taken and precipitated with cold 5% TCA, filtered, and counted in a Beckman LS-8000 liquid scintillation counter to determine the amount of activity incorporated in each reaction. The remaining RNA was precipitated by adding 2.5 volumes 95% ethanol and 0.1 volume 3 M sodium acetate, and storing at -20°C overnight. The samples were centrifuged at high speed for 5 min and the pellet was washed once with ice-cold 80% ethanol and dried.

Electrophoresis of run-off transcripts through formaldehyde-denaturing gels. Formaldehyde-denaturing RNA gels were prepared and run according to Maniatis et al. (1982) as modified by Gary Merrill (personal communication), in a mini-sub electrophoresis cell (BioRad). RNA electrophoresis buffer (10X MOPS) contains: 0.2 M morpholinopropanesulfonic acid (MOPS), pH 7.0, 50 mM sodium acetate, and 5 mM

EDTA pH 8.0. For a 1.1% agarose gel, 0.27 g agarose was added to 18 ml sterile distilled water and melted. After the agarose solution had cooled to about 65°C, 2.5 ml 10X MOPS buffer and 4.5 ml formaldehyde were added to give 1X and 2.2 M final concentrations, respectively, and the gel (10 x 6.5 x 0.3 cm) was cast under a fume hood.

Samples for electrophoresis were prepared by mixing together the following in sterile microfuge tubes: 3.4 µl RNA (volume adjusted with sterile double-distilled water), 1.5 µl 10X MOPS buffer, 2.6 µl formaldehyde, and 7.5 µl deionized formamide. The samples were then heated to 65°C for 15 min, and 1.5 µl sterile loading buffer (50% glycerol, 5 mM EDTA, 0.05% xylene cyanol, and 0.05% bromphenol blue) were added and the samples were loaded onto the gel. The gel was electrophoresed at 100 volts in 1X MOPS buffer containing 2.2 M formaldehyde until the bromphenol blue dye had migrated about two-thirds down the gel. The gel was then washed for 30 min with several changes of distilled water to remove the formaldehyde. Lanes containing unlabelled ribosomal RNA were stained for 1 hr in 0.1 M ammonium acetate containing 7.5 µg ethidium bromide, and photographed. The labelled RNA from the remainder of the gel was blotted to a nitrocellulose filter, which had been presoaked for 1 min in distilled water and 5 min in 20X SSC (3 M NaCl and 0.3 M sodium citrate, pH 7.0), using 20X SSC as the blotting buffer. Transfer of RNA is complete after 3-4 hrs, and the nitrocellulose filter was then washed in 3X SSC for 5 min and air dried for 1 hr. The filter was baked at 80°C between two pieces of Whatman 3MM filter paper for 2 hrs under vacuum, after which Kodak X-Omat AR5 film was placed against the filter for autoradiography for 24-72 hrs. The sizes of

the transcripts were determined using ^{32}P-labelled control transcripts (125, 557, and 1386 bases, the gift of G. Merrill) and E. coli ribosomal RNA as standards.

RESULTS

The results of the purification procedure are summarized in Table 2. The procedure resulted in a 420-fold purification of the RNA polymerase from Rhizobium meliloti. During the purification process RNA polymerase assays were performed as described in the Materials and Methods section. The elution profiles for the major proteins and for enzyme activity are shown in Fig. 2, 3, and 4. The majority of the contaminating proteins were removed either in the initial DEAE-cellulose chromatography step or by failure to bind to DNA-agarose. Greater than 90% of the original activity is eluted from the DEAE column with 0.25 M. KCl as shown in Fig. 2. The elution profile in Fig. 3 indicated the typical results obtained by elution of the enzyme from DNA-agarose. Fully one-third of the enzyme activity is removed when the column is washed with 0.45 M KCl. A number of other elution steps were attempted in order to minimize this early elution of enzyme, but all were unsatisfactory in that they resulted in a larger number of contaminating proteins in the final elution step. The peak fractions were examined by SDS-polyacrylamide gel electrophoresis. Representative peak fractions of the R. meliloti RNA polymerase are shown in Fig. 5. DNA-agarose-purified holoenzyme and core enzyme from the phosphocellulose peak fractions showed similarities with the profiles obtained with R. leguminosarum RNA polymerase (Lotz et al. 1981).

Molecular weight determination and subunit structure. The typical gram-negative RNA polymerase has the subunit structure $\alpha_2\beta\beta'$ for the core polymerase. In addition, E. coli has been shown to

Table 2. Purification of RNA polymerase from Rhizobium meliloti.

EXTRACTION STEP	VOL (ml)	PROTEIN (µg/ml)	TOTAL ACTIVITY[a]	SP. ACTIVITY[b]
DEAE CELLULOSE	35	7500.0	1.270	0.00425
DNA-AGAROSE	40	45.6	0.690	0.38
PHOSPHOCELLULOSE	14	5.3	0.133	1.8

[a]Expressed as nmoles [^{3}H]-UMP incorporated in 10 min., under the assay conditions described in Materials and Methods.

[b]Expressed as nmoles [^{3}H]-UMP incorporated per mg protein in 10 min.

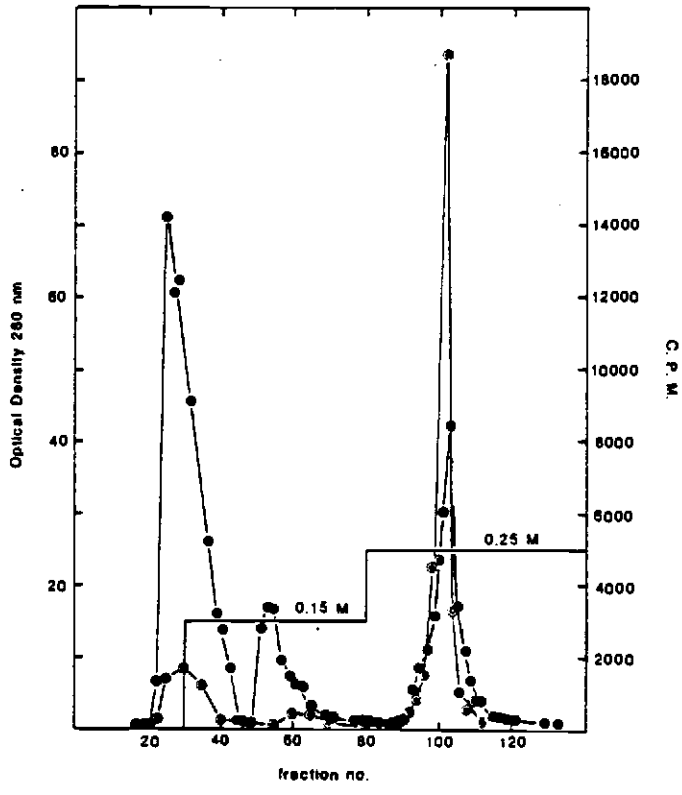

Fig. 2. Elution profile of the DEAE-cellulose column. RNA polymerase extracted from free-living Rhizobium meliloti was chromatographed over a 32 x 2.5 cm column and the fractions eluted with a step gradient of KCl, as shown. Activity of each fraction was measured using a poly(dAdT) template. $O.D._{280}$, ●; enzyme activity in C.P.M., ◉.

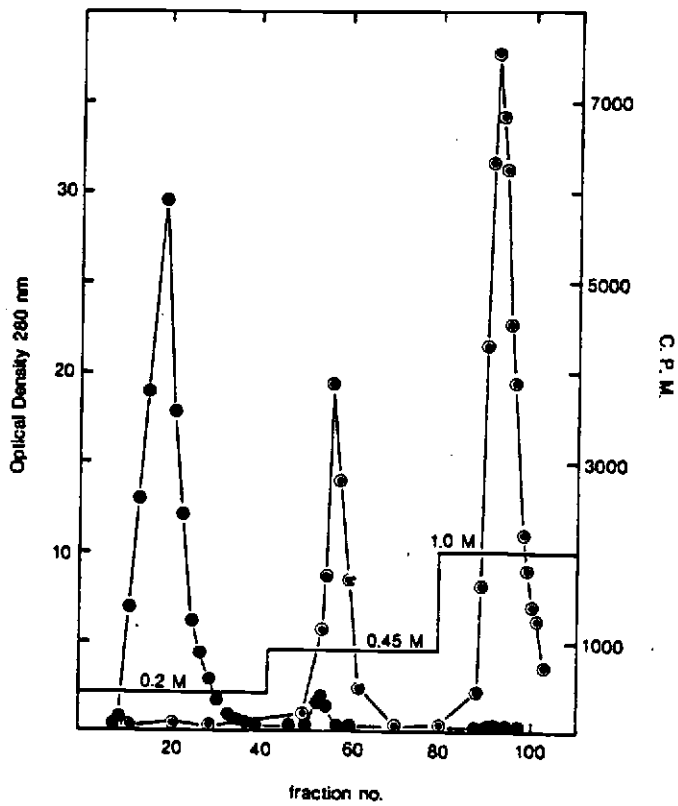

Fig. 3. Elution profile of the DNA-agarose column. Peak fractions (97-105 shown in Fig. 2) were pooled, dialyzed and loaded onto the column. Step elution was with 0.2 M, 0.45 M, and 1.0 M KCl. $O.D._{280}$, ●; Activity in C.P.M., ◉.

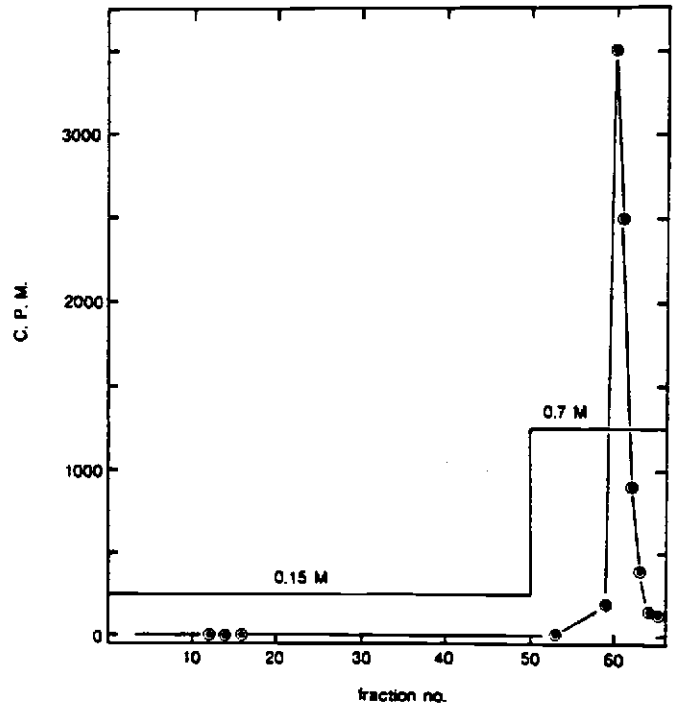

Fig. 4. Stepwise elution profile of RNA polymerase activity from a phosphocellulose column. Column was eluted with 0.15 M and 0.7 M KCl.

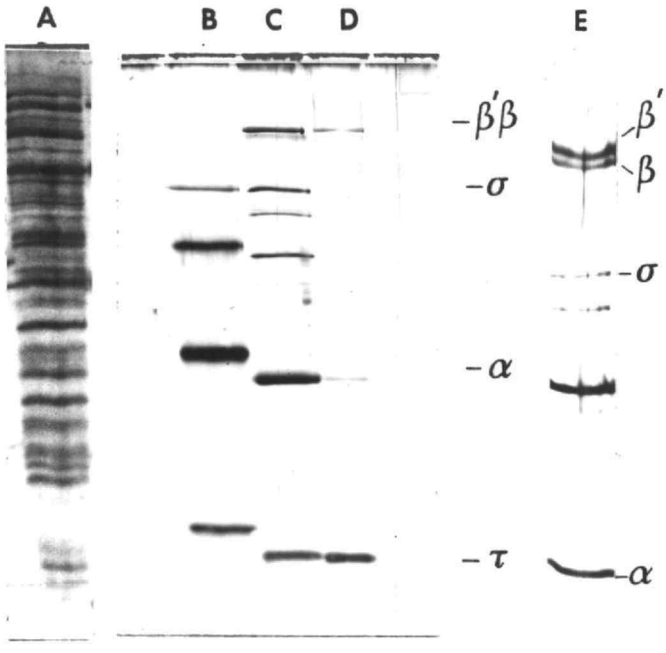

Fig. 5. SDS-polyacrylamide gel electrophoresis of peak fractions of R. meliloti RNA polymerase purification. Samples were electrophoresed on 10% (lanes a-d), or 7.5% (lane e), SDS-polyacrylamide gels, and the gels were silver stained as described in Materials and Methods. Lane a, DEAE-cellulose column fraction; lane b, molecular weight standards from top to bottom: phosphorylase b, 93,000 m.w.; bovine serum albumin, 66,000 m.w.; ovalbumin, 45,000 m.w.; and carbonic anhydrase, 31,000 m.w. Lane c, DNA-agarose column fraction, labelled bands from top to bottom: β and β′subunits, 151,000 and 155,000 m.w., respectively; σ subunit, 93,000 m.w.; 86,000 m.w. polypeptide; 63,000 m.w. polypeptide; α subunit, 43,000 m.w.; and τ polypeptide, 29,000 m.w.; lane d, phosphocellulose column fraction, labelled bands from top to bottom: β and β′ subunits, α subunit, and τ subunit; lane e, 7.5% acrylamide gel to show separation of β and β′subunits.

have a small molecular weight subunit, omega (ω), which consistently copurifies with the bacterial RNA polymerase. The subunits identified with R. meliloti RNA polymerase are shown in Figure 5. A comparison of the mobility of these protein subunits in SDS-polyacrylamide gels with proteins of known molecular weight lead to the tentative identification of the following subunits: β′, 155,000; β, 151,000; α, 43,000; σ, 93,000; and τ, 29,000. Although this latter subunit may be analogous to the ω subunit in other gram-negative RNA polymerase molecules, its high molecular weight, 29,000, makes this unlikely. Unlike the RNA polymerase from Bradyrhizobium japonicum (Rhizobium japonicum) the β and β′ subunit of the R. meliloti RNA polymerase can be readily separated, as shown in Figure 5E.

Stoichiometry of subunits in the purified RNA polymerase complex. Results from soft laser scanning densitometry of a sample that had been subjected to gel electrophoresis and then stained with Coomassie Blue are shown in Figure 6. A determination made from a number of RNA polymerase preparations indicated that the stoichiometry for the average RNA polymerase was 2α:1β:1β′:0.7σ:1τ. The subunit that showed the most variation in relative amounts was σ with a range of 0.5-0.9 for the six different preparations. This was not unexpected because σ can be stripped from the holoenzyme complex by passage over a phosphocellulose column, indicating that it is less tightly bound to the RNA polymerase complex. The variation in the σ subunit concentration is consistent with results obtained with other gram-negative organisms (Engbaek et al. 1976, Iwakura et al. 1974).

Positive identification of the sigma subunit. Many of the preparations of RNA polymerase obtained following DNA affinity chroma-

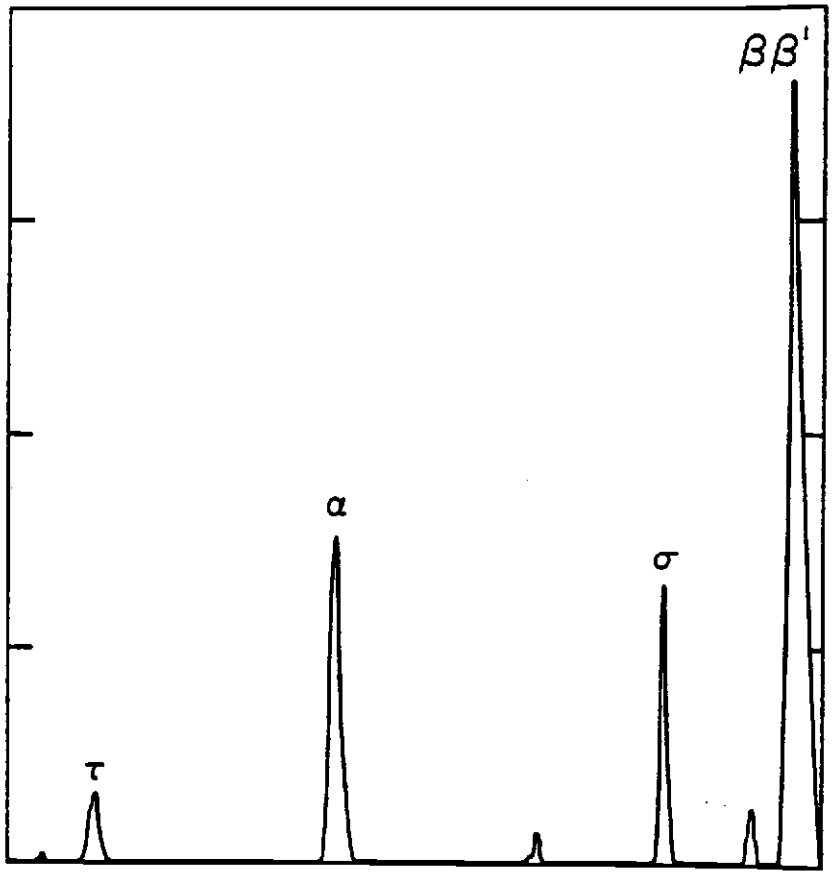

Fig. 6. Stoichiometry of R. meliloti RNA polymerase holoenzyme, as determined by soft laser scanning densitometry of a Coomassie blue-stained SDS-polyacrylamide gel. Analysis of this data yields a calculated stoichiometry of $2\alpha:1\beta:1\beta':0.7\sigma:1\tau$, with two minor contaminants.

tography contained, in addition to the proteins tentatively identified as RNA polymerase subunits, two proteins with molecular weights of 86,000 and 63,000 as determined by mobility characteristics in SDS-polyacrylamide gels. To determine if these proteins would affect RNA polymerase activity, individual proteins were obtained by preparative gel eletrophoresis and a reconstitution system was established similar to that devised by Hager and Burgess (1980). Three proteins, with molecular weights of 93,000, 86,000, and 63,000, were eluted from a gel, denatured in the presence of 6 M guanidine hydrochloride, and then allowed to renature at room temperature. The effect of the addition of the 93,000 molecular weight protein to enzyme assays containing phosphocellulose-purified core polymerase and specific templates is shown in Figure 7. Clearly the 93,000 molecular weight protein showed stimulatory activity on each of the individual templates. The 86,000 and 63,000 molecular weight proteins had no effect on the reactions. This subunit-specific activation of RNA polymerase on a number of templates is characteristic of the σ subunit.

Optimal conditions for enzyme activity and for storage of R. meliloti RNA polymerase. Optimum conditions for the in vitro transcription assays were found to be 34°C, 5 mM $MgCl_2$ and 120 mM NaCl for plasmid and phage DNA templates and 150 mM NaCl for poly(dAdT). The RNA polymerase showed polymerizing activity when assayed on plasmid pRK290 or pLAFR1 DNA, and on phage T3 DNA and M13 mp8 DNA. The RNA polymerase purified through DNA-Agarose can be stored for more than 6 months in buffer A + 0.1 M KCl containing 50% glycerol at -20°C at a protein concentration of 50 µg/ml or greater.

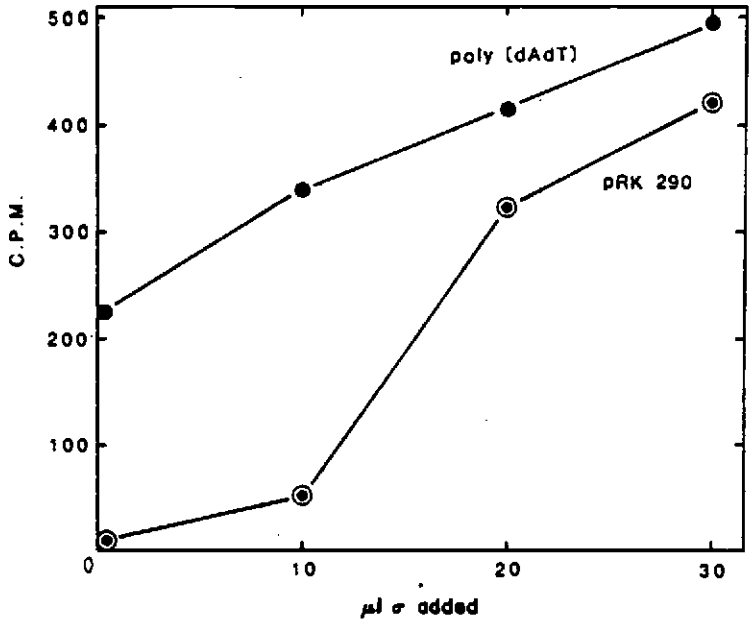

Fig. 7. Stimulation of core RNA polymerase activity upon addition of the 93,000 m.w. σ subunit when assayed on poly(dAdT)(●) or broad host range plasmid pRK290 (◉) templates. The 93,000 m.w. protein was obtained from purified holoenzyme by elution from SDS-polyacrylamide gels as described in Materials and Methods. The 86,000 and 63,000 m.w. proteins also obtained in this way showed no stimulation of core RNA polymerase activity under these conditions.

The relative activity on various templates. Table 3 shows the relative activity of the purified RNA polymerase on a number of different DNA templates. Activity on templates known to contain E. coli promoters indicates that the polymerase has the ability to initiate RNA synthesis on these templates but does not imply that the promoter recognition sequences used by the E. coli and by R. meliloti polymerases are necessarily identical. The synthesis from the specific E. coli phage templates where a limited number of possible start sites for the polymerase are possible does make it highly likely that the RNA polymerase extracted from free-living cells of R. meliloti can utilize some E. coli promoters.

Drug sensitivity of the purified enzyme. To measure the sensitivity of the purified RNA polymerase to RNA polymerase inhibitors, in vitro reactions were used. The relative activity remaining after rifampicin, streptolydigin and lipiarmycin addition to the in vitro reactions is shown in Figure 8. The polymerase is clearly sensitive to these antibiotics, as are most gram-negative polymerases. Enzyme extracted from rifampicin-resistant mutants (presented below) is fully resistant to rifampicin in the in vitro assay. Thus, it appears that the R. meliloti polymerase is similar to other gram-negative enzymes in that one of the subunits can be mutated to yield rifampicin-resistant strains.

Identification of the subunit responsible for rifampicin sensitivity. Individual RNA polymerase subunits were purified from 7.5% SDS-polyacrylamide slab gels of holoenzyme from wild type rifampicin-sensitive and HYP9 rifampicin-resistant mutant strains of R. meliloti,

Table 3. Relative activity of RNA polymerase on various templates.

Template	Properties of plasmids	% of pBR322 Activity with Holoenzyme[a]
pBR322	$Tet^r Amp^r$	100%
pSP19	*E. coli* T4 Phage Dihydrofolate Reductase gene in pBR322	193%
1.1 kb HindIII DHFR fragment[b]		58%
HindIII-cutpBR322[b]		55%
pDC2	*R. meliloti nif* P2 and P3 operons in pBR322 derivative, Tet^r, Amp^r	271%
pDC4	*R. meliloti nifHDK* structural genes in pBR322 derivative, Tet^r, Amp^r	162%
pRM23-2	From *R. meliloti* clone bank in pLAFR1, Tet^r, met^+	119%
T3 phage		71%
M13mp8		238%

[a]Standard enzyme assays were performed as described in Methods, but 1.1 µg of the specified DNA template replaced the poly(dAdT).

[b]Purified from low melting point agarose gels.

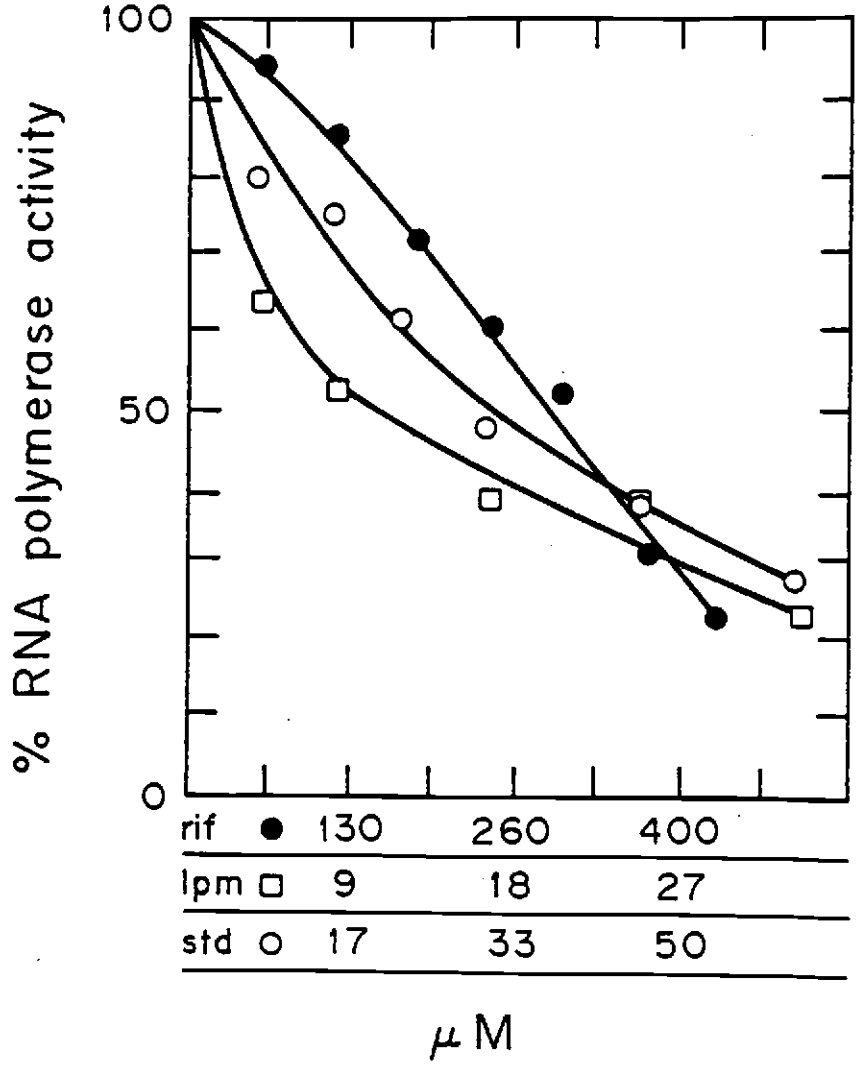

Fig. 8. Sensitivity of purified R. meliloti RNA polymerase to RNA polymerase inhibitors in vitro. Rifampicin (●), lipiarmycin (□), and streptolydigin (○) were added to standard RNA polymerase assays at the concentrations shown, and reactions were incubated for 10 min at 34°C. The relative activity remaining is shown as a percent of RNA polymerase activity in the absence of any inhibitor.

as described in the Materials and Methods section. The efficiency of recovery of the enzyme subunits and RNA polymerase activity is presented in Table 4. Purified subunits from these two enzymes were mixed in different combinations and allowed to renature for 12 hrs. The reconstituted enzyme was assayed with poly(dAdT) as template. The results of these assays are presented in Table 5. Rifampicin was added to a concentration of 260 μM where indicated, which is sufficient to show greater than 50% inhibition of rifampicin-sensitive RNA polymerase (Fig. 7). In Table 5, β is used to designate the smaller of the two large subunits, consistent with the identification of β in E. coli RNA polymerase (Heil and Zillig 1970). As can be seen from this data, this smaller subunit is responsible for rifampicin resistance. Therefore, the designation of this subunit as β is correct. A stimulation of activity in the presence of rifampicin with reconstituted RNA polymerase containing the β subunit from the rifampicin-resistant RNA polymerase can also be noted.

Assays of reconstituted holoenzyme lacked the τ subunit, but activity was recovered on poly(dAdT) and plasmid pRK290 templates, so it appears that this subunit is not required for activity. However, the τ subunit may play a role in enzyme specificity or promoter selection. Additional reconstitution experiments using truncated DNA fragments will need to be used to determine if τ has a role in promoter selection.

Unusual characteristics of the rifampicin-resistant RNA polymerase. Two unexpected observations were found when purifying the RNA polymerase from the HYP9 rifampicin-resistant R. meliloti strain.

Table 4. Efficiency of recovery of RNA polymerase subunits from SDS-polyacrylamide gels, and activity of the reconstituted enzyme.

	α_2	β
Molecules recovered[a]	1.75×10^{13}	6.8×10^{12}
% Subunit recovered[b]	83%	33%

	Activity/Molecule
Holoenzyme	5.6×10^{-9} C.P.M.
Reconstituted enzyme[c]	2.9×10^{-9} C.P.M.
% Specific activity recovered[d]	52%

[a]Calculated by estimating the protein concentration of the eluted subunit on SDS-polyacrylamide mini-gels, and comparing with protein standards of known concentration.
[b]From 2.1×10^{13} molecules of holoenzyme used for subunit isolation.
[c]Calculated using the value of molecules recovered for the β subunit which is limiting for enzyme renaturation.
[d]Shown as a percent of the original holoenzyme activity.

Table 5. Rifampicin resistance of RNA polymerase reconstituted from individual subunits purified from wild type and rifampicin resistant strains of Rhizobium meliloti leads to the identification of the β subunit.

RECONSTITUTED ENZYME [a]	-RIFAMPICIN	+RIFAMPICIN [b]
$\alpha^r\beta^r\beta'^r\sigma^r$	100 [c]	110
$\alpha^r\beta^r\beta'^s\sigma^r$	140	230
$\alpha^r\beta^s\beta'^r\sigma^r$	130	25
$\alpha^s\beta^s\beta'^s\sigma^s$	150	30
$\alpha^s\beta^r\beta'^s\sigma^s$	90	120
$\alpha^s\beta^s\beta'^r\sigma^s$	70	20

[a] In this table, the 151,000 m.w. subunit was arbitrarily identified as β, consistent with the designation of the β subunit in E. coli, and β′ represents the 155,000 m.w. subunit. The superscript r and s identify subunits purified from the rifampicin-resistant HYB9 and rifampicin-sensitive RM41 strains, respectively, as described in the Materials and Methods section.

[b] Rifampicin was added to these assays at 260 μM.

[c] Activity is recorded as TCA-precipitable C.P.M. per 20 min assay.

First, purified holoenzyme and core enzyme showed a stimulation of activity on poly(dAdT) (Fig. 9) or pRK290 (Fig. 10) templates with increasing concentration of rifampicin. A similar stimulation was also observed in reconstitution assays containing the β subunit from rifampicin-resistant RNA polymerase, as already noted. Second, partially purified RNA polymerase from the rifampicin-resistant strain showed a drop in activity to 60-70% of the original activity over the same range in rifampicin concentration (Fig. 9 & 10). To determine if this phenomenon is due to a polypeptide present in partially purified RNA polymerase which alters rifampicin resistance, groups of 2 to 4 polypeptides from the partially purified enzyme fraction were eluted from slab gels, renatured, added to rifampicin-resistant holoenzyme, and assayed in the presence or absence of rifampicin. Only one of the four groups, when added to holoenzyme, caused a decrease in activity in the presence of rifampicin, but not in its absence. The two polypeptides in this group were then individually eluted from gels and renatured. Fig. 11 shows that these eluted proteins, with molecular weights of 63,000 and 65,000, were free from any other polypeptides, as determined by silver-stained SDS-polyacrylamide mini-gels. Only the 65,000 molecular weight protein was found to cause a decrease in RNA polymerase activity when added to rifampicin-resistant holoenzyme and assayed in the presence of 260 μM rifampicin (Fig. 12). No decrease in activity was observed in the absence of rifampicin when the 65,000 molecular weight protein was added to holoenzyme.

RNA polymerase from another rifampicin-resistant mutant R. meliloti strain, selected for the ability to grow on nutrient plates

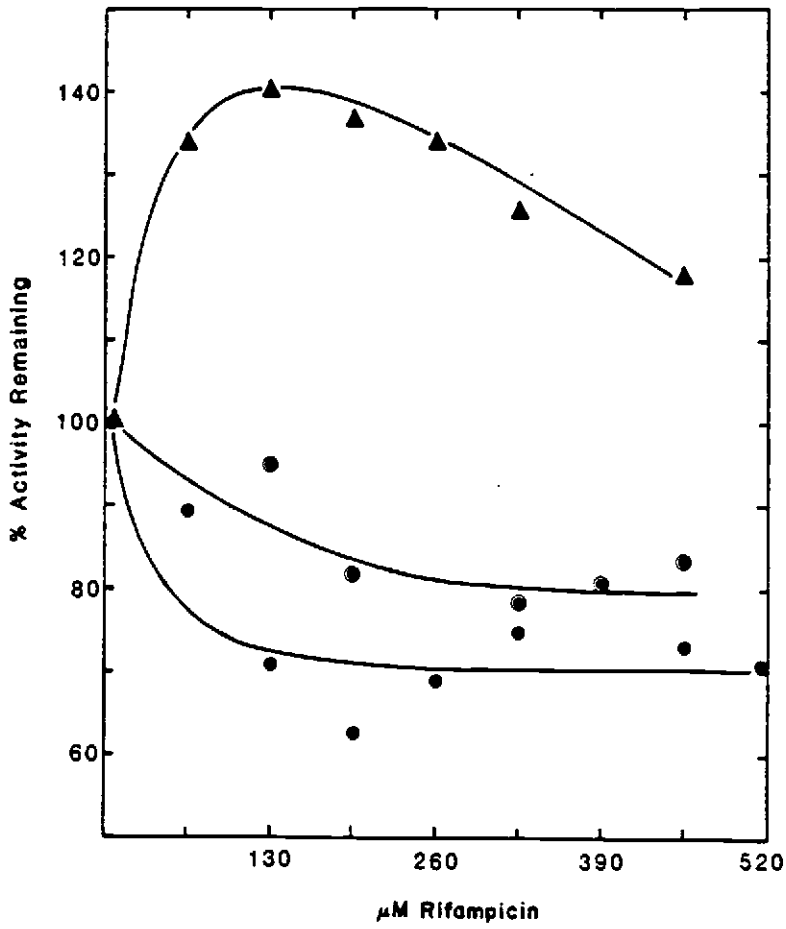

Fig. 9. Rifampicin resistance of partially and fully purified RNA polymerase from the HYP9 rifampicin-resistant strain of Rhizobium meliloti, on a poly(dAdT) template. Rifampicin was added to duplicate RNA polymerase assays (see Materials and Methods) at the concentrations shown, and the assays were incubated for 20 min at 34°C. The percent of RNA polymerase activity remaining as compared with enzyme assays lacking rifampicin was plotted. ◉, RNA polymerase from the DEAE-cellulose column step of the purification; ●, RNA polymerase from the 0.45 M KCl wash of the DNA-agarose column; ▲, fully purified RNA polymerase from the 1.0 M KCl wash of the DNA-agarose column.

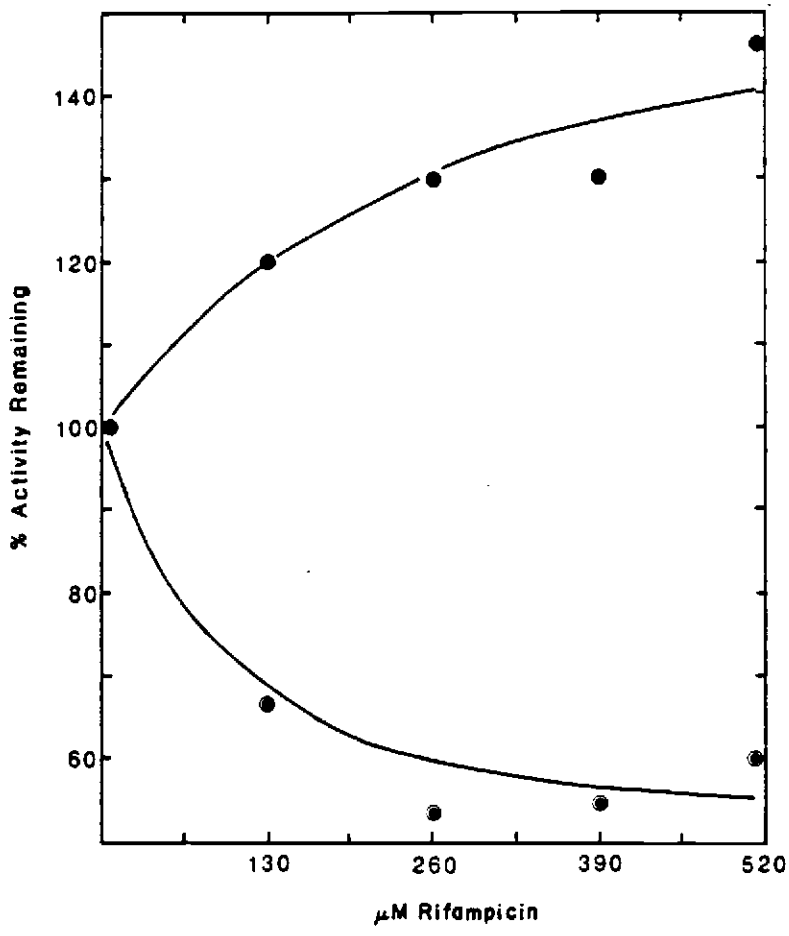

Fig. 10. Rifampicin-resistance of partially and fully purified RNA polymerase from the HYP9 rifampicin-resistant strain of Rhizobium meliloti as assayed on plasmid pRK290 as template. Assays were performed as in Fig. 9. ● , fully purified RNA polymerase from the DNA-agarose column. ◉ , RNA polymerase from the DEAE-cellulose column step of the purification.

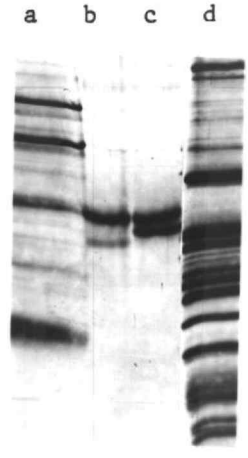

Fig. 11. Purification of individual polypeptides from the 0.45 M KCl fraction of the DNA-agarose column purification of HYP9 RNA polymerase. Proteins were purified by elution from preparative SDS-polyacrylamide gels, and analyzed for purity on a 10% SDS-polyacrylamide mini-gel stained with the silver stain, as described in Materials and Methods. Lane a contains the following molecular weight markers: β-galactosidase, 116,250 m.w.; phosphorylase b, 93,500 m.w.; bovine serum albumin, 66,200 m.w.; and ovalbumin, 45,000 m.w. Lane b shows a 63,000 m.w. protein purified from the enzyme fraction described above, and lane c shows a 65,000 m.w. protein from the same enzyme fraction. These samples contain bovine serum albumin (BSA) to stabilize the protein. Lane d shows the 0.45 M KCl wash from the DNA-agarose column from which these polypeptides were isolated.

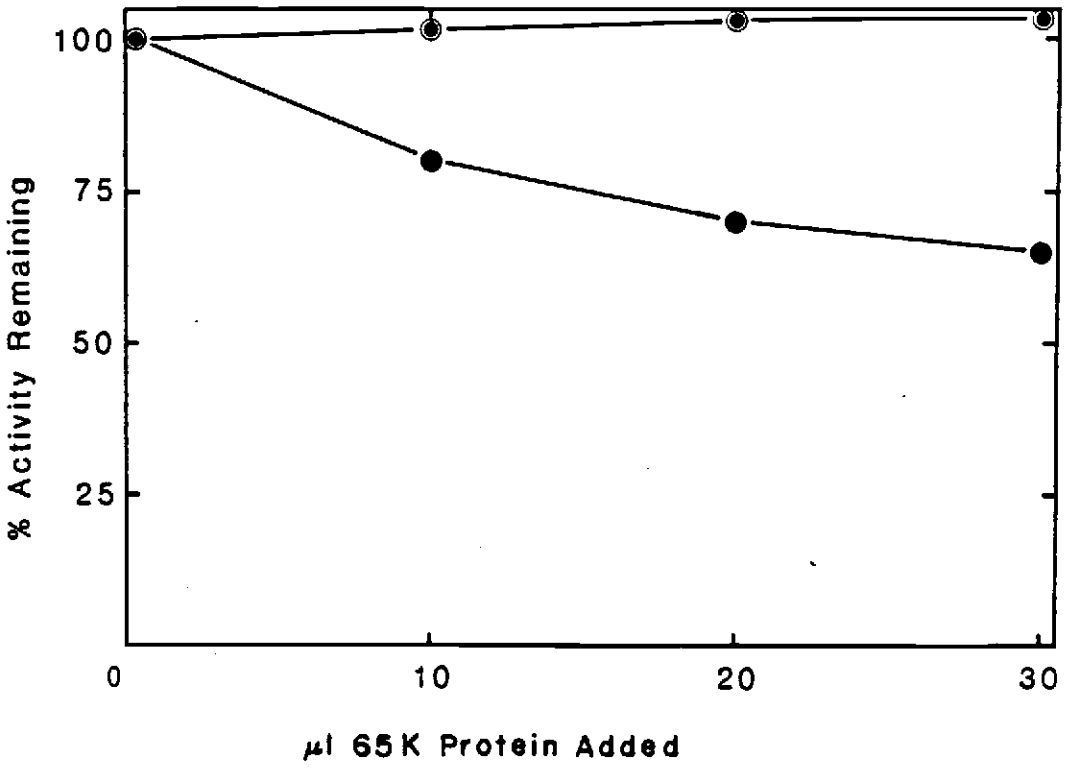

Fig. 12. The effect of the addition of purified 65,000 m.w. protein to rifampicin-resistant HYP9 RNA polymerase holoenzyme, assayed in the presence or absence of rifampicin. The level of RNA polymerase activity in RNA polymerase assays obtained when increasing amounts of the purified 65,000 m.w. protein were added is plotted as a percent of the activity obtained when no 65,000 m.w. protein was present. Reactions were carried out as described in Materials and Methods in the absence (◉) or presence (●) of 260 µM rifampicin.

containing 15 µg/ml rifampicin, showed no stimulation of the purified holoenzyme by rifampicin (Fig. 13). Partially purified enzyme showed no significant alteration in activity by fampicin addition, although some slight effects can be seen. The addition of the purified 65,000 molecular weight protein to this second mutant RNA polymerase did not have any effect on the resistance of this enzyme to rifampicin.

RNA polymerase in bacteroids. Fig. 14 shows a silver-stained polyacrylamide gel containing a nodular protein fraction (lane a), a bacteroid protein fraction (lane c), and purified RNA polymerase from free-living cells of R. meliloti (lane b). Several proteins unique to either the nodule or bacteroid fractions can be seen, and the nifHDK structural genes are identified by their molecular weight (Zimmerman et al. 1983). Polypeptides showing the same rate of migration as the subunits of purified RNA polymerase can be seen in the bacteroid fraction. Separation of the two large proteins migrating the same as the β and β' subunits can also be seen in the bacteroid fraction.

Subcloning of nif gene promoter fragments into M13. In order to prepare subclones of individual nif gene promoter regions for transcriptional analysis, restriction fragments from plasmids pDC2 and pDC4, which contain nif DNA from R. meliloti (Better et al. 1983; Corbin et al. 1983), were ligated into M13mp8. Fig. 15 shows the BglII-HindIII fragments from pDC4 and pDC2 which were subcloned into the BamHI-HindIII site of the polylinker region in M13mp8 to produce pNB1 and pNB2. Purification of DNA from these clones and digestion with HindIII, XhoI, and EcoRI confirmed the presence of these frag-

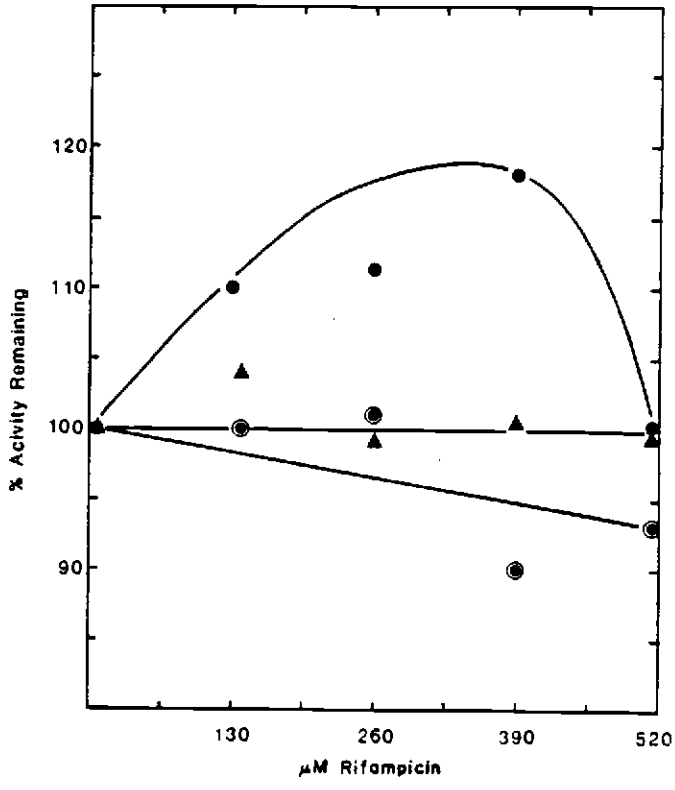

Fig. 13. Rifampicin resistance of partially and fully purified RNA polymerase from a spontaneous rifampicin-resistant strain of R. meliloti assayed on poly(dAdT). Assays were performed as in Fig 9. ● , RNA polymerase from the DEAE-cellulose column step of the purification; ◉ , RNA polymerase from the 0.45 M KCl wash of the DNA-agarose column; ▲ , fully purified RNA polymerase from the 1.0 M KCl wash of the DNA-agarose column.

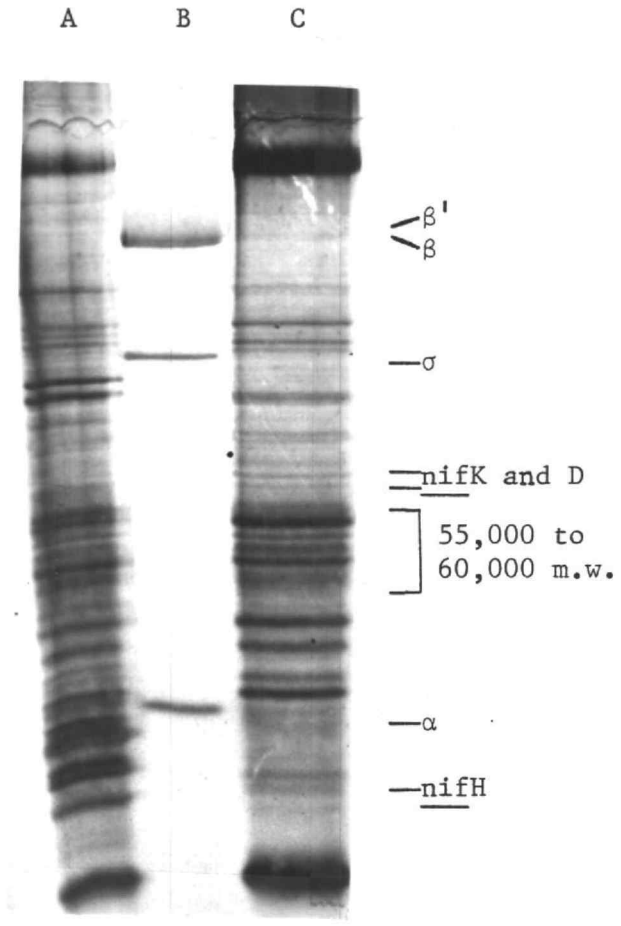

Fig. 14. Alfalfa nodule and Rhizobium meliloti bacteroid protein profiles. Nodular and bacteroid protein fractions from 4 nodules were separated according to Zimmerman et al. (1983; see Materials and Methods), and analyzed on a silver-stained 10% SDS-polyacrylamide mini-gel. Lane a contains nodular proteins. Lane b contains purified R. meliloti RNA polymerase from free-living cells, with subunits, from top to bottom: β', 155,000 m.w.; β, 151,000 m.w.; σ, 93,000 m.w.; and α , 43,000 m.w. Lane c shows R. meliloti bacteroid proteins. The locations of the nifHDK structural gene proteins are indicated. The 55,000-60,000 m.w. proteins containing the proposed nif regulatory protein eluted for use in in vitro transcription stimulation assays are also identified.

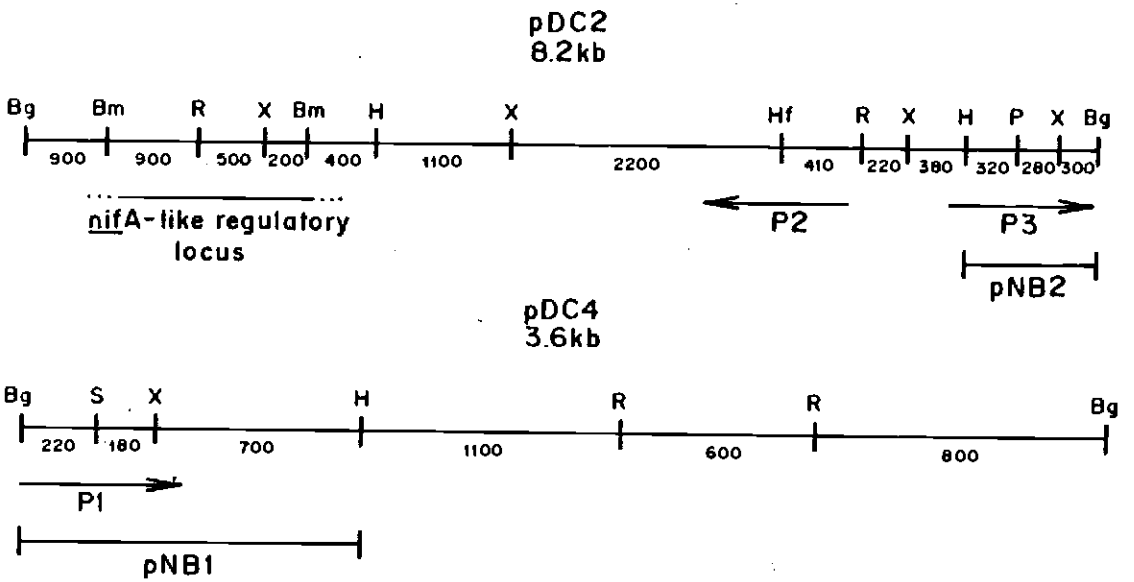

Fig. 15. Subcloning of nifP1 and nifP3 gene regions from pDC4 and pDC2 into M13mp8. Plasmids pDC2 and pDC4 were digested with BglII and HindIII restriction endonucleases, and the resulting fragments were purified from low melting point agarose gels. The fragments designated pNB1 and pNB2 in the figure were separately cloned into HindIII-BamHI-digested M13mp8 as described in Materials and Methods. Restriction endonuclease analysis of recombinant phage confirmed the presence of these two fragments in the respective clones.

ments in the clones. These subclones contain the 5' regions of the nifP1 and nifP3 operons, but pNB1 lacks 86 bases at the 5' end of the 160 base pair upstream region conserved among Rhizobium nif gene promoters, and pNB2 lacks 120 bases at the 5' end of this region (Better et al. 1983).

Hybridization of in vitro transcripts to Southern blots of plasmid pDC4, which contains the nifHDK operon. Because the R. meliloti HYP9 strain causes a higher plant yield than the wild type strain when used as inoculant, and as the mutation in this strain has been localized to the β subunit, the possibility that the HYP9 RNA polymerase may show different specificity of nif gene transcription was examined by Southern blot hybridization, and compared with the pattern of transcription obtained with the wild type enzyme. Autoradiograms of probes prepared with either wild type or mutant HYP9 RNA polymerase on supercoiled plasmid pDC4, which contains most of the nifHDK operon, hybridized to Southern blots of BglII-EcoRI and BglII-SalI-digested pDC4 DNA are shown in Fig. 16. The bands containing the transcription start site and the adjacent downstream region of the nifP1 operon show much greater hybridization of probe prepared with the mutant HYP9 RNA polymerase as compared with wild type enzyme. All bands showed some hybridization, presumably due to transcription read-through from pBR322 vector promoters. These autoradiograms were scanned with a Zeineh Soft Laser Scanning Densitometer, and data was analyzed with an Electrophoresis Reporting Integration Program (Biomed Instruments, Fullerton, CA) on an Apple IIe computer to quantitate the amount of probe hybridizing to each band. The results of this analysis are recorded in Table 6. The

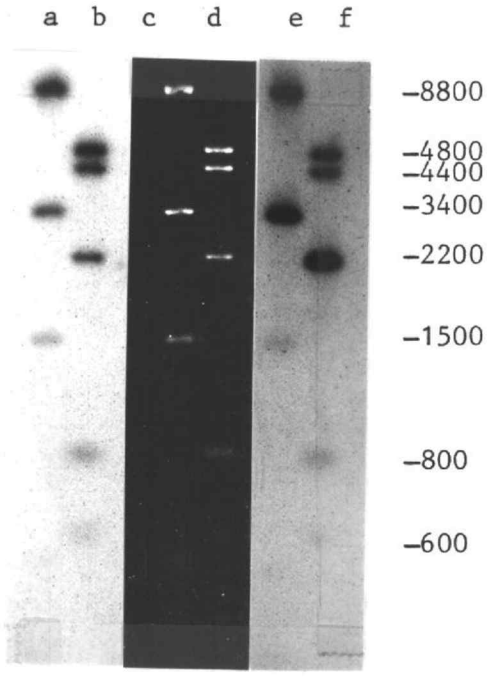

Fig. 16. Hybridization of <u>in vitro</u> transcription probes produced from plasmid pDC4 by purified wild type and HYP9 RNA polymerase to Southern blots to compare transcriptional specificity of the two enzymes. [α^{32}P]-UTP-labelled transcripts were prepared <u>in vitro</u> from supercoiled plasmid pDC4 as template, as described in Materials and Methods. These probes were then hybridized to Southern blots of BglII-SalI- or BglII-EcoRI- digested plasmid pDC4. Lane c shows the fragments detected by ethidium bromide staining of a electrophoresis gel containing BglII-SalI restriction fragments blotted for hybridization (see Fig. 1). Lane d shows the fragments generated by BglII and EcoRI digestion. Lanes a and b show the results of hybridization of the transcription probe prepared by wild type enzyme on the plasmid template. Lanes e and f show the results of hybridization of the probe produced by the HYP9 RNA polymerase. The size of each fragment is labelled.

Table 6. Hybridization of *in vitro* transcripts prepared using wild type and mutant RNA polymerase to Southern blots of plasmid pDC4. [α^{32}P]-UTP-labelled transcript probes were prepared from the supercoiled plasmid template as described in Materials and Methods.

Restriction enzymes used	DNA fragment size[a]	Amount of WT probe hybridizing[b]	Amount of HYP9 probe hybridizing	HYP9/WT
BglII and EcoRI	4800 b vector DNA	4600	4600	1.0
	4400 b vector DNA	3140	3140	1.0
	2200 b *nifP1* 5' region	1700	10200	6.0
	850 and 800 b vector and *nifP1* 3' region	980	1200	1.2
	600 b *nifP1* internal region	300	280	1.0
BglII and SalI	8800 b vector DNA	8900	8900	1.0
	3400 b *nifP1* coding region	4200	14100	3.4
	1500 b vector DNA	940	910	1.0

Autoradiograms resulting from hybridization of transcripts to Southern blots of plasmid pDC4 digested with the enzymes shown in the first column were scanned with a Zeineh Soft Laser Scanning Densitometer and data was analyzed with an Electrophoresis Reporting Integration Program on an Apple IIe computer, and the data was tabulated.

[a]See Fig. 1 for location and relation of fragments on plasmid pDC4.

[b]Values are in arbitrary units based on the areas under the corresponding peaks for each DNA fragment detected on the autoradiogram.

fragments which contain the nifH structural gene at the 5' end of the nifP1 operon in plasmid pDC4 show a 3.4-6.0 fold greater hybridization of probe prepared with the HYP9 RNA polymerase as compared with wild type. Other bands corresponding to the nifDK region of the operon did not show any differential binding of the probe (Table 6). This suggests that the mutant enzyme is capable of initiating transcription from the nifH region of the nifP1 operon, but that this transcription terminates before reaching the nifD portion of the operon. However, this does not provide any evidence that the increased transcription activity with HYP9 RNA polymerase is due to initiation from the nifP1 promoter transcription start site used by nifA-stimulated wild type RNA polymerase.

A partially purified protein extract from bacteroids, containing the proposed nif regulatory protein, stimulates ^{32}P-incorporation by wild type RNA polymerase on nifP1 and nifP3 linear templates. Transcription of the nif genes is known to be activated by a positive regulatory protein with a molecular weight of 55,000 to 60,000 in wild type cells of R. meliloti. To determine if proteins of 55,000 to 60,000 molecular weight from nitrogen fixing bacteroids can stimulate nif gene transcription, in vitro incorporation assays were performed. The results of these assays, using linear templates containing the nifP1 or nifP3 promoters are shown in Fig. 17. From each 100 µl reaction carried out as described in Materials and Methods, 5 µl was withdrawn, precipitated with cold 5% TCA, and filtered to determine incorporation of [α^{32}P]-UTP. When the 1400 base nifP1 fragment or the 980 base nifP3 fragment were used as template (see Fig. 18), both of which contain the entire 160 base pair

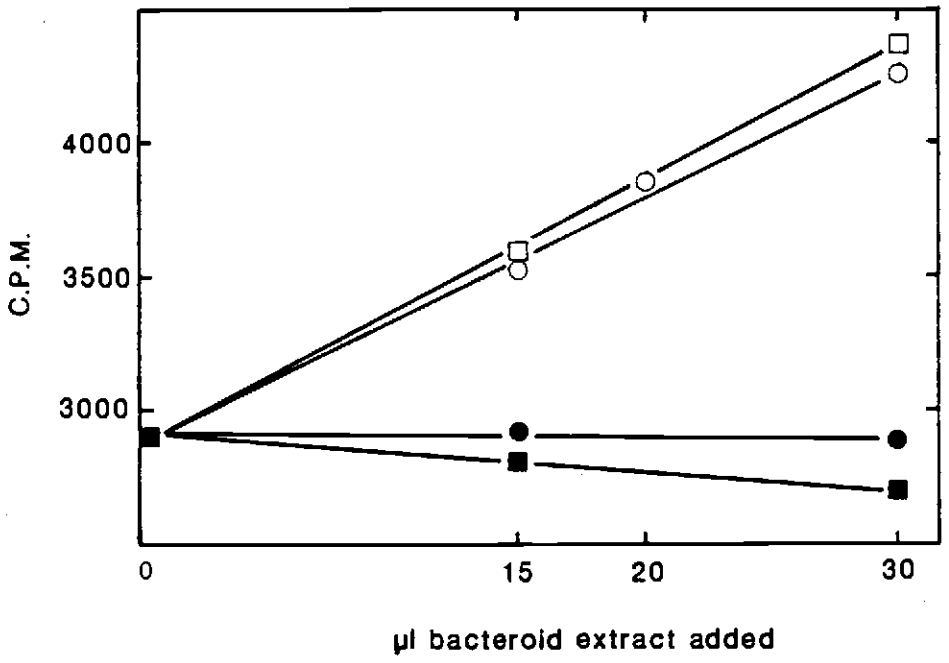

Fig. 17. Stimulation of ^{32}P-UTP incorporation with R. meliloti RNA polymerase on nifP1 and nifP3 linear DNA templates by a partially purified bacteroid protein extract. The 55,000 to 60,000 molecular weight proteins from isolated bacteroids were eluted from SDS-polyacrylamide gels by the method of Hager and Burgess as described in Materials and Methods and in Fig. 14. This bacteroid extract, which presumably contains the nif regulatory protein, was added to RNA polymerase run-off transcription reactions on nifP1 (circles) or nifP3 (squares) templates in a 100 µl reaction as described in Materials and Methods, and 5 µl was withdrawn, TCA precipitated, and filtered to determine the amount of ^{32}P incorporated. Templates used are (see Fig. 18): ●, 1100 base pair nifP1 fragment lacking 86 bases of the 160 base pair conserved region upstream of the transcription start site; ○, 1400 base pair nifP1 fragment containing the entire 160 base pair upstream region; ■, 900 base pair nifP3 fragment lacking 110 bases of the upstream region; and □, 980 base pair nifP3 fragment containing the entire 160 base pair upstream region.

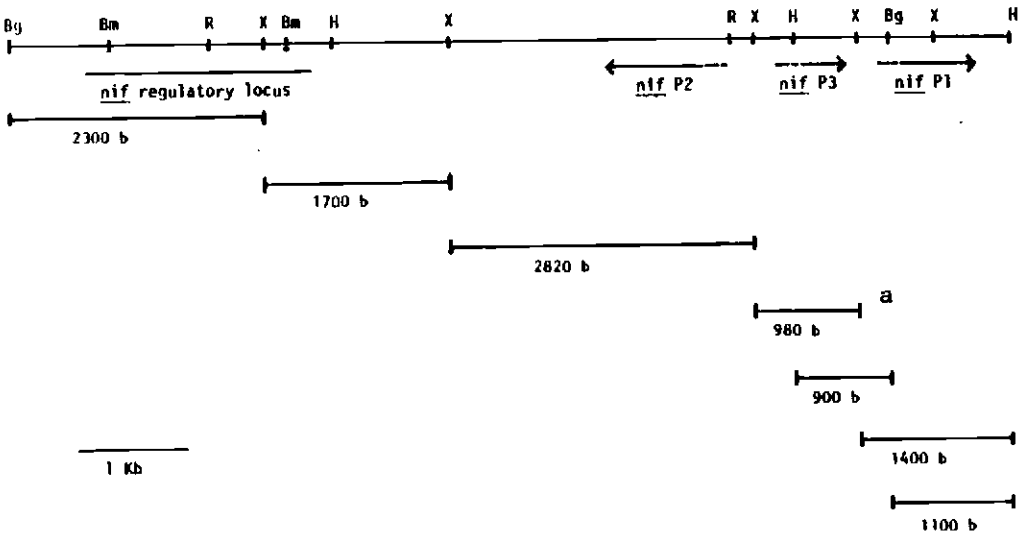

Figure 18. Restriction fragments from plasmids pDC2 and pDC4 used for *in vitro* transcription run-off assays. Plasmids pDC2 and pDC4 were digested with BglII and either HindIII or XhoI, and the resulting fragments were separated on a low melting point agarose gel and purified as described in the Materials and Methods section. The 1400 base pair fragment covering the *nif*P1 promoter region was constructed hy ligating a 300 base XhoI-BglII fragment from the 3' end of the cloned region in pDC2 with a 1100 base BglII-HindIII fragment from the 5' end of the pDC4 subclone region. The arrows indicate the initiation site and direction of transcription of the *nif* promoters, with the 160 base pair upstream conserved region shown as a broken line. The location of the *nif* regulatory locus is also shown.

[a]This fragment has recently been determined to be 917 base pairs by nucleotide sequence analysis (Better et al. 1983).

conserved region upstream of the transcription start site, the addition of the bacteroid protein extract containing 55,000 to 60,000 molecular weight proteins, including the nifA-like activator protein (Szeto et al. 1984), resulted in stimulation of ^{32}P incorporation, compared with templates lacking portions of the upstream 160 base pair region, as shown in Fig. 17. These shorter templates show no stimulation by the bacteroid protein extract, and addition of other bacteroid proteins outside this molecular weight range to control assays also resulted in no stimulation (not shown). The locations of the cut sites generating the shortened templates are shown in Fig. 19 for nifP1 and in Fig. 20 for nifP3.

Analysis of in vitro transcripts on formaldehyde-agarose gels. One method of detecting specific transcription from a particular promoter in vitro is to use truncated linear DNA templates containing individual promoters to measure on gels whether run-off transcripts of the expected size are made. In order to determine whether this system would work for analysis of transcripts made with R. meliloti RNA polymerase, a linear fragment containing the tetracycline resistance promoter was used to generate a run-off transcript, which was then analyzed on a formaldehyde-denaturing agarose gel. This gene is expressed in both E. coli and R. meliloti (Ditta et al. 1980), so it should serve as a good template for in vitro analysis. Transcription run-off from this fragment yielded the expected 880 base transcript, as well as some smaller RNA of about 250-350 bases (Fig. 21 lane b).

The results of transcription run-off assays using a 2319 base linear fragment from plasmid pBR322 containing the RNA primer for

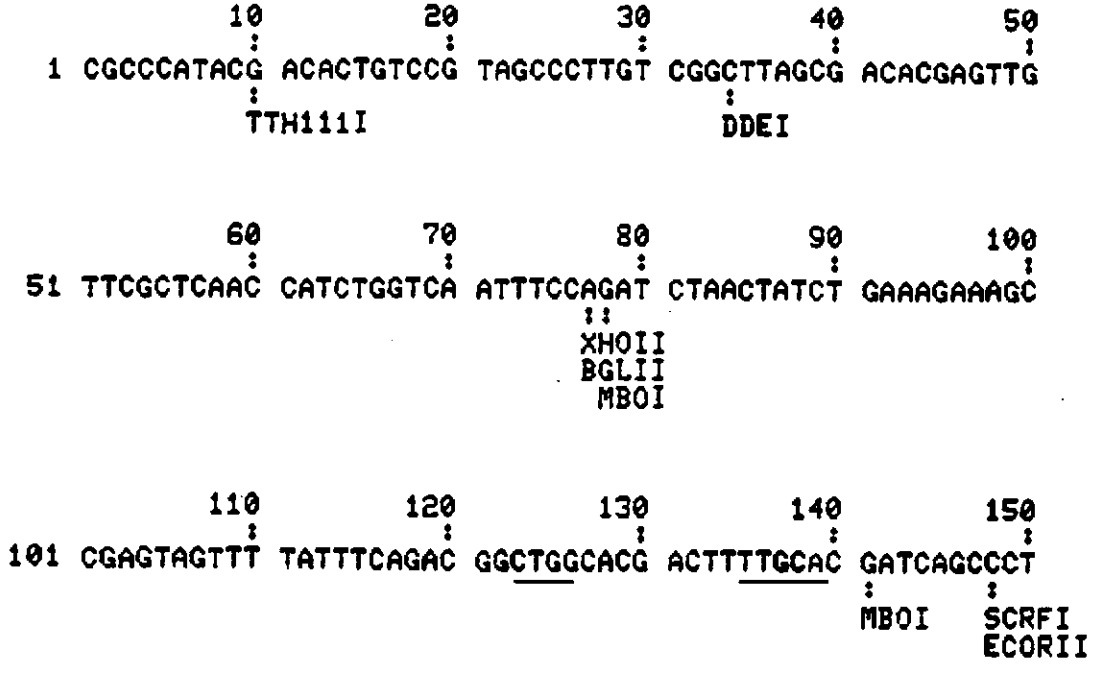

Fig. 19. Nucleotide sequence of the conserved upstream region of the nitrogenase structural gene (nifP1) promoter from Rhizobium meliloti (Better et al. 1983). The sequence reads 5' to 3' from left to right, and the last base shown is the start site of transcription. The nif promoter homologous regions at -26 to -23 and -14 to -10 are underlined. The first bases of restriction endonuclease recognition sites determined by computer analysis are designated below the sequence.

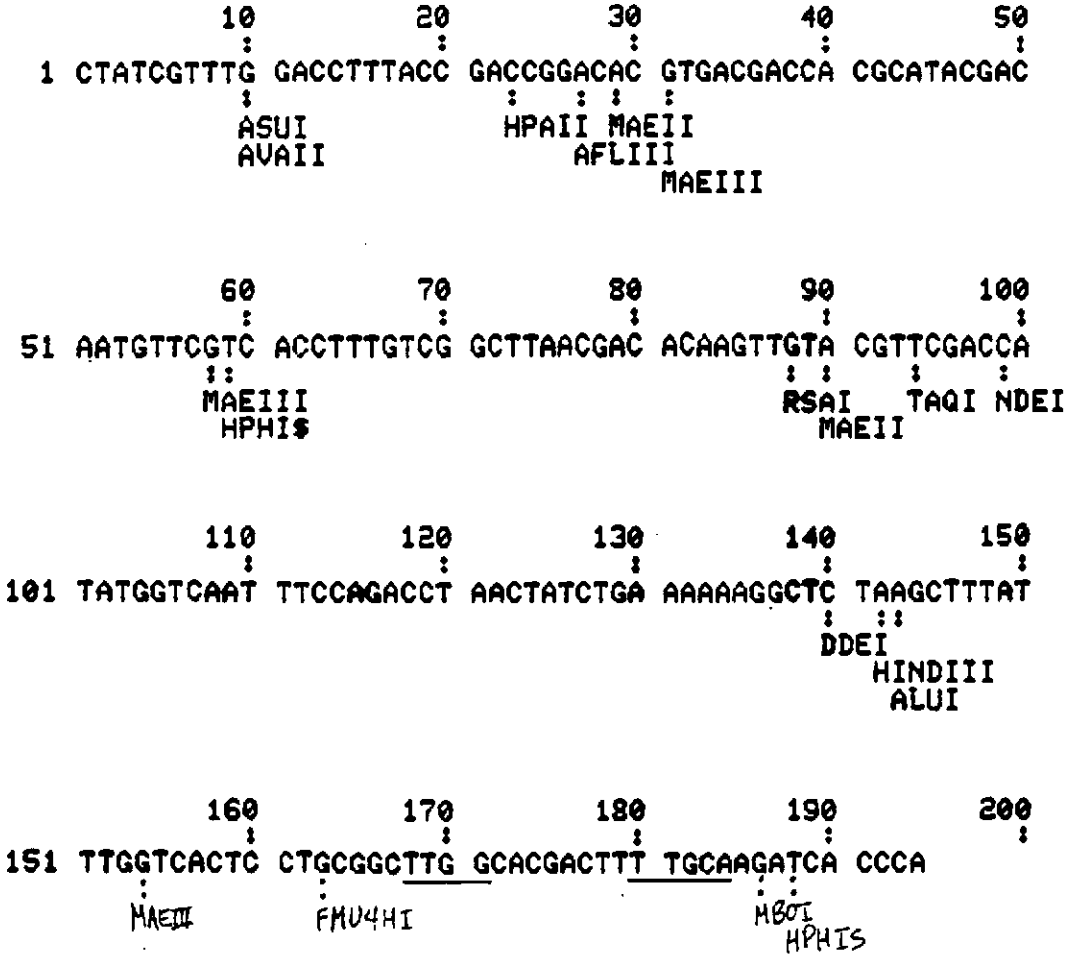

Fig. 20. Nucleotide sequence of the upstream conserved region of the nifP3 promoter from Rhizobium meliloti (Better et al. 1983). The format is the same as in Fig. 19.

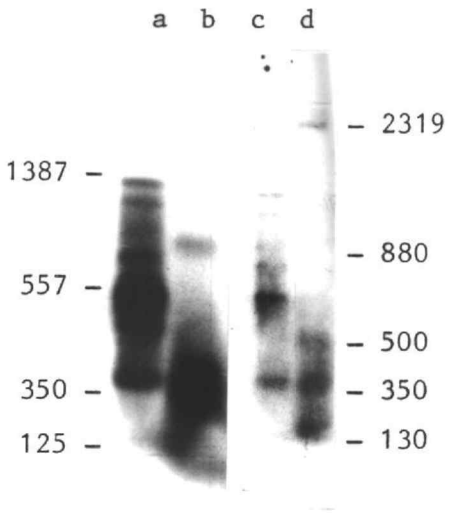

Fig. 21. In vitro run-off transcripts prepared by purified RNA polymerase on templates containing E. coli consensus promoters. Run-off transcripts were prepared as described in Materials and Methods, and separated on formaldehyde-denaturing mini-sub agarose gels. Lanes a and c show RNA molecular weight standards, with the sizes of the major bands labelled. Lane b shows the 880 base run-off transcript expected from a 930 base pair EcoRI-BglI fragment containing the tetracycline resistance gene. Lanes c and d are from another gel. Lane d shows the run-off transcripts generated from a 2319 base pair BglI fragment from plasmid pBR322 which contains the 104 base RNA-1 promoter and the RNA primer for DNA replication. The sizes of the transcripts are listed as the number of bases measured by comparison with the standards. Theses transcripts were prepared with wild type R. meliloti RNA polymerase.

DNA replication, the 104 base RNA-1 repressor RNA, and promoter P4, which requires the cAMP-repressor protein for activation (Rodriguez and Tait 1983), are shown in lane d of Fig. 21. A 2319 base end-to-end transcript can be seen, as well as 3 distinct smaller bands with measured sizes of 350, 200, and 130 bases. Run-off transcription from the 104 base RNA-1 promoter would generate a 500 base transcript, while run-off transcription from the RNA primer promoter would yield a 1930 base transcript, although the normal size of these transcripts in vivo is 104 and 550 bases, respectively. The transcripts observed on the gel may be due to premature termination of transcription from one or more of these promoters.

Analysis of run-off transcripts from nif promoter fragments. This system was then used to measure transcripts generated from linear templates containing nif gene promoters. Fig. 22 shows the transcripts generated when fragments containing the nifP3 promoter were used as templates. For this promoter, only end-to-end transcripts were observed with either of the two templates used when wild type or mutant RNA polymerase alone was used to prepare the transcripts. However, when the 55,000 to 60,000 molecular weight bacteroid proteins, including the proposed nif gene activator protein, was added, specific transcripts of 550 and 350 bases were observed only with the template that contains the entire 160 base pair region upstream of the nifP3 transcription start site (lane c). The other template, cut with HindIII, lacks all but 50 bases of this region (Fig. 20), and shows no specific activation of transcription (Fig. 22 lane f). These results are consistent with the stimulation data presented in Fig. 17 for the ^{32}P incorporation assays.

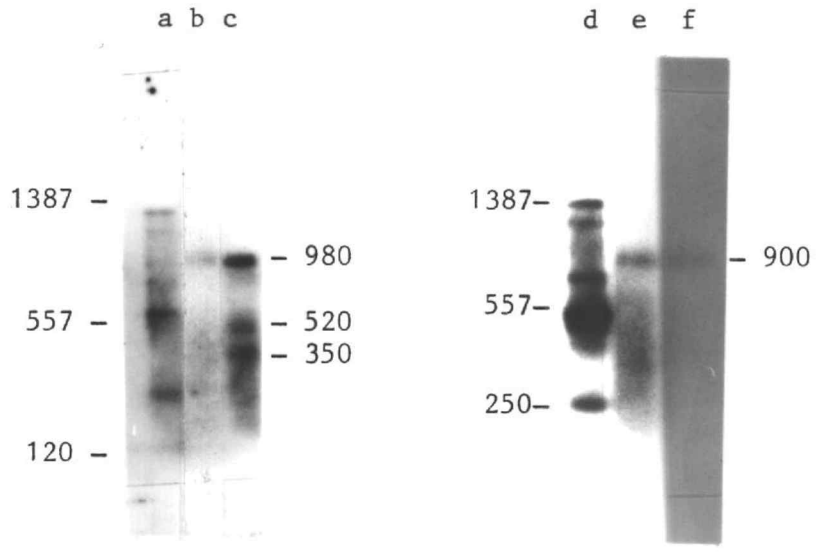

Fig. 22. In vitro run-off transcription on linear templates containing the nifP3 promoter. Run-off transcripts were prepared and analyzed as described in the legend to Fig. 21. Lanes a and d contain RNA molecular weight standards, with the lengths of the major bands labelled. Lane b shows run-off transcription from a 980 base pair XhoI fragment from pDC2 containing the entire 160 base pair upstream conserved nif gene promoter region (see Fig. 18) by wild type RNA polymerase in the absence of any additional factors. Lane c shows specific run-off transcription resulting in transcripts of 520 and 350 bases on the same template, but with the addition of a partially purified bacteroid protein extract which contains the presumed R. meliloti nifA regulatory protein. Lanes d and e show run-off transcription from a 900 base HindIII-BglII fragment from pDC2 which lacks all but the promoter-proximal 50 base pairs of the upstream conserved region of nifP3 (Fig. 18), in the absence (lane e) or presence (lane f) of the bacteroid protein extract. Lane f was exposed longer to show the end-to-end transcript. The length of the transcripts is shown as the number of base pairs. The XhoI fragment used as template has been found by nucleic acid sequencing to be 917 base pairs Better et al. 1983).

The *in vitro* incorporation analysis of the *nifP1* templates in Fig. 17 showed similar results as for the *nifP3* templates, suggesting that specific transcription from the *nifP1* promoter was occurring, similar to the case for the *nifP3* promoter. However, when the 1400 and 1100 base pair linear fragments covering the *nifP1* operon (Fig. 18) were used as templates for transcription run-off, no specific bands could be observed on agarose gels, although considerable activity could be seen in the lower molecular weight region of the gels (not shown), suggesting that prematurely terminated RNA transcripts of short, nonspecific length are being generated. This was found for several attempts with both the wild type and HYP9 RNA polymerases, and with or without the addition of the bacteroid protein extract.

The template containing the *nifP2* promoter (Fig. 18) showed no specific transcription other than end-to-end transcription with either wild type or HYP9 RNA polymerase (Fig. 23 lanes b and c), while addition of the 55,000 to 60,000 molecular weight bacteroid proteins caused a stimulation of transcription activity which was observed on the gel in the 400-500 base region rather than as a distinct band of 2600 bases which would be expected from transcription run-off. The *nifP2* promoter region does not contain convenient restriction enzyme cut sites within the 160 base pair upstream region (Fig. 24) to do similar cuts as with the *nifP3* operon to determine if all of this region is required for activation by the regulatory protein.

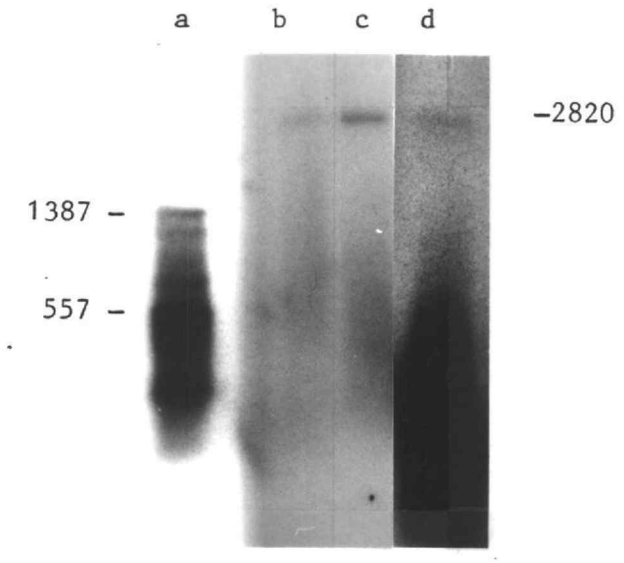

Fig.23. In vitro run-off transcription on a linear template containing the R. meliloti nifP2 promoter. Run-off transcripts were prepared and analyzed as described in the legend to Fig. 21. Lane a contains RNA standards, with the lengths of the major bands labelled. This lane was exposed for a shorter time in order to better resolve the bands. Lanes b and c show the end-to-end transcript generated from a 2820 base pair XhoI fragment of pDC2 (Fig. 18) using HYP9 and wild type RNA polymerase, respectively. Lane d shows the transcription generated when the bacteroid nifA-like protein extract was added to wild type enzyme on this template. This lane is overexposed in order to show the end-to-end transcript.

```
  1  CCTATACGCA AAGAAACCCG CCGTCGAGCG GATGATTGCC GCAGCCATAT
                           :     :       :   :    :
                          TAQI  FOKI     FNU4HI   NDEI
                                           BBVI
                                           FNU4HI

 51  GACATTGCCG TCGCCTCTGT CGGCCCCTCG ACAGATTGTT CCTTCAAGCA
                 :         :   : :                 :  ::
                HNLI      ASUI  TAQI              TTH111IL
                          HAEIII                    SPHI
                          NLAIV                     NSP7524I
                              MNLI                   NLAIII

101  TGCGGCCAAT TTCCCGATCT AACTATTTGA AAGAAAGCAA TTAGCATTAT
      :::              :
     FNU4HI          MBOI
      GDIII
      CFRI
       HAEIII

151  TTCAGTCACC TCTGCGACCT GGCACGACTT TTGCACGATC ATCCCC
        ::  :          :                  :  :
       MAEIII         ECORII             MBOI
        HPHI          SCRFI                 FOKI
           MNLI
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Fig. 24. Nucleotide sequence of the upstream conserved region of the nifP2 promoter from Rhizobium meliloti (Better et al. 1983). The format is the same as in Fig. 19.

DISCUSSION

The recent analysis of the promoter structure of the nitrogen fixation genes in Klebsiella pneumoniae and Rhizobium meliloti indicates that they exhibit very poor homology to the normal gram-negative consensus promoter sequence (Beynon et al. 1983; Hawley and McClure 1983; Better et al. 1983). However, these nitrogen fixation genes show almost complete homology with each other at the -14 and -26 regions, and in R. meliloti there is 85% sequence homology over the 160 base pairs upstream of the transcription start site. Several plasmid-encoded antibiotic resistance factors are expressed in both E. coli and R. meliloti (Ditta et al. 1980), indicating that the RNA polymerase from both organisms is able to utilize the same promoters. Recent experiments indicate that the nifA gene product is required for the expression of all other nitrogen fixation genes in a number of species (Drummond et al. 1983; Ow and Ausubel 1983; Sundaresan et al. 1983). Additional evidence indicates that the entire 160 base pair upstream region conserved among the nif gene promoters is required for maximal expression of the nif genes (Drummond et al. 1983; Better et al. 1984). The mechanism by which the nifA protein enables the RNA polymerase, which normally recognizes and transcribes genes containing homology at -10 and -35 to E. coli consensus promoters, to recognize the nif gene promoters showing a very different homology, is a complex question. The nifA protein and/or other protein(s) may bind directly to an RNA polymerase subunit to alter the structure and also the promoter specificity of the enzyme, or the protein(s) may bind to a specific site

in the conserved upstream region of the nif gene promoters to facilitate effective RNA polymerase binding. To approach this problem, the RNA polymerase from free-living cells was purified, and the subunit composition of the enzyme was determined.

RNA polymerase purification. The method used for purifying the RNA polymerase resulted in a 420-fold purification of the enzyme from R. meliloti. Although up to one-third of the enzyme activity was lost with the 0.45 M KCl wash of the DNA agarose column, lower salt concentration washes were insufficient for removing several contaminating proteins which were removed with the 0.45M KCl wash. SDS-polyacrylamide gel analysis of the 1.0M KCl wash of the DNA-agarose column showed the presence of 7 protein bands in the holoenzyme fraction (Fig. 5), rather than the four present in the general structure $\alpha_2\beta\beta'\sigma$ found in other bacteria. However, densitometric tracing of Coomassie Blue stained gels showed that two of the additional polypeptides (86,000 and 63,000 molecular weight) in this fraction are present in much lower than stoichiometric amounts (Fig. 6). These are possibly minor contaminants carried through the purification procedure, as the addition of these proteins eluted from gels to purified RNA polymerase caused no alteration of enzyme activity on pRK290 or poly(dAdT). The other extra polypeptide has a molecular weight of 29,000, which we have designated as τ, and is present in stoichiometric amounts. The stoichiometry of the holoenzyme determined by densitometry is $2\alpha:1\beta:1\beta':0.7\sigma:1\tau$. The subunit structure of the RNA polymerase from R. meliloti is compared with the enzyme from other members of the Rhizobiaceae in Table 7. The measured molecular weights of the subunits are similar, but not

Table 7. Molecular weight and subunit composition of RNA polymerase of Rhizobiaceae

Subunit	Rhizobium meliloti[a]	Rhizobium leguminosarum[b]	Rhizobium japonicum[c]	Agrobacterium tumefaciens[d]
β'	155,000	149,000	150,000	160,000
β	151,000	143,000	150,000	150,000
α	43,000	42,000	41,000	41,000
σ	93,000	93,000	96,000	98,000[e]

[a]This report

[b]Lotz, Fee, Wohlleben and Burkhardt (1981)

[c]Regensburger and Hennecke (1983)

[d]Knopf (1974)

[e]Reported as χ subunit; sigma-like properties

identical, among these enzymes.

Identification of the σ subunit. The 93,000 molecular weight protein associated with R. meliloti holoenzyme fractions was shown to stimulate the activity of the core enzyme on specific templates, which is characteristic of σ subunit function. The subunit predicted to be σ from the work on R. leguminosarum and Agrobacterium tumefaciens appears to be correct on the basis of size (Lotz et al. 1981; Knopf 1974).

Densitometric analysis of holoenzyme in polyacrylamide gels showed that the σ subunit is present in less than a stoichiometric amount, ranging from 0.5 to 0.9 relative to the other subunits. The average value for σ among several preparations was 0.7. This lower value for σ in relation to the other subunits is not unusual, as σ is not tightly bound to the RNA polymerase complex, and is removed from core polymerase by passage of the enzyme through the phosphocellulose column. A similar variation in σ subunit concentration has been observed in enzyme purifications of RNA polymerase from other gram-negative organisms (Engbaek et al. 1976; Iwakura et al. 1974).

Identification of the β subunit. The β subunit of bacterial RNA polymerase contains the binding site for the ribonucleoside triphosphate substrates as well as rifampicin, a transcription inhibitor (Ishihama and Ito 1972; Palm et al. 1975). In E. coli, the smaller of the two large subunits binds rifampicin (Rabussay and Zillig 1969), while in Bacillus subtilis, the largest subunit binds rifampicin (Halling et al. 1977). Therefore, when examining the RNA polymerase from a new species it is not always correct to

identify a particular polypeptide as the β subunit on the basis of molecular weight relationship alone. By reconstituting enzyme from various combinations of individual subunits isolated from both rifampicin-sensitive and rifampicin-resistant strains of R. meliloti, and assaying the reconstituted enzyme in the presence and absence of rifampicin, the subunit which confers rifampicin resistance on the enzyme can be identified (Heil and Zillig 1970). Reconstitution assays performed in this way led to the identification of the smaller of the two large subunits as the subunit responsible for rifampicin resistance in R. meliloti, so this was designated as the β subunit (Table 5). Identification of the subunit responsible for rifampicin sensitivity in the RNA polymerase from other members of the Rhizobiaceae family has not been completed, and the designation of β and β′ in the enzyme from R. leguminosarum and A. tumefaciens is based on size alone, analogous to the E. coli enzyme structure (Lotz et al. 1981; Knopf 1974).

The τ polypeptide. The τ polypeptide that copurified through both DNA-agarose and phosphocellulose columns with the RNA polymerase is much larger than the 12,000 molecular weight ω protein associated with E. coli polymerase (Burgess 1969). The presence of τ in stoichiometric amounts in R. meliloti RNA polymerase preparations indicates that it may well be a subunit of the RNA polymerase, but reconstitution assays containing all of the other enzyme subunits except for τ indicated that the τ subunit is not required for polymerizing activity. As soon as a sufficient number of R. meliloti promoters are identified, transcription run-off assays to test if the τ subunit is involved in promoter specificity can be performed.

RNA polymerase activity on various templates. Purified RNA polymerase from R. meliloti showed polymerizing activity on several plasmid and phage DNA templates (Table 3) which contain genes with closely homologous promoter sequences to the consensus E. coli promoter sequence (Hawley and McClure 1983). This data suggests that R. meliloti RNA polymerase can recognize and transcribe at least some E. coli genes, although all of the recognition and initiation sites are not the same in these two organisms. The RNA polymerase from R. leguminosarum and R. japonicum was found to bind specifically to E. coli phage and plasmid gene promoter regions by electron microscopy (Lotz et al. 1981) and filter binding assays (Regensburger and Hennecke 1983), which also suggests that the enzyme from Rhizobium has similar promoter specificity to the enzyme from E. coli. Transposon Tn5-encoded streptomycin resistance is expressed in R. meliloti but not in E. coli, suggesting a difference in the regulation of expression of this gene in these two organisms (Putnoky et al. 1983; O'Neill et al. 1984). There is some evidence that in R. meliloti the streptomycin resistance gene may be transcribed from its own promoter which is not recognized by E. coli RNA polymerase (Mazodier et al. 1983). Other experiments analyzing polar mutations in this region suggest that this regulation is post-transcriptional, and that Tn5-encoded streptomycin resistance in R. meliloti is expressed from the adjacent upstream kanamycin resistance gene promoter of the transposon (Putnoky et al. 1983; Selvaraj and Iyer 1984). It is apparent that there may be some difference in the promoter specificity of the RNA polymerase from R. meliloti and E. coli, as the nifHDK genes are not transcribed when a R. meliloti megaplasmid is

transferred to E. coli (Hirsch et al. 1984), although this recipient strain was able to form pseudonodules on alfalfa. Analysis of in vitro transcription from specific Rhizobium gene promoters, when more are identified, should resolve what is important for Rhizobium RNA polymerase promoter recognition.

Sensitivity of the purified enzyme to transcription inhibitors. The activity of purified R. meliloti RNA polymerase was reduced by the addition of rifampicin, streptolydigin, or lipiarmycin (Fig.8), which are inhibitors of transcription in bacteria. In E. coli, rifampicin was found to block the translocation step which follows the formation of the first phosphodiester bond during transcription initiation (McClure and Cech 1978). Streptolydigin blocks the elongation step of transcription by interfering with the binding of the ribonucleoside triphosphate substrates to the elongation site of the enzyme (McClure 1980). Lipiarmycin prevents the formation of the first phosphodiester bond, but does not affect RNA polymerase binding to the promoter (Sonnenshein and Alexander 1979). The sensitivity of the RNA polymerase from R. meliloti to these transcription inhibitors suggests that the structure and mechanism of activity of the enzymes in this organism is similar to the RNA polymerase of E. coli and other gram-negative bacteria. In addition, the isolation and characterization of enzyme subunits from the HYP9 R. meliloti strain showed that the next largest subunit of the enzyme was responsible for rifampicin resistance, similar to the enzyme from E. coli (Heil and Zillig 1970).

Rifampicin-resistant RNA polymerase. The RNA polymerase from the high plant yielding hyp9 mutant rifampicin-resistant R. meliloti

strain was unexpectedly found to be stimulated by increasing amounts of rifampicin in *in vitro* transcription assays. This stimulation was also observed in reconstitution assays when the β subunit from the rifampicin-resistant RNA polymerase was present. A similar stimulation was found by Sumida-Yasumoto and Doi (1977) in rifampicin-resistant mutant strains of *Bacillus subtilis*. They suggest that the RNA polymerase, although resistant to inhibition by the drug, may still bind rifampicin, and that the mutant enzyme is not fully active. They postulate that the stimulation observed may actually be a correction of the mutant enzyme conformation to a more native and active form. Affinity studies could be used to determine whether rifampicin actually binds to the mutant enzyme.

Alteration of rifampicin resistance by a 65,000 molecular weight protein. Another unexpected observation was that partially purified RNA polymerase from the rifampicin-resistant *R. meliloti* strain showed some sensitivity to rifampicin, decreasing to about 60-70% of the original activity at 520 μM rifampicin. By individually purifying the extra proteins in the partially pure fraction and adding these proteins back to purified rifampicin-resistant holoenzyme, a 65,000 molecular weight protein was identified as being responsible for this phenomenon. This protein causes a drop in holoenzyme activity in the presence of rifampicin, but not in its absence. This suggests that the 65,000 molecular weight protein may interact with the RNA polymerase to alter its configuration and make it susceptible to inhibition by rifampicin. This is consistent with the hypothesis presented above that rifampicin is still able to bind to the rifampicin-resistant RNA polymerase.

RNA polymerase purified from a spontaneous rifampicin-resistant mutant R. meliloti strain did not show a stimulation of activity in the presence of rifampicin, or a decrease in activity of partially purified enzyme when rifampicin was added. The 65,000 molecular weight protein purified from the hyp9 RNA polymerase fractions caused no change in the resistance of this enzyme, suggesting that the site of the rifampicin resistance mutation in the β subunit is different in these two strains.

Any possible role of the 65,000 molecular weight protein in promoter specificity of R. meliloti RNA polymerase is not known, but would be interesting to examine. If this protein binds the β subunit of the RNA polymerase, which is the subunit that binds rifampicin, it may alter the conformation enough to change the promoter specificity of the enzyme. Although the σ subunit of the RNA polymerase is responsible for the specificity of the enzyme, β subunit mutants showed different promoter selectivity in an in vitro mixed transcription system using mixtures of truncated DNA templates carrying specific gene promoters when compared with wild type enzyme (Nomura et al. 1984). This suggests that the β subunit can be involved in promoter selection and specificity. It is possible, then, that the rifampicin-resistance mutation in this strain and the modification of this resistance by the 65,000 molecular weight protein may alter the promoter specificity of the enzyme.

Analysis of bacteroid RNA polymerase. Separation of R. meliloti bacteroids from nodular material by sucrose gradient centrifugation led to clear identification of proteins unique to the nodule or to the bacteroid fractions (Fig.14). The β and β′ subunits of RNA poly-

merase were clearly separated in the bacteroid fraction, and showed the same migration rate as the β and β' subunits from RNA polymerase purified from free-living R. meliloti cells. Proteins of the same size as the β and β'subunits could also be seen in the bacteroid fraction. Although this suggests that the same form of RNA polymerase present in free-living cells is also present in bacteroids, the correlation between rifampicin resistance and ability to fix nitrogen (Pankhurst et al. 1982; Werner 1978) suggests that RNA polymerase structure may be involved in the expression of the nitrogen fixation genes. To resolve this, purification of RNA polymerase directly from bacteroids needs to be completed. Research to identify and purify nif gene activating factors from nitrogen-fixing cells, and to elucidate the mechanism of nif gene control can then be carried out.

The amount of protein obtained from bacteroids isolated from just four nodules is sufficient for analysis by this procedure. The sensitivity of the silver stain allows detection of as little as 2-5 ng of protein in an individual band. This sensitivity, as well as the ability to isolate bacteroids by sucrose gradients, will make this stain technique very useful for examining bacteroid and nodule proteins produced by various plant and bacterial mutants.

Subcloning of nif gene promoter fragments. Attempts to subclone nif gene promoter regions from plasmids pDC2 and pDC4 into M13mp8 resulted in the construction of recombinants pNB1 and pNB2 (Fig. 15), containing portions of the nifP1 (nifHDK) and nifP3 operons, respectively. This was done in hopes of preparing separate supercoiled plasmid templates containing individual nif gene promoters

for *in vitro* transcription studies. pNB1 lacks 86 bases (see Fig. 19), and pNB2 lacks 120 bases (Fig. 20) of the 5' end of the 160 base pair upstream conserved region of the *nif* gene promoters required for maximal activation of *nif* gene transcription (Drummond et al. 1983; Better et al. 1984).

Mutant HYP9 RNA polymerase stimulates transcription of the nifH gene as analyzed by Southern blot hybridization. To determine if there is a difference in the transcriptional specificity of the wild type and HYP9 mutant RNA polymerases, hybridization of mRNA prepared *in vitro* to Southern blots was carried out. Hybridization of RNA probes prepared using wild type or HYP9 mutant RNA polymerase on supercoiled plasmid pDC4, which carries a portion of the *nif*HDK operon, to Southern blots of restriction-digested pDC4 showed that the HYP9 enzyme caused 3.4 to 6.0 fold greater hybridization to fragments containing the *nif*P1 transcription initiation site and *nif*H coding region when compared to wild type enzyme (Fig. 16 and Table 6). All fragments showed some hybridization to probe, apparently due to readthrough transcription from strong promoters on the vector (Hawley and McClure 1983). At least some of these strong promoters can be recognized and transcribed by *R. meliloti* RNA polymerase, as shown by run-off transcription analysis (Fig. 21). The lack of increased hybridization by the HYP9 probe to the promoter-distal 800 base and 600 base BglII-EcoRI fragments containing the *nif*D and *nif*K coding regions of the *nif*P1 operon in the subclone indicates premature termination *in vitro* of transcription of the *nif*HDK template occurred prior to these sequences. The possibility of premature transcription termination will be discussed later.

In vitro incorporation of radiolabel with M13 recombinant pNB1 DNA as template showed significantly higher activity then the M13 control template when the HYP9 RNA polymerase was used (not shown). pNB1 contains the first 1100 bases of the pDC4 fragment containing the *nifH* gene, which is the same region that showed enhance transcription by Southern blot hybridization. These results, which were obtained without the addition of any other protein factors, suggest that the HYP9 RNA polymerase does not require the *nifA*-like activator protein to initiate transcription from the *nifH* gene region of the *nifP1* operon.

The rifampicin resistance mutation in the HYP9 strain was found to be in the RNA polymerase β subunit, as discussed earlier. Although the σ subunit of RNA polymerase normally confers transcriptional specificity upon the enzyme, Nomura et al. (1984) have found that the β subunit is also involved in template specificity, which is consistent with the evidence presented here. This evidence also is consistent with the correlation that has been found between rifampicin resistance of *Rhizobium* inoculant strains, which is a RNA polymerase β subunit function, and the ability to express nitrogenase reviewed earlier (Pankhurst 1977; Pain 1979; Werner 1978; Pankhurst et al. 1982). We have consistently observed increased plant size of seedling inoculated with the HYP9 strain as early as 1 week after infection compared with seedlings inoculated with the wild type strain, and this size difference is consistent with the observed increase in the dry weight of plants harvested at 6 to 8 weeks.

The results from the Southern blot hybridization experiment do not indicate whether the increased transcription observed is due to initiation from the correct nifP1 (nifHDK) start site or from another site in this region of the pDC4 plasmid, although no other transcription units have been found (Corbin et al. 1983). Analysis of in vitro run-off transcripts should allow determination of whether a transcript of the correct size is generaged by the HYP9 RNA polymerase. Run-off transcription by HYP9 RNA polymerase on a linear 1100 base pair nifHDK template containing the same 5' end as the pDC4 subclone, and extending from -74 to +1026 of the nifHDK operon, which covers the nifH and part of the nifD coding regions, failed to show the 1026 base transcript expected by run-off transcription from the correct transcription start site. Possible reasons for this will be discussed later.

The 55,000 to 60,000 molecular weight bacteroid protein extract containing the nif regulatory protein stimulates incorporation activity on linear templates of the nifP1 and nifP3 operons. As was shown in Fig. 17, a partially purified bacteroid protein extract containing the 55,000 to 60,000 molecular weight proteins causes a significant stimulation of TCA-precipitable ^{32}P incorporation on either the nifHDK or nifP3 templates which contain the entire 160 base pair upstream region implicated in nif gene activation (Drummond et al. 1983; Better et al. 1984), with wild type RNA polymerase. This group of proteins probably includes the R. melliloti nifA-like regulatory protein, which has a molecular weight of 55,000 to 60,000 (Szeto et al. 1984). The level of incorporation obtained without addition of the bacteroid proteins is due to end-to-end transcription

of the template, which can occur with fragments containing short single stranded overlaps generated by restriction enzyme digestion. This was observed in the analysis of in vitro nifP3 transcripts on gels, as shown in Fig. 22, resulting from nonspecific transcription initiated from RNA polymerase bound to the short single stranded overlaps. No stimulation by the bacteroid protein extract was observed on a nifHDK template lacking 86 bases at the 5' end of the 160 base upstream region, or on a nifP3 template lacking 110 bases at the 5' end of this region (Fig. 17). The stimulation observed is clearly dependent on the presence of greater than 74 bases of the 160 base pair upstream region and upon the addition of the 55,000 to 60,000 dalton proteins, as eluted bacteroid proteins with higher or lower molecular weights were unable to cause any noticable stimulation of incorporation.

Run-off transcripts from strong E. coli promoter-containing fragments. Transcription run-off from a linear fragment containing the tetracycline resistance promoter yielded the expected 880 base transcript, which would be generated by specific initiation from this promoter. Some smaller RNA of 250-350 bases, apparently the result of premature transcription termination, was also observed with this template.

Analysis of in vitro transcripts generated by wild type R. meliloti RNA polymerase on a linear 2319 base pair restriction fragment of plasmid pBR322 allowed the identification of three specific transcripts, of 350, 200, and 130 bases, in addition to the 2319 base end-to-end transcript. This fragment contains the RNA primer of DNA replication, the 104 base RNA-1 promoter, and

promoter P4, which requires the cAMP-repressor protein for activation. The 130 base transcript observed could correspond to the 104 base RNA, although run-off transcription from this promoter would yield a 500 base transcript. Run-off transcription from the RNA primer promoter would yield a 1930 base transcript, although the normal size of this transcript is 550 bases (Rodriguez and Tait 1983). A 1930 base transcript was not observed, but the shorter 350 and 200 base transcripts could be generated by premature termination of transcription from either of these two promoters. These two pBR322 promoters and the tetracycline resistance promoter show close homology to the consensus E. coli promoter sequences at -10 (TATAAT) and -35 (TTGACA) (Hawley and McClure 1983; Mulligan et al. 1984). These results, along with promoter-binding studies with R. japonicum RNA polymerase (Regensburger and Hennecke 1983) and R. leguminosarum RNA polymerase (Lotz et al. 1981), strongly suggest that Rhizobium RNA polymerase can recognize, bind, and initiate transcription from at least some E. coli promoters. However, S1 nuclease protection experiments would be required to conclusively show that transcription initiation occurs at the same site with Rhizobium RNA polymerase as with the E. coli enzyme.

Run-off transcripts from nif gene promoter fragments. Analysis of the nifP3 promoter gave the clearest results showing activation of transcription by the bacteroid protein extract, yielding a transcript of the expected size. The expected 550 base transcript was only obtained when the fragment containing the entire 160 base pair conserved region was used as template, and when the bacteroid protein extract containing the 55,000 to 60,000 molecular weight proteins,

including the nifA regulatory protein, was added to the in vitro transcription reaction. A 350 base transcript was also observed under these same conditions, and is apparently generated by premature termination of transcription from the nifP3 promoter rather than being transcribed from its own promoter, as this transcript was not observed with the template lacking all but 50 of the upstream conserved bases of nifP3. This is consistent with the results obtained on the incorporation of label in in vitro transcription reactions (Fig. 17).

Fig. 17 also showed stimulation of incorporation with nifP1 templates similar to that obtained with nifP3 upon addition of the nifA-like protein-containing bacteroid fraction, but analysis of run-off transcription from the nifP1 templates unexpectedly failed to identify the specific transcripts. The activity present in the samples was observed as a smear in the 100-250 base region of the gel (not shown), indicating that transcription was initiating but terminating prematurely at non-specific sites.

Analysis of in vitro transcripts from a template carrying the entire nifP2 promoter failed to show the expected 2600 base transcript which would be obtained if specific run-off transcription occurred, when either the wild type or mutant HYP9 RNA polymerase alone was present in the reactions. However, when the bacteroid extract was added to RNA polymerase on this template, transcripts in the 400 to 500 base region were obtained. These transcripts were not present as sharp bands, indicating that premature termination of transcription was occurring. As no other promoters are known to be on this fragment (Better et al. 1983), this transcription,

which is dependent upon the addition of the bacteroid protein extract presumably containing the nif regulatory protein, may be initiated from the nifP2 promoter.

Analysis of in vitro run-off transcripts did not yield the expected results for several of the templates used, as has been mentioned. There are three possible reasons for this. First, DNA supercoiling has been found to be required for transcription from some, but not all, E. coli promoters (Yang et al. 1979; Shmerling and Gragerov 1982). The transcription run-off assays utilized a linear DNA template, while the probe prepared for the Southern blot hybridization experiment, which showed differential transcription activity by the HYP9 RNA polymerase, was prepared using a supercoiled plasmid DNA template. It is not known whether DNA supercoiling is involved in nif gene expression.

Second, a transient pausing of transcription at random sites occurs up to 70% of the time during in vitro elongation of T7 phage mRNA, λ phage mRNA, and E. coli lac mRNA, primarily at GC-rich regions (Kassavetis and Chamberlin 1981). This pausing may allow premature dissociation of the RNA polymerase from the template. Analysis of sequences around the nifH coding region in the nifP1 operon (Torok and Kondorosi 1981) showed a GC-rich region followed by a short poly A sequence typical of pause sites where transcription termination can occur. This site is between the nifH and nifD coding regions.

Third, the coupling of nif gene transcription with translation may be required for complete transcription of the nif genes. The formation of RNA secondary structure has been shown to be involved

in transcription termination, and the presence of ribosomes on the growing transcript may prevent formation of RNA secondary structures which could result in premature transcription termination (Rosenberg and Court 1979; Yanofsky 1981; von Hippel et al. 1984). This could serve as an economical control mechanism to prevent transcription of the nif genes under conditions unfavorable for translation of the oxygen-labile nif structural proteins. Further work needs to be done to clarify which factors are important in nif gene expression.

Clear results were obtained for specific nifA-activated transcription from the nifP3 promoter, but the importance of this operon is not known. The nifP3 region is not required for nitrogen fixation as determined by transposon mutagenesis in this region, but it is transcribed specifically in root nodules (Better et al. 1983). The 68 base leader mRNA segment of the nifP3 transcript shows 88% homology to the leader mRNA of the nifP1 transcript, and the first 69 bases of the coding region are also highly conserved between these transcripts. There is no evidence that the nifP3 transcript is translated, and no protein product in this region has been identified, although a 620 base open reading frame located 248 bases downstream from the transcriptional start site could code for a 24,000 dalton protein (Better et al. 1983). Although it is not clear what role, if any, the nifP3 region has in the development of symbiotic nitrogen fixation, this promoter is apparently regulated similarly to the nifP1 and nifP2 promoters. The nifP2 and nifP3 promoter 160 base pair upstream sequences are 75-85% homologous to the nifP1 upstream sequences (Better et al. 1983). In lining up homologous bases, however, there are an extra 9 bases present in nifP2 and nifP3

immediately upstream of the -23 to -20 sequences (Better et al. 1983). The presence of these additional bases may suggest that these promoters may not be regulated the same as the the nifP1 promoter under all conditions.

The role of the nif regulatory protein. It is clear from the results presented that the 55,000 to 60,000 molecular weight bacteroid protein extract is capable of stimulating transcriptional activity on nifP1 and nifP3 templates containing the entire 160 base conserved region upstream of the transcription start site. This extract also activated specific transcription from the nifP3 promoter when the template contained the conserved upstream region but not when part of the upstream region was lacking. This bacteroid protein extract presumably contains the nif regulatory protein, as discussed earlier, and the results suggest that no other factors of higher or lower molecular weight are required for activation of R. meliloti nif gene transcription in vitro. In the model for nif gene regulation in Klebsiella pneumoniae, the product of a general nitrogen regulatory gen, glnF (ntrA), is required along with the nifA regulatory gene product to activate transcription of all other nif gene operons (Ausubel 1984). In K. pneumoniae, the glnF, glnL, and glnG gene products regulate the nitrogen assimilation genes such as nif, hut, and put in response to the level of available fixed nitrogen. In R. meliloti, however, the nif genes are activated only under symbiotic conditions, and not merely by nitrogen limitation of free-living cells (Ausubel 1984). Also, the addition of NH_4^+ to nitrogen-fixing bacteroids did not repress nif gene expression (Van den Bos et al. 1983), suggesting the lack of a general nitrogen

regulatory mechanism being involved in the control of nif gene expression in Rhizobium. Since the bacteroid protein extract used in these experiments contained several proteins in the 55,000 to 60,000 molecular weight range, it is possible that more than one of these proteins may be involved in the activation of nif gene transcription. In addition, one or more proteins outside this molecular weight range may be involved in maximizing nif gene expression. The identification and purification of the product of the R. meliloti nif regulatory gene should allow a more precise determination of the mechanism of nif gene control in this organism. The mode of action of this regulatory protein is not known, but the availability of the purified protein would allow the identification of its binding site, whether it is within the 160 base region upstream of the transcription start site, or whether it binds directly to the RNA polymerase to modulate promoter specificity, similar to the binding of phage proteins to RNA polymerase which occurs during bacteriophage T4 infection of E. coli to activate the expression of phage genes (Geiduschek et al. 1983).

SUMMARY AND CONCLUSIONS

The RNA polymerase was purified from wild type and rifampicin resistant mutant strains of Rhizobium meliloti by chromatography on DEAE-Cellulose, DNA agarose, and phosphocellulose. Characterization of the subunits showed that the enzyme had subunit composition and stoichiometry similar to that of the RNA polymerase from other species of the family Rhizobiaceae and other gram-negative bacteria, although some differences in the molecular weights measured were observed. In addition to the α, β, β' and σ subunits normally found in bacterial RNA polymerases, a small, 29,000 molecular weight protein was found associated with both core and holoenzyme through all steps of the purification. We have designated this polypeptide as τ, and although it was found to not be required for polymerase activity in reconstitution assays, it may be involved in promoter specificity of the enzyme. At present a sufficient number of Rhizobium promoters have not been identified, but when they become available a possible role for the τ polypeptide can then be examined. Positive identification of the σ subunit, which is responsible for promoter specificity of the enzyme, was made due to its ability to stimulate core polymerase activity. The purification of RNA polymerase from a rifampicin-resistant strain allowed the identification of the β subunit, which contains the rifampicin binding site, by reconstitution of enzyme from individual subunits purified from the wild type and rifampicin resistant enzymes. In R. meliloti, the subunit is the smaller of the two large β and β' subunits, analogous to the E. coli enzyme.

The rifampicin resistant RNA polymerase from the R. meliloti HYP9 strain showed some unusual characteristics. This strain confers a 20% higher yield of dry plant weight on alfalfa seedlings inoculated with it. The RNA polymerase holoenzyme from this strain actually showed a stimulation in polymerase activity in the presence of rifampicin, perhaps due to correction of the mutant enzyme configuration by the drug binding to the β subunit, rather than inhibiting the enzyme as occurs with wild type RNA polymerase. Partially purified RNA polymerase from this strain showed a drop in activity in the presence of rifampicin to 70% of the level obtained in the absence of the drug. Analysis of the additional proteins in this fraction led to the identification of a 65,000 molecular weight protein which caused a similar reduction in activity when added to purified holoenzyme in the presence of rifampicin but not in the absence of the drug. This protein may modify promoter specificity of the RNA polymerase holoenzyme, or otherwise modify its activity, and this analysis needs to be done with the nif gene promoters and other Rhizobium promoters as they become available.

Southern blot hybridization showed that the HYP9 RNA polymerase caused a specific stimulation of transcription of the nifH gene region of plasmid pDC4, which contains the nifHDK operon, while the wild type enzyme caused no such stimulation. This increased transcription did not continue through the nifD and nifK regions of the plasmid, but a typical GC-rich transcription pause site was found in the region between the nifH and nifD genes by analysis of the nucleotide sequence (Torok and Kondorosi 1981), which could cause

transcription termination (Kassavetis and Chamberlin 1981). Attempts to measure *in vitro* run-off transcripts from a linear template containing this *nifH* region, to determine if specific transcription from the same promoter used during symbiosis occurs with the HYP9 RNA polymerase, were unsuccessful. This transcriptional activity of the *nifH* region by HYP9 RNA polymerase may require supercoiling of the template. Analysis of the *in vitro* transcripts generated by HYP9 RNA polymerase on supercoiled plasmid templates should help determine the site from which this enhanced transcription initiates. These results suggest the RNA polymerase structure may be involved in the expression of nitrogen fixation, consistent with the correlation found by others between RNA polymerase structure and rifampicin resistance and the ability of a *Rhizobium* strain to fix nitrogen (Pain 1979; Werner 1978; Pankhurst et al. 1982).

The addition of the partially purified bacteroid protein extract presumably containing the *R. meliloti nifA*-like regulatory protein (Szeto et al. 1984) to linear DNA templates containing the *nifP1* or *nifP3* promoters, which are regulated similarly during symbiosis (Better et al. 1983), caused a significant stimulation of *in vitro* incorporation of label by the RNA polymerase, suggesting that specific transcription initiation was occurring from these *nif* gene promoters. A specific transcript of the expected size was obtained from the *nifP3* template in the presence of the bacteroid protein extract, as measured by run-off transcription analysis. This stimulation was only observed when the template contained more than 74 bases of the upstream conserved *nif* gene promoter sequence which has been found by others to be important for *nifA* activation

(Drummond et al. 1983; Better et al. 1984), and only the bacteroid proteins in the 55,000 to 60,000 molecular weight range were required. Although further characterization needs to be done, this suggests that the glnF gene product may not be required for nif gene transcription in R. meliloti as it is in K. pneumoniae, where the glnF protein has a molecular weight of 75,000-85,000 (Dixon 1984; Ausubel 1984). This is not unreasonable, as nif gene expression in R. meliloti is limited to symbiosis, rather than being activated by nitrogen limitation as occurs by the gln nitrogen regulatory genes in K. pneumoniae. The identification and purification of the R. meliloti nifA-like regulatory protein is required in order to gain a better understanding of nif gene control in R. meliloti. When this purified protein is available, identification of other bacteroid proteins involved in nitrogen fixation can be approached by in vitro studies with purified RNA polymerase. This should lead to a better understanding of the control of nitrogen fixation expression in R. meliloti.

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