Molecular Characterization of Tn5-Induced Symbiotic (Fix-) Mutants of \textit{Rhizobium meliloti}

J. LYNN ZIMMERMANN,† WYNNE W. SZETO,‡ AND FREDERICK M. AUSUBEL*  

Department of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts 02138

Received 11 July 1983/Accepted 12 September 1983

To investigate the expression of specific symbiotic genes during the development of nitrogen-fixing root nodules, we conducted a systematic analysis of nodule-specific proteins and RNAs produced after the inoculation of alfalfa roots with a series of \textit{Rhizobium meliloti} mutants generated by site-directed transposon Tn5 mutagenesis. The mutagenized region of the \textit{Rhizobium} genome covered ~10 kilobases and included the region encoding the nitrogenase polypeptides. All mutant strains that were analyzed produced nodules, but with several strains the nodules failed to fix nitrogen (Nod* Fix- phenotype). All Fix- nodules accumulated reduced levels of the host plant protein leghemoglobin. In addition, Tn5 insertions in the nitrogenase operon (nifHDK genes) eliminated some or all of the nitrogenase polypeptides and nifHDK RNA transcripts, depending on the site of insertion. Finally, mutation of a region ~5 kilobases upstream from the nitrogenase operon resulted in the absence of all three nitrogenase polypeptides and their corresponding RNAs, suggesting that this region may serve a regulatory function during nitrogen fixation. The studies presented here indicate that site-directed mutagenesis coupled with biochemical analysis of nodule proteins and RNAs allows the identification of products of specific gene regions as well as the assignment of specific functions to previously unidentified regions of the \textit{R. meliloti} genome.

\textit{Rhizobium meliloti} fixes nitrogen in symbiosis with its host plant alfalfa (\textit{Medicago sativa}). As in all \textit{Rhizobium}-legume symbioses, the production of nitrogen-fixing nodules on the roots of the host plant represents the culmination of a complex developmental sequence requiring the temporally defined expression of specific genes encoded both in the bacterial endosymbiont and in the host plant.

In \textit{R. meliloti}, at least some of the genes necessary for both nodulation (nod genes) and for the enzymatic reduction of nitrogen (nif genes) are located on a large (> 300-megadalton) "megaplasmid" (1, 10, 24; W. J. Buikema, S. R. Long, S. E. Brown, R. C. van den Bos, C. D. Earl, and F. M. Ausubel, J. Mol. Appl. Genet., in press). Overlapping cosmids clones spanning ca. 90 kilobases (kb) of the \textit{R. meliloti} megaplasmid have been isolated (Buikema et al., in press). A portion of this 90-kb region is known to encode the nitrogenase structural genes \textit{nifH}, \textit{nifD}, and \textit{nifK} (6, 25, 26, 28). Molecular genetic and DNA sequence analyses have shown that these three genes are adjacent and most likely constitute an operon (5, 28).

Other experiments have shown that a region ~20 kb downstream from the \textit{nifH} promoter encodes functions necessary for nodulation (1, 16), and a second region, covering at least 5 kb and located ~1.5 kb upstream from the \textit{nifHDK} promoter, has been suggested to contain one or two operons (5, 28) that specify functions essential for nitrogen fixation but not nodule formation.

To more specifically define the symbiotic functions encoded in the region of the megaplasmid that surrounds the nitrogenase operon, we conducted a systematic molecular characterization of several mutant strains of \textit{R. meliloti} produced by site-directed Tn5 mutagenesis (26). All of these strains form nodules which do not fix nitrogen (Nod* Fix- phenotype). We analyzed the proteins and RNAs produced in nodules after inoculation with these mutant strains and observed differences in both host plant expression and bacterial gene expression when compared with nodules produced by wild-type \textit{R. meliloti} (strain 1021). The Fix- phenotype of some of these mutants is clearly due to the absence of some or all of the nitrogenase polypeptides caused by the insertion of Tn5 into the
nitrogenase operon. Other mutants show no dramatic changes in their protein or RNA accumulation profile. Finally, we identified a region of the megaplasmid which appears to encode a product involved in the regulation of the nitrogenase operon.

MATERIALS AND METHODS

Mutagenesis of R. meliloti. The method of site-directed mutagenesis of R. meliloti with transposon Tn5 has been described previously (26). The strains analyzed were constructed by G. Ruvkun, W. Bui-kema, or S. Gibbons in our laboratory. The position of each Tn5 insertion analyzed in this study, its numerical designation, and its fixation phenotype are indicated in Fig. 1. Also shown in Fig. 1 is the site of insertion of an endogenous insertion sequence element, ISRm1, which when inserted at this location results in a nod* Fix* phenotype (27).

Assay of plants for nitrogen fixation ability. The ability of R. meliloti strains to produce effective nitrogen-fixing nodules was determined by standard acetylene reduction assays (7, 21).

Growth of plants for preparative nodule isolation. Alfalfa seeds (variety Iroquois) were germinated in sterile, moist vermiculite (coarse grade, ProGro, Inc.) underlaid with a 1-in. (ca. 2.54-cm) layer of Super Coarse Perlite (Griffin Greenhouse Supplies, Inc.). Two days after germination, logarithmically growing cultures of R. meliloti were diluted with sterile water (1:1) and poured over the plants in a sterile transfer hood. Five days post-inoculation, plants were watered with a 1:1 dilution of the solution of Warner and Kleinhof without nitrate (33); thereafter, plants were watered as needed with sterile water. Nodules were hand picked 4 to 5 weeks post-inoculation, frozen immediately in liquid nitrogen, and stored at −80°C until use.

Isolation of plant-specific and bacteroid-specific nodule proteins. Nodule proteins were extracted and fractionated by the scheme shown in Fig. 2. The resulting protein fractions were stored at −80°C until use.

Isolation of RNA from nodules. Nodules were ground in liquid nitrogen for protein preparation as described in Fig. 2. The powder was suspended in an extraction buffer (12) containing 10 mM vanadyl-ribonucleoside complex (3, 15). The suspension was filtered through two layers of sterile Miracloth (Calbiochem) prewetted with extraction buffer. The filtrate was placed on ice and adjusted to 1% Sarkosyl with moderate vortexing.

The solution was extracted three times with phenolchloroform and once with chloroform (containing 1/24 volume of isoamyl alcohol), and nucleic acid was precipitated with ethanol at −20°C. The pellet was dissolved in sterile 10 mM Tris-hydrochloride (pH 7.4)–10 mM NaCl–10 mM MgCl2, DNase (RNase-free grade, Worthington Diagnostics) was treated with iodoacetate to inactivate RNase (S. Henikoff, Ph.D. thesis, Harvard University, Cambridge, Mass., 1977). Iodoacetate was added in a ratio determined empirically for each batch of enzyme (in these experiments, 0.01 μg of DNase per μg of nucleic acid was optimal), and the solution was incubated for 30 min at 37°C. The sample was extracted twice with phenol-chloroform, extracted once with chloroform, precipitated with ethanol, and stored as a precipitate until use.

Protein gel analysis. Proteins were analyzed on sodium dodecyl sulfate-polyacrylamide gels according to O’Farrell (22). Gels were stained (9), and the amount of protein per band (expressed as a fraction of the total protein per lane) was quantitated by scanning each gel in a Kratos SD3000 spectrodensitometer (Schoeffel Instruments) equipped with a Hewlett-Packard integrator.

DNA biochemistry. Plasmid DNA was isolated as described by Godson and Vapnek (11). DNA was digested with selected restriction enzymes, displayed on a 1% agarose gel, and transferred to Gene Screen (New England Nuclear Corp.). All procedures for hybridization and washing of the transfer were as specified by the manufacturer (New England Nuclear Corp., instruction manual no. NEF-972). Prehybridization and hybridization were conducted in the presence of 10% dextran sulfate (Pharmacia Fine Chemicals, Inc.)–50% formamide (Fluka) and appropriate salts. After hybridization, filters were air dried and exposed for autoradiography.

Labeling RNA with [γ-32P]ATP. RNA samples were radioactively labeled by using polynucleotide kinase essentially as described by Maizels (19). Labeled RNA was separated from unincorporated 32P by the spincolumn procedure (20).

RESULTS

Nodule morphology. Nodules formed after inoculation with R. meliloti Fix− mutants showed characteristic differences in size, color, and distribution from nodules formed by the wild type (strain 1021) or by strains carrying Tn5 insertions in regions not essential for nitrogen fixa-
FIG. 2. Protocol for the isolation of plant- and bacteroid-specific nodule proteins. 1Nodule extraction buffer: 50 mM Tris (pH 7.4), 20 mM KCl, 10 mM MgCl₂, 0.5 M mannitol, 0.1% polyvinylpyrrolidone, 1 mM phenylmethylsulfonyl fluoride. 2Obtained from Calbiochem. 3Bacteroid protein extraction buffer: 25 mM Tris, 1 mM dithiothreitol, 20 mM Na₂S₂O₄, 2% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride.
or of wild-type nodules associated with the presence of the protein leghemoglobin. However, as will be discussed in detail below, nodules formed by Fix\textsuperscript{−} mutants contained substantial, although reduced, levels of leghemoglobin.

**Separation of protein fractions for nodule extracts.** To analyze the effect of Tn5 insertions in different regions of the *R. meliloti* megaplasmid on both plant- and bacteroid-specific gene expression within the nodule, it was necessary to sufficiently separate the plant and bacteroid fractions in total nodule extracts. Using the method outlined in Fig. 2, we achieved adequate separation of plant-specific and bacteroid-specific nodule fractions, as evidenced by the obvious differences between the proteins isolated from each of these fractions (Fig. 4). A comparison of the two center lanes [labeled "Nodule (Plant)" and "Nodule (Bacteroid)""] shows that the proteins isolated from the plant- and bacteroid-specific fractions of the same total nodule preparation are essentially free of contamination with proteins from each other. Furthermore, a comparison of proteins from Nodule (Plant) with uninfected root proteins or a comparison of bacteroid proteins with free-living *R. meliloti* proteins reveals that, although there are many bands common to both (as would be expected), there are several obvious plant and bacteroid protein bands unique to the differentiated state of the nodule.

In the plant-specific fraction, the most abundant protein migrated at \( \sim 14,000 \) daltons (Fig. 4, lane 2). This protein corresponds to the plant-encoded nodule protein leghemoglobin (Lb in Fig. 4), as evidenced by its molecular size and abundance in nodule extracts (31). Moreover, the 14,000-dalton protein comigrated with a protein extracted from nodules by the method of Jing et al. (13; data not shown) which possessed...
the spectral properties of purified leghemoglobin (data not shown).

In the bacteroid-specific proteins, several unique bands ranging from ~35,000 to greater than 92,000 daltons (Fig. 4, lane 3) are evident. Three of these protein bands, migrating at 64,000, 60,000, and 40,000 daltons, were shown by mutational analyses (see below) to be the products of the nitrogenase operon. Our studies on various Tn5 mutants demonstrated that the 40,000-dalton protein band corresponds to the \( \text{nifH} \) gene. However, as will be discussed below, it was not possible from our data to unambiguously determine which of the other two protein bands (64,000 and 60,000 daltons) corresponded to the \( \text{nifD} \) gene and which corresponded to the \( \text{nifK} \) gene. In light of this, the 64,000- and 60,000-dalton proteins are designated \( \text{nifDK} \) polypeptides.

It is apparent, then, that the nodule fractionation procedure described in Fig. 2 allows the detection of proteins produced specifically in the plant and bacteroid fractions of the nodule. Therefore, we reasonably expected to observe changes in these protein patterns in extracts from ineffective nodules produced by various Fix\(^-\) mutants.

**Plant-derived nodule proteins.** Polyacrylamide gel analysis of plant-derived proteins obtained from nodules formed after inoculation with Fix\(^-\) \( R. \) meliloti strains showed relatively few differences from nodule proteins resulting from wild-type infection (data not shown). The most obvious change was the reduced level of leghemoglobin in all nodules produced by Fix\(^-\) mutants. The amount of leghemoglobin ranged from 35 to 88% of wild-type levels as determined by quantitative densitometry of stained gels. (In each case, the amount of leghemoglobin was calculated as a percentage of the total amount of protein in a particular lane.) The relative amounts of leghemoglobin in nodules induced by each mutant strain are summarized in Fig. 7. (It should be noted that the gel system used in these experiments was a denaturing system in which the apoprotein and heme portions of the functional leghemoglobin molecule are dissociated. The quantitation refers only to the amount of apoprotein observed on these gels.)

**Bacteroid-specific nodule proteins.** Analysis of bacteroid-specific proteins from nodules induced by mutants representing each Fix\(^-\) region revealed a number of differences from the wild-type protein gel pattern (Fig. 5).

By focusing first on Tn5 insertions in the presumptive nitrogenase operon (strains 1491, 1312, and 1308; see Fig. 7 for insertion locations), it is apparent that insertion into the first gene of the operon, \( \text{nifH} \) (strain 1491), results in the absence of all three nitrogenase polypeptides (\( \text{nifH} \), \( \text{nifD} \), and \( \text{nifK} \)). The band remaining at the \( \text{nifH} \) position in strain 1491 may represent low levels of \( \text{nifH} \) protein accumulation, but more likely represents a protein that comigrates with \( \text{nifH} \) and is only obvious in the absence of \( \text{nifH} \). Insertion of Tn5 into the \( \text{nifD} \) gene, the second gene of the operon (strain 1312), results in bacteroids which produce \( \text{nifH} \) protein but which lack the \( \text{nifDK} \) polypeptides. Because Tn5 insertions are usually polar (2), these observations support previous results which indicated that the \( \text{nifHDK} \) cluster is transcribed as a single unit beginning at \( \text{nifH} \) (28). Surprisingly, strains carrying Tn5 insertions in \( \text{nifK} \) (represented by strain 1308) contained low amounts (which varied from experiment to experiment) of the 64,000-dalton polypeptide and no detectable 60,000-dalton polypeptide. This result suggests that the 64,000-dalton band corresponds to \( \text{nifD} \). However, since this band is variable and its mobility is slightly slower than the 64,000-dalton band, we cannot rule out the possibility that it is a degradation product of some other polypeptide and not really \( \text{nifD} \). It is also possible that this band represents a \( \text{nifD-Tn5} \) or \( \text{nifK-Tn5} \) fusion peptide. The reason for the reduced level of \( \text{nifD} \) polypeptide in strain 1308 is unknown; however, since the \( \text{nifD} \) and \( \text{nifK} \) polypeptides are subunits of the iron-molybdenum (FeMo) nitrogenase protein (23), it is possible that the \( \text{nifD} \) subunit is unstable in the absence of the \( \text{nifK} \) subunit. This hypothesis is supported by the RNA hybridization data presented below. Similar observations have been made with Klebsiella pneumoniae \( \text{nifK} \) insertion mutants (23). The bacteroid proteins from strains 1491 and 1308 also appear to contain polypeptides in the 21,000- to 31,000-dalton range that are absent in the bacteroids of strains 1021 and 1312. The origin of these bands is unknown.

FIG. 5. Proteins isolated from the bacteroid-specific fraction of nodules induced by Fix\(^-\) \( R. \) meliloti strains (see the text for details).
Tn5 insertions into a region spanning ca. 4 kb and starting ~1.4 kb to the right of the nifH promoter (strains 1334, 80, and 1350) or insertion of an endogenous insertion sequence (strain 1066) in this region resulted in no obvious changes in the proteins produced by the bacteroids. The protein profile is represented by mutant strain 80 in Fig. 5. On the other hand, an unexpected profile was observed in a mutant carrying a Tn5 insertion 5 kb to the right of the nitrogenase operon (strain 1354). The bacteroids of this mutant failed to accumulate detectable levels of any of the nitrogenase polypeptides (Fig. 5). Genetic complementation analysis (28) indicated that this mutant strain does not contain a secondary mutation in the nifH promoter. Moreover, independent Tn5 insertions adjacent to strain 1354 which have the same phenotype have recently been identified (W. Szeto, unpublished data). It is likely, therefore, that the mutation in strain 1354 affects a gene which functions in regulating the expression of the nitrogenase operon, presumably by encoding a trans-acting product. A more detailed analysis of the strain 1354 regulatory region will be presented elsewhere (W. W. Szeto, J. L. Zimmerman, V. Sundaresan, and F. M. Ausubel, manuscript in preparation).

The last Tn5 insertion mutation studied is located ~7 kb to the right of the nifH promoter (strain 112). The bacteroids produced by this mutant accumulated wild-type levels of all major proteins, including the nifHDK polypeptides (Fig. 5). This mutant, then, serves to delimit the potential regulatory region marked by strain 1354.

Transcription of the nitrogenase operon in Fix\textsuperscript{−} mutants. To determine whether the absence of the nitrogenase polypeptides in bacteroid proteins extracted from mutant-induced nodules is due to the absence of their corresponding mRNAs (i.e., Tn5 insertion disrupts the transcription of the region into which it is inserted), we analyzed the hybridization of \textsuperscript{32}P-labeled nodule RNA to a cloned DNA fragment containing the nifHDK operon (clone pRmR29T2 [28]). DNA from pRmR29T2 was digested with HindIII and EcoRI to generate unique fragments corresponding to each nitrogenase gene, as shown on the restriction map of the clone in Fig. 6b. The sites of Tn5 insertion corresponding to each of the mutants analyzed are also indicated in Fig. 6b. After electrophoresis through agarose and transfer to Gene Screen, the immobilized DNA was hybridized with \textsuperscript{32}P-labeled RNA isolated from nodules. The results of these hybridizations are shown in Fig. 6a and are tabulated in Fig. 6b. They can be summarized as follows. (i) RNA extracted from wild-type nodules (produced by strain 1021) hybridized to the nifHDK DNA fragments, although hybridization to the 0.9-kb fragment adjacent to nifK was barely detectable and was variable. This hybridization pattern serves as the basis for comparison with those obtained with RNA from the mutant strains. The 2.1-kb terminal restriction fragment represents a region which does not encode any essential nif function. It is transcribed in all strains tested and serves as a positive control for hybridization of nodule RNA. (ii) Hybridization with RNA from strain 1491, which carries a Tn5 insertion in the middle of the nifH gene (the first gene of the operon), resulted in hybridization to the nifH fragment and to the terminal 2.1-kb fragment but did not show detectable hybridization to any of the other fragments. Hybridization to the nifH fragment was expected since Tn5 is inserted into the middle of the gene, allowing the first half of the gene to be transcribed. This result supports the model that these three genes are organized as an operon and that the operon is transcribed from nifH to nifK (28). (iii) RNA isolated from nodules induced by strain 1312 contained nifH mRNA as well as transcripts complementary to nifD. Surprisingly, trace amounts of nifK transcripts were also detected. This could be a result of the fact that polarity caused by Tn5 is sometimes not absolute and is dependent on the position of insertion (5). (iv) Insertion of Tn5 in the nifK gene (strain 1308) resulted in nodule RNA containing wild-type amounts of transcripts from both nifH and nifD and trace amounts from nifK. This result is particularly interesting in light of the analysis of bacteroid proteins extracted from strain 1308-induced nodules. These bacteroids contained very low levels of nifD polypeptides, although it is clear from the RNA analysis that there is some accumulation of nifD transcripts (Fig. 5). This supports the interpretation that the absence of the nifD protein in strain 1308 bacteroids is due to the instability of the nifD polypeptide in the absence of the nifK polypeptide. (v) Insertion of Tn5 in a Fix\textsuperscript{−} region upstream from the nifHDK operon, exemplified by strain 80, resulted in nodule RNA containing transcripts complementary to all three nitrogenase genes, as would be predicted from the protein profile of mutants in this region. However, the RNA from strain 80, as well as from all other mutant strains analyzed, does not appear to hybridize to the 0.9-kb band corresponding to the region flanking the nifK gene. Since this band shows extremely light and variable hybridization with RNA from wild-type nodules (strain 1021), it is possible that this transcript is too rare in the mutant nodules to be detected by this assay. RNA purified from nodules formed by strain 112 (which carries a Tn5 insertion ~3 kb to the right of strain 80) showed the same hybridization pattern as that of strain
80. (vi) Finally, and most interestingly, RNA isolated from nodules induced by strain 1354, the strain carrying a Tn5 insertion in the putative regulatory region, showed no hybridization to any of the nifHDK gene fragments, although hybridization to the 2.1-kb terminal fragment was observed, as it was in all other strains tested. This result indicates that the absence of the nifHDK polypeptides in the bacteroid proteins produced by this strain is due to the absence of their corresponding mRNAs.

**DISCUSSION**

**Nodule morphology.** A summary of characteristics of the nodules produced by each of the *R.
meliloti strains analyzed is presented in Fig. 7. It is clear that the nodules produced by all Fix strains are smaller and less vigorous than those produced by wild-type R. meliloti (strain 1021) or by Fix strains carrying Tn5 insertions in regions not involved in an essential symbiotic function (strain 1431). It is also apparent that nodules produced by all Fix strains contain significantly less leghemoglobin than do nodules produced by wild-type R. meliloti. Although it is not clear how bacterial mutations affect the production and accumulation of this plant-encoded protein, similar reductions in the amount of leghemoglobin protein (14) and RNA (32) have been observed in soybean nodules produced by a Fix strain of Rhizobium japonicum.

Effects of mutations in the nitrogenase operon on proteins and dRNA. The patterns of transcription and translation of the nifHDK genes by mutant strains 1491 and 1312 are as expected for an operon transcribed in the direction nifH to nifK (28). The unexpected observation that strain 1308 does not accumulate the nifD polypeptide but does accumulate nifD mRNA indicates either that the nifD mRNA is not translated (which seems unlikely) or that the nifD polypeptide is unstable in these bacteroids. The absence of nifD polypeptides may reflect the instability of either subunit of the FeMo protein in the absence of the other.

Identification of a potential nif regulatory region. Mutant strain 1354 produces bacteroids which fail to accumulate any of the nitrogenase polypeptides and which do not contain detectable levels of mRNA for these proteins. Thus, it is likely that this region regulates the expression of nifH, nifD and nifK at the level of either transcription or mRNA stability. It is not possible to determine whether this regulation is direct (i.e., a product of the strain 1354 region acts directly on the nifH promoter) or secondary via the regulation of an intermediate. However, recent data from this laboratory have demonstrated that the R. meliloti nifH promoter can be activated by either the nifA or glnG (ntrC) proteins of K. pneumoniae, an enteric bacterium that fixes nitrogen in a free-living state (29, 30). The K. pneumoniae nifA gene encodes the activator of all of the K. pneumoniae nif operons (4, 8, 17). The glnG (ntrC) gene product regulates the major nitrogen utilization pathways of enteric bacteria (18). In these interspecies activation experiments, the R. meliloti nifH promoter was fused to the Escherichia coli lacZ gene, and

<table>
<thead>
<tr>
<th>Nodule morphology</th>
<th>Nod²Fix²</th>
<th>Nod²Fix⁻</th>
<th>Nod²Fix⁺</th>
<th>Nod²Fix⁻</th>
<th>Nod²Fix⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>size</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>color</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>pink</td>
<td>white/straw</td>
</tr>
<tr>
<td>no./plant</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td>&gt;3-5</td>
<td>&gt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>distribution</td>
<td>primary + some lateral roots</td>
<td>primary + some lateral roots</td>
<td>primary + most lateral roots</td>
<td>primary + most lateral roots</td>
<td>primary + some lateral roots</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Leghemoglobin polypeptide</th>
<th>61% wt</th>
<th>89% wt</th>
<th>81% wt</th>
<th>100% wt</th>
<th>64% wt</th>
<th>38% wt</th>
<th>54% wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RNA hybridizing to nitrogenase DNA</th>
<th>H</th>
<th>D</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>K</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

FIG. 7. Summary of physical and biochemical characteristics associated with nodules induced by Fix⁻ R. meliloti strains. The single column under mutant strains 1334, 80, 1066, and 1350 indicates that nodules formed by all of these strains exhibited the same set of characteristics. Symbols: ↓, position of Tn5 insertion; ∨, position of ISRm1 insertion; nt, not tested.
activation of the nifH promoter was monitored by the production of β-galactosidase in E. coli. When the nifH lacZ gene was placed in strains of E. coli which constitutively produced nifA or gltG proteins, the nifH promoter was activated. We are currently investigating whether the region mutated in strain 1354 encodes a nifA- or gltG-type regulator in R. meliloti (Szeto et al., manuscript in preparation).

In conclusion, we think that the data presented here demonstrate that the approach we have taken in analyzing the proteins and RNA from fractionated and whole nodules induced by a series of defined Fix− Rhizobium mutants is a viable and productive one for identifying abundant proteins of specific gene regions, for assigning functions to previously undefined regions, and potentially for understanding the interaction of bacteroid- and plant-specific gene products leading to effective symbiotic nitrogen fixation.

ACKNOWLEDGMENTS

We thank W. Buikema, S. Gibbons, and G. Ruvkun for providing us with the strains analyzed here and R. Hyde for the preparation of this manuscript.

This work was supported by National Science Foundation grant PCM-8104492 and an FMC Corporation grant awarded to F. M. A., J. L. Z. and W. W. S. were supported by National Research Service Awards GM07415 and GM07479, respectively.

LITERATURE CITED

