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Cloning and Characterization of *Rhizobium meliloti* Loci Required for Symbiotic Root Nodule Invasion

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An immunological assay of root nodule polypeptides was used to analyze the nodules induced by 25 symbiotically defective *Rhizobium meliloti* mutants. Differences in polypeptide accumulation in these nodules were used to divide the mutants into three subsets. One subset, containing two mutant strains, was further analyzed. Nodules induced by these mutant strains lack both infection threads and bacteria. The kinetics of nodule formation by these mutant strains, by an *exo*B mutant, and by mixed mutant inocula suggest that the gene products required for nodule invasion may also influence nodule meristem induction. One of the two mutants characterized in this study contains a transposon Tn5 insertion in the *ndv*B locus, which probably results in the loss of β-glucan synthesis. The second mutant contains a transposon in a previously uncharacterized locus. RNA analysis suggests that the newly identified locus is transcribed in free-living cultures of *ndv*B and *exo*B strains, as well as in the parental *R. meliloti* strain. Southern blot analysis suggests that at least a portion of this locus is duplicated. This duplication may explain the apparently leaky phenotype of the mutant strain.

Additional keywords: duplicated DNA, nitrogen fixation, nodule-specific polypeptides, *Medicago sativa*.

Symbiotic nitrogen-fixing root nodules develop in a series of morphologically identifiable steps. Nodule development may be arrested at any one of these steps as a result of genetic defects in either *Rhizobium* or its leguminous host. Analysis of *Rhizobium* mutants that result in the arrest of nodule development at a particular step can help to identify the mechanisms by which the symbionts interact. For instance, the *Rhizobium* genes that induce the formation of a nodule meristem (*nod* and *hsn*) are themselves activated only in the presence of specific flavonoids exuded from the roots of an appropriate leguminous plant (reviewed by Downie and Johnston 1986). Once the nodule meristem has been initiated, there are four major morphological events in nodule development (Vincent 1980). These are the formation of infection threads through which the rhizobia gain entry into the nodule, the release of bacteria from the infection thread into nodule cells, the differentiation of the released bacteria into a form capable of reducing nitrogen, and the eventual senescence of nodule cells containing bacteria. No specific bacteria-plant interaction during these steps has yet been characterized.

In several recent studies, there has been an attempt to correlate the accumulation or synthesis of nodule proteins with particular morphological events in nodule development. *Rhizobium* invasion of the nodule via infection threads appears to be required for the induction of many of the nodule-specific polypeptides synthesized by the plant (Lang-Unnasch *et al.* 1985; Vandenbosch *et al.* 1985; Stanley *et al.* 1986; Leigh *et al.* 1987; Lullien *et al.* 1987; Moerman *et al.* 1987; Morrison *et al.* 1987; Dickstein *et al.* 1988; Dunn *et al.* 1988; Norris *et al.* 1988). *Rhizobium* mutants defective in the synthesis of calcofluor-binding exopolysaccharides (*exo*) induce nodules devoid of infection threads and bacteria (Finan *et al.* 1985; Leigh *et al.* 1985; Vandenbosch *et al.* 1985; Djordjevic *et al.* 1987; Leigh *et al.* 1987). These "empty" nodules contain some nodule-specific polypeptides, but lack others, including leghemoglobin (Lang-Unnasch *et al.* 1985; Vandenbosch *et al.* 1985; Leigh *et al.* 1987; Lullien *et al.* 1987; Dickstein *et al.* 1988; Dunn *et al.* 1988; Norris *et al.* 1988).

Other studies indicate that *Rhizobium* mutants with normal acidic exopolysaccharides may also induce "empty" nodules (Dylan *et al.* 1986; Geremia *et al.* 1987). In one case, the mutant is known to have lost the ability to synthesize neutral β-1,2-glucan (Geremia *et al.* 1987). In the reports describing each of these mutants, the effect on protein accumulation in the "empty" nodules was not described.

In this report, we describe two *R. meliloti* Dangeard transposon mutants that induce nodules which contain the same set of nodule-specific polypeptides found in the "empty" nodules induced by *R. meliloti exo* mutants. Bacteriological and microscopic assays indicate that the new mutants also induce "empty" nodules. One of these strains, Rm 1142, is phenotypically similar to the β-glucan mutant described by Geremia *et al.* (1987) and contains the transposon Tn5 in the *ndv*B locus described by Dylan *et al.* (1986). Our data suggest that the *ndv*B locus is required for stimulating the initiation of the nodule meristem as well as the formation of infection threads by the plant.

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The second mutant strain, Rm 1045, is defective in leucine biosynthesis and in root nodule invasion. This mutant may be similar to one of the leucine auxotrophs described by Truchet *et al.* (1980). Analysis of the DNA adjacent to the transposon in Rm 1045 indicates that it is transcribed in cultures of the free-living parental strain, Rm 1021, and in cultures of other mutant strains which induce "empty" nodules. Transcripts from this region are present at reduced levels in Rm 1045. It appears that at least part of the locus defined by Rm 1045 is duplicated in the *R. meliloti* genome. The impact of this duplication on the characteristics of the mutant and on the symbiotic functioning of *R. meliloti* is discussed.

MATERIALS AND METHODS

Strains and media. The strains and plasmids used in this study are listed in Table 1. Bacterial media were *Rhizobium*-defined medium (RDM) (Vincent 1970), tryptone-yeast extract (TY) (Beringer 1974), Luria-Bertani (LB), and minimal M9 (Meade and Signer 1977). In some cases, RDM was supplemented with leucine (40 μg/ml). Antibiotics used were neomycin sulfate (100 μg/ml) and rifampicin (50 μg/ml). LB agar medium containing 0.02% calcofluor white M2R was prepared as described by Leigh *et al.* (1987). Soft agar for motility assays was 0.3% agar in TY.

The neomycin resistance marker on the transposon Tn5 in strain Rm 1142 or in strain Rm 1045 was transduced to both Rm 1021 and Rm 5000 using the generalized transducing phage, φM12 (Finan *et al.* 1984).

Root nodules. Alfalfa seeds (*Medicago sativa* L. cv. Iroquois) were surface-sterilized in sulfuric acid for 10 min. Alfalfa seedlings were grown on nitrogen-free agar slants and inoculated with 10^7 *R. meliloti* as previously described (Meade *et al.* 1982). For mixed strain inoculations, equal numbers of each bacterial strain were used. Nitrogen fixation was determined by acetylene reduction as previously described (Meade *et al.* 1982). Nodules or roots harvested for immune assay were immediately frozen in liquid nitrogen and stored at −80° C.

The bacterial content of nodules was determined following surface sterilization as previously described (Leigh *et al.* 1985). In nodules induced by mixed strain inocula, we observed the following: Rm 1021 colonies were identified by sensitivity to neomycin; Rm 7094 colonies were identified by their lack of fluorescence on LB-calcofluor plates; and Rm 1142 colonies were identified by their small colony morphology on soft agar.

Microscopy. Nodules harvested 4 wk after inoculation were immediately fixed at 4° C in 0.1 M phosphate buffer containing 4% glutaraldehyde, pH 6.8. Fixed nodules were embedded in Spurr's Resin (Electron Microscopy Sciences, Fort Washington, PA). Thick sections (3 μm) were stained with 0.1% toluidine blue.

The motility of *R. meliloti* strains was analyzed microscopically using fresh cultures grown in TY broth.

Western blot analysis. Nodule and root extracts were prepared as previously described (Lang-Unnasch and Ausubel 1985). Polypeptides were separated by electrophoresis on a 13% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS) and electrophoretically transferred to nitrocellulose. The nitrocellulose blot was incubated with a 1:100 dilution of "nodule-specific" immune serum and then ^{125}I-labeled protein A. It was expected that 10 alfalfa polypeptides and one bacterial polypeptide would be detected by the "nodule-specific" immune serum. These polypeptides were previously characterized (Lang-Unnasch and Ausubel 1985). To control for the possibility of incomplete preadsorption of the immune serum with root proteins, uninfected root extracts were included in all experiments. Extracts of effective nodules grown under the same conditions as the ineffective nodules were also included in each experiment to control for variations in nodule polypeptides due to growth conditions.

DNA manipulations and analysis. Total genomic DNA from *R. meliloti* strains was isolated by the method of Dhaese *et al.* (1981). Southern blots and nick-translated DNA probes were prepared as previously described (Maniatis *et al.* 1982). Hybridizations were conducted in 5× SSC, 50% formamide, 10× Denhardt's solution containing 100 μg/ml of herring sperm DNA. The hybridization temperature was 42° C for all DNA probes except pCD523. Hybridization with the pCD523 probe was conducted at 37° C. The blots probed with DNAs other than pCD523 were washed at 50° C in 0.1× SSC, 0.1% SDS.

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	Source or reference
Rhizobium meliloti		
Rm 1021	Str^r derivative of SU47, Nod$^+$, Fix$^+$	Meade *et al.* 1982
Rm 1045	Rm 1021, Tn5$^+$, Nod$^+$, Fix$^-$	Buikema *et al.* 1983 and this work
Rm 1142	Rm 1021 *ndv*B::Tn5, Nod$^+$, Fix$^-$	Buikema *et al.* 1983 and this work
Rm 1308	Rm 1021 *nif*k::Tn5, Nod$^+$, Fix$^-$, Nop$^-$	Zimmerman *et al.* 1983
Rm 5000	Rif^r derivative of SU47, Nod$^+$, Fix$^+$	E. Signer, Massachusetts Inst. of Technol., Cambridge
Rm 7023	Rm 1021 *exo*A::Tn5, Nod$^+$, Fix$^-$, Inf$^-$	Leigh *et al.* 1985
Rm 7094	Rm 1021 *exo*B::Tn5, Nod$^+$, Fix$^-$, Inf$^-$	Leigh *et al.* 1985
Rm 7154	Rm 1021 *exo*H::Tn5, Nod$^+$, Fix$^-$, Bar$^-$	Leigh *et al.* 1987
Rm 102F26	Nod$^+$, Fix$^-$, Bad$^-$	J. Burton (Nitragin Co., Milwaukee, WI) and Vance *et al.* 1980
Plasmids		
pKC7	pBR322 with 1.8-kilobase *Hin*dIII-*Bam*HI Tn5 segment, Ap^r, Nm^r	Rao and Rogers 1979
pCD523	*chv*AB region of *Agrobacterium* in pLAFR1	Douglas *et al.* 1985
pRK290.112	*ndv*AB region of *R. meliloti* in pRK290	Dylan *et al.* 1986
pBC2.30	16s chloroplast ribosomal DNA from flax	Coates and Cullis 1987

[a] Nod = nodule formation; Fix = nitrogen fixation; Inf = infection thread formation; Bar = bacterial release; Bad = bacterial development; Nop = nodule persistence; Ap = ampicillin; Nm = neomycin sulfate; Str = streptomycin; Rif = rifampicin; and r = resistant.

The blot probed with pCD523 was washed at room temperature in 2× SSC, 0.1% SDS. (1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0. 1× Denhardt's solution = 0.02% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin.)

A genomic library from a *Sau*3A partial digest of Rm 1045 DNA was constructed in λEMBL4 (Frischauf *et al.* 1983). Phages containing the neomycin resistance gene of Tn5 were detected by hybridization to pKC7 (Maniatis *et al.* 1982). One phage isolate, λD241, was shown to contain *R. meliloti* DNA by its ability to hybridize to a genomic Southern blot of Rm 1021 DNA. The 2.8-kilobase (kb) *Eco*RI fragment of λD241 was isolated from an agarose gel using Glassmilk (Bio-101, La Jolla, CA) and ligated into pBR325 to produce subclone pBL1.

RNA blot analysis. RNA was isolated from stationary-phase *R. meliloti* cultures grown in TY medium using the guanidinium isothiocyanate procedure described by Maniatis *et al.* (1982). To prepare slot blots, the RNA was denatured with formaldehyde and transferred to a nylon membrane using a slot blot apparatus as described by the manufacturer (Schleicher & Schuell, Keene, NH). Hybridization and wash conditions were the same as described above for Southern blot analysis. The intensity of the exposed grains corresponding to each of the slots in a slot blot experiment was quantitated using a dual-wavelength TLC scanner CS-930 densitometer (Shimadzu Corp., Kyoto, Japan).

RESULTS

An immunological assay of nodule development. The patterns of immunologically detectable nodule polypeptides that accumulated in nodules induced by *R. meliloti nif* mutants were previously shown to be consistent with morphological data obtained by electron microscopy of root nodules induced by the same *R. meliloti* mutant strains (Lang-Unnasch and Ausubel 1985; Hirsch *et al.* 1983; Hirsch and Smith 1987). To test the potential of the previously devised immunoassay to augment microscopy, we obtained Fix$^-$ *R. meliloti* mutants known to induce nodules that fall into each of four morphological categories. Nodules induced by each mutant strain were harvested approximately 4 wk after seedling inoculation and subjected to western blot analysis (Fig. 1A). The pattern of nodule polypeptide accumulation in each of these Fix$^-$ nodules was different from the pattern observed in Fix$^+$ nodules. Three of the immunoreactive polypeptides were informative in this experiment: a 66-kDa constitutively synthesized *Rhizobium* polypeptide (B66); a 50-kDa nodule-specific alfalfa polypeptide (N50); and leghemoglobin (Lb), a small family of 15-kDa nodule-specific alfalfa polypeptides.

At 4 wk after inoculation, nodules containing undifferentiated bacteroids (Bad$^-$) had levels of B66 similar to those found in Fix$^+$ nodules. The level of N50 was somewhat reduced and the level of Lb was clearly reduced in Bad$^-$ nodules as compared to Fix$^+$ nodules. In contrast, no B66, N50, or Lb was detected within nodules in which infection threads did not form (Inf$^-$); bacteria were not released from infection threads (Bar$^-$); or premature senescence occurred (Nop$^-$). Thus, at this stage of nodule development, only nodules with a Bad$^-$ morphology were clearly distinguishable from other Fix$^-$ nodules by immunoassay. Previous studies have shown that at early stages of nodule development, the immunoassay distinguishes Inf$^-$ or Bar$^-$ nodules from Nop$^-$ and Bad$^-$ nodules (Lang-Unnasch and Ausubel 1985; Lang-Unnasch *et al.* 1985). Thus, by immunoassay of nodules two times during development, three of the four morphological categories of Fix$^-$ nodules could be differentiated.

Using the results of these analyses as a guide, we assayed nodules induced by 25 different Fix$^-$ *R. meliloti* mutants. These mutants had been previously identified by screening *R. meliloti* Tn5-induced mutant strains for their ability to induce nodules on alfalfa and for the ability of those nodules to reduce acetylene (Meade *et al.* 1982). By immunoassay, two of these mutants induced nodules that appeared Inf$^-$ or Bar$^-$, five mutants induced nodules that appeared Bad$^-$, and eight mutants induced nodules that appeared Nop$^-$. The remaining mutant strains induced nodules that seemed to be either Bad$^-$ or Nop$^-$. The two strains that appeared to induce nodules of the Inf$^-$ or Bar$^-$ type were chosen for further characterization.

Characterization of nodules induced by two *R. meliloti* mutants. The two *R. meliloti* mutants that induce nodules which appeared Inf$^-$ or Bar$^-$ by immunoassay are Rm 1142 and Rm 1045. Early in development, the nodules induced by these two strains lacked B66, B50, and Lb, as did nodules induced by Rm 7094 (*exo*B), a strain known to induce

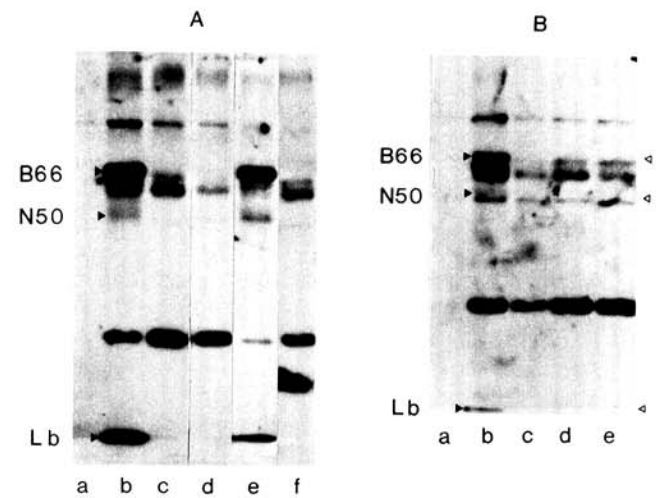

Fig. 1. A, Polypeptides extracted from uninfected roots (a) and from nodules of known ultrastructure (b–f) were subjected to sodium dodecyl sulfate (SDS)-PAGE, transferred to nitrocellulose, and probed with "nodule-specific" immune serum. The positions of the three polypeptides discussed in the text are indicated. All nodules were harvested 4 wk after inoculation. The *Rhizobium meliloti* strain used to induce Fix$^+$ nodules was Rm 1021 (b), Inf$^-$ nodules was Rm 7023 (c), Bar$^-$ nodules was Rm 7154 (d), Bad$^-$ nodules was Rm 102F26 (e), and Nop$^-$ nodules was Rm 1308 (f). **B**, Polypeptides extracted from uninfected roots (a) and from nodules (b–e) harvested 18 days after inoculation were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with nodule-specific immune serum. The *R. meliloti* strains used to induce the nodules were Rm 1021 (b), Rm 7094 (c), Rm 1142 (d), and Rm 1045 (e). The key polypeptides are identified at left.

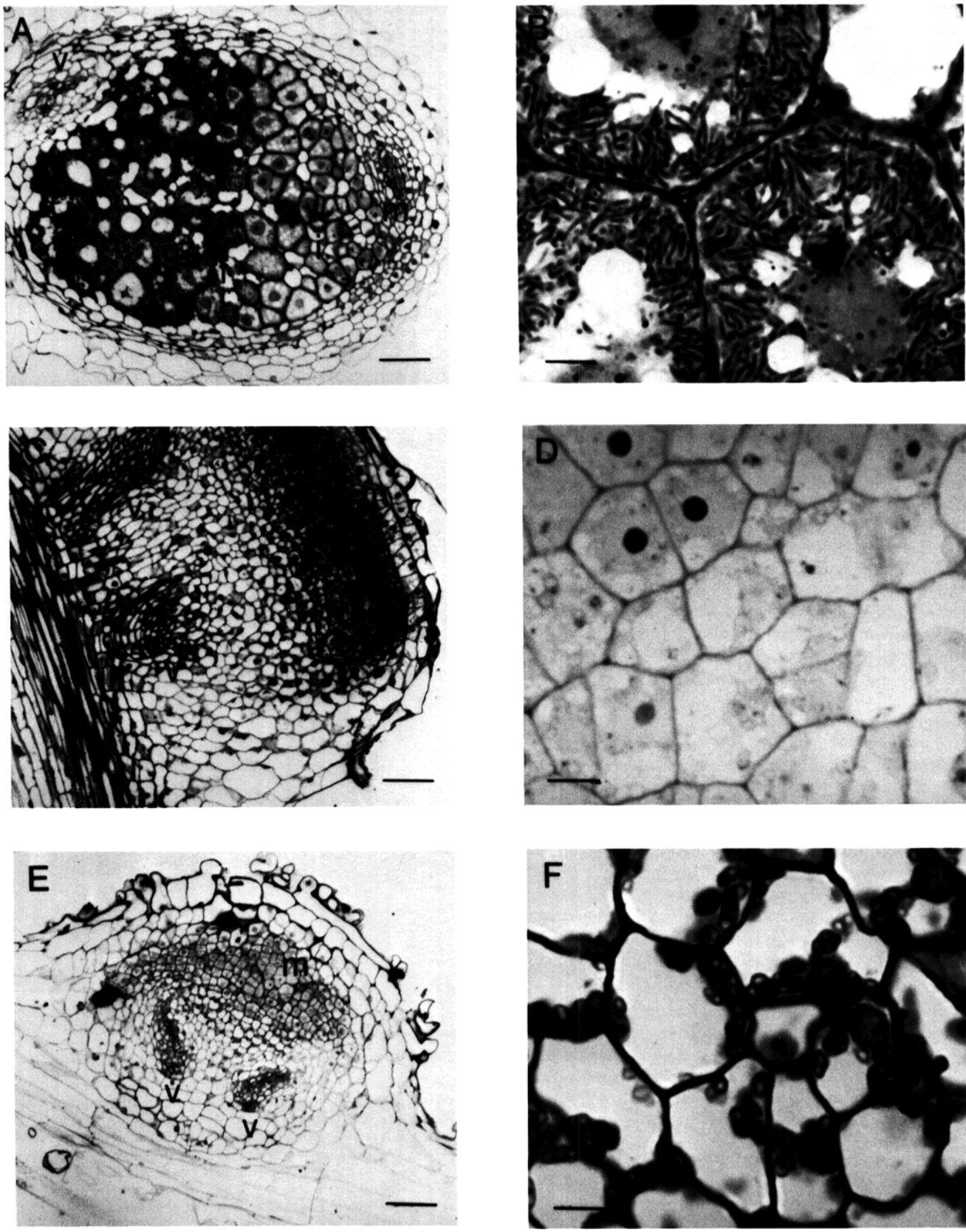

Fig. 2. Light microscopy of nodules induced by the parental strain, Rm 1021 (**A** and **B**), and by mutant strains Rm 1142 (**C** and **D**) and Rm 1045 (**E** and **F**). Longitudinal sections (**A**, **C**, and **E**) are through nodules harvested 4 wk after inoculation. (Bar = 100 μm). The meristematic zone (m), the zone of infection (i), the zone of fixation (f), and vascular bundles (v) are indicated when present in the section. Higher magnification (**B**, **D**, and **F**) of the submeristematic regions of each of these nodules is shown. (Bar = 10 μm).

Inf⁻ nodules (Fig. 1B). At later times during development, nodules induced by Rm 1045 sometimes contained small but immunologically detectable amounts of these three polypeptides, though nodules induced by Rm 1142 or Rm 7094 did not (data not shown). Two other techniques were used to clarify the ambiguity in the immunological results on nodules induced by Rm 1045 and to confirm the morphological phenotype of nodules induced by Rm 1142.

First, the number of *R. meliloti* within nodules induced by these strains was determined by surface sterilizing and then by squashing nodules to release internal bacteria. Nodules induced by strain Rm 1142 or by an Inf⁻ control strain, Rm 7094, consistently contained fewer than 10 rhizobia per nodule. About 75% of the nodules induced by Rm 1045 also contained fewer than 10 rhizobia per nodule. The remaining Rm 1045-induced nodules contained up to 10^4 rhizobia. In contrast, Rm 1021-induced Fix⁺ nodules routinely contained 10^5 to 10^6 rhizobia. The rhizobia isolated from Rm 1045-induced nodules were resistant to neomycin, suggesting that they had not lost the transposon. In addition, no Fix⁺ plants were detected among alfalfa seedlings inoculated with Rm 1045. The presence of higher numbers of rhizobia in some of the nodules suggested that the amount of immunologically detectable B66, N50, and Lb in nodules induced by Rm 1045 was due to a small proportion of nodules sampled which contained bacteria, and that this proportion may increase in older nodules.

The second technique used to confirm the morphological phenotype of nodules induced by Rm 1045 and Rm 1142 was light microscopy. As seen in Figure 2, both strains induced nodules with normal tissue organization. The chief distinction between these nodules and Fix⁺ nodules was the absence of infected cells. In serial sections of five nodules induced by Rm 1142, neither bacteria nor infection threads were observed. Though it is possible that infection threads aborted in the root hair would not have been seen, these analyses suggest that strain Rm 1142 induced Inf⁻ nodules. In serial sections of six nodules induced by Rm 1045, we observed the following: three were devoid of bacteria and infection threads; one contained a single infection thread but no released bacteria; and two contained both infection threads and released bacteria. Taken together, these results suggest that Rm 1045 bacteria induce a leaky, noninvasive phenotype and that the small proportion of nodules invaded by Rm 1045 do not go on to fix nitrogen.

Nodulation by noninvasive *R. meliloti* mutants. To determine if the mutations in *R. meliloti* strains that induce empty nodules also affect earlier stages of nodule development, we quantitated the ability of these strains to nodulate alfalfa seedlings. In a typical experiment (Fig. 3A), the appearance of nodules induced by an *exo*B mutant, Rm 7094, was slightly delayed in comparison to nodules induced by the parental strain, Rm 1021. This result is consistent with previous observations of Leigh and coworkers (1985). A slightly longer delay in the appearance of nodules was apparent when alfalfa seedlings were inoculated with mutant Rm 1045. Both Rm 1045 and Rm 7094 eventually nodulated all or nearly all of the alfalfa seedlings inoculated, as did the parental strain, Rm 1021. In contrast, Rm 1142 nodulated alfalfa seedlings with a pronounced delay and never nodulated all of the seedlings.

Physiology of noninvasive *R. meliloti* mutants. Rm 1045 and Rm 1142 grew on medium containing neomycin, as would be expected for strains containing a Tn5 insertion. Like the parental strain, neither mutant autoagglutinated in broth culture. The colony morphology of both mutants was like that of the parental strain, Rm 1021, except that Rm 1142 colonies were smaller than those of Rm 1045 or Rm 1021. This small colony size was apparently not due to a metabolic deficiency in strain Rm 1142 because its doubling time in TY broth culture was equal to that of Rm 1021. All three strains grew on minimal M9 plates; however, both mutant strains grew more poorly than did the parental strain in liquid minimal medium (RDM). Strain Rm 1142 reached stationary phase at less than half the optimal density of the parental strain though the log phase growth rate was similar. Strain Rm 1045 grew at half the rate of Rm 1021 or Rm 1142 in RDM. When this medium was supplemented with leucine, the growth rate of Rm 1045 was approximately equal to that of Rm 1021 and Rm 1142. Strain Rm 1142 also grew to a higher density following the addition of leucine to the medium. The growth of the parental strain was unaffected by the addition of leucine.

Two *R. meliloti* molecules previously shown to be important for nodule invasion are an acidic exopolysaccharide that binds to the fluorescent dye, calcofluor

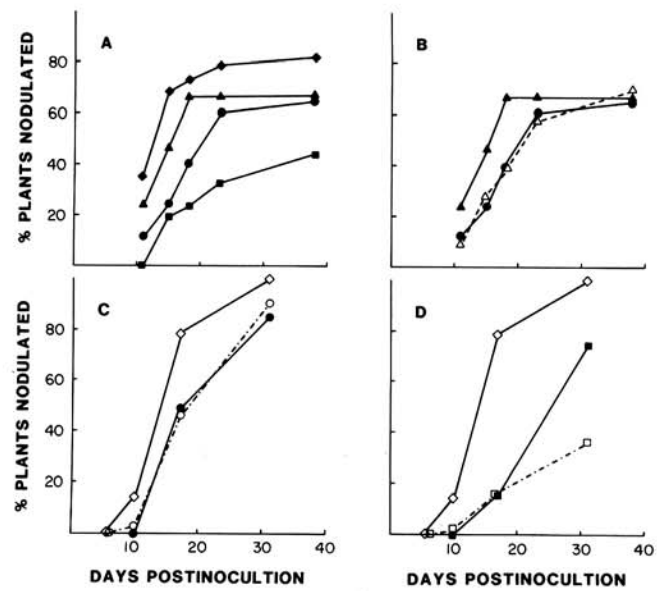

Fig. 3. Kinetics of nodule induction by inoculation with mutant (**A**) or mixed mutant (**B**) strains, and by neomycin-resistant Rm 5000 transductants from Rm 1045 (**C**) or Rm 1142 (**D**). At each time point, the percent of alfalfa seedlings nodulated by the indicated strain or strains is plotted. **A**, Strain symbols are as follows: ◆ = parental strain Rm 1021; ▲ = *exo*B mutant Rm 7094; ■ = Rm 1142; and ● = Rm 1045. **B**, Strain symbols are as follows: ▲ = Rm 7094; ● = Rm 1045; and △ = Rm 7094 + Rm 1045. **C**, Strain symbols are as follows: ◇ = parental strains, pooled data for Rm 1021 and Rm 5000; ● = Rm 1045; and ○ = transductants from Rm 1045 to Rm 5000. **D**, Strain symbols are as follows: ◇ = parental strains, pooled data for Rm 1021 and Rm 5000; ■ = Rm 1142; and □ = transductants from Rm 1142 to Rm 5000.

(Finan *et al.* 1985; Leigh *et al.* 1985; Leigh *et al.* 1987), and β-(1→2) glucan (Geremia *et al.* 1987). Using the calcofluor fluorescence assay of Leigh and coworkers (1985, 1987), it was demonstrated that both Rm 1142 and Rm 1045 fluoresced when grown on calcofluor-containing medium and produced a halo of fluorescence in the surrounding medium (Fig. 4A). In contrast, the *exo*B mutant, Rm 7094, produced no fluorescence and the *exo*H mutant, Rm 7154, produced no halo of fluorescence in the adjacent medium. Strain motility was tested to determine if either strain was phenotypically similar to known β-glucan mutants, because β-glucan-deficient strains of both *Rhizobium* (Geremia *et al.* 1987) and *Agrobacterium* (Puvanesarajah *et al.* 1985) are amotile. As shown in Figure 4B, Rm 1142, but not Rm 1045, appears to have reduced motility as compared to the parental strain.

Genetic analyses of noninvasive *R. meliloti* mutants. Southern blot analysis was used to detect the presence of Tn5 DNA sequences in each of the mutant strains, Rm 1142 and Rm 1045, and to test for alterations in genes previously associated with β-glucan synthesis (Fig. 5). The bacterial DNAs were digested with *Eco*RI, which does not cut within Tn5. Using the neomycin resistance gene as a probe for Tn5, a single *Eco*RI restriction fragment was detected in each mutant strain. This restriction fragment was approximately 12-kb in Rm 1142 and 18-kb in Rm 1045 (Fig. 5A). These results suggested the presence of only a single transposon insertion in each strain.

To confirm the association of the Tn5 insert with the phenotypes of Rm 1142 and Rm 1045, we used the *R. meliloti* transducing phage, φM12, as described by Finan and coworkers (1984). Transductants containing the Tn5 insert of the donor strain were selected by their resistance to neomycin. Transductants of Rm 5000 were shown to be resistant to rifampicin as well as to neomycin. Transductants arose at a frequency of 3×10^{-6} for strain Rm 1142 and 7×10^{-7} for strain Rm 1045. Genomic DNAs from several of the transductants from Rm 1045 were compared to Rm 1045 DNA. In each case, the same two *Sal*I restriction fragments hybridized with the Tn5 neomycin resistance gene probe, pKC7 (data not shown).

Nodules induced by transductants from Rm 1142 or Rm 1045 were Fix$^-$ as measured by their inability to reduce acetylene. These transductants also nodulated alfalfa seedlings with a delay similar to the original mutants (Fig. 3, C and D). Transductants derived from Rm 1142 were amotile by microscopic assay. Thus, the transductants displayed all of the mutant phenotypes for which they were tested.

Because the phenotype of Rm 1142 was similar to that of known *ndv* mutants, this strain was tested for alterations in genes previously associated with β-glucan synthesis. A Southern blot prepared with bacterial DNAs digested with *Eco*RI was probed with an *Agrobacterium* cosmid containing the *chv*A and *chv*B loci and with an *R. meliloti* cosmid containing the *ndv*A and *ndv*B loci (Fig. 5, B and C). Both probes hybridized with a 6-kb *Eco*RI fragment in strains Rm 1021 and Rm 1045. This fragment was missing in Rm 1142 DNA. Rm 1142 DNA contained an *Eco*RI restriction fragment of about 12 kb that hybridized with the *chv*AB and *ndv*AB probes and that was not present in DNA from either Rm 1021 or Rm 1045. This 12-kb fragment exactly comigrated with the *Eco*RI fragment of Rm 1142 that hybridized to the probe for Tn5 DNA. It is known that the *R. meliloti ndv*B locus is in a 6.2-kb *Eco*RI fragment (Dylan *et al.* 1986), so the predicted size of an *ndv*B::Tn5 *Eco*RI fragment is 11.6-kb. Thus, it appears that the Tn5 insert in Rm 1142 is in the *ndv*B locus.

DNA sequences adjacent to the transposon in Rm 1045 are transcribed. As a first step in characterizing the locus defined by the mutation in Rm 1045, the DNA adjacent

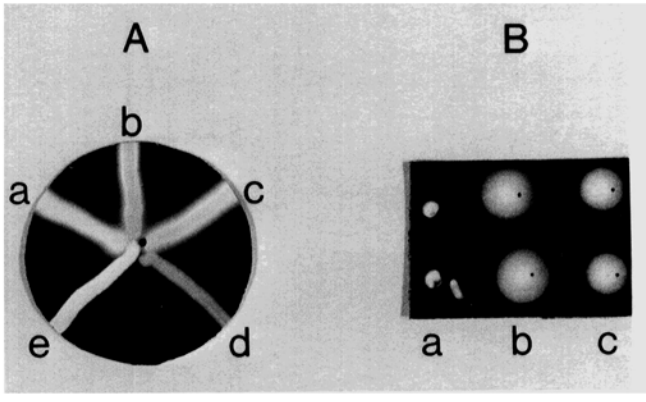

Fig. 4. Characteristics of the free-living mutants. **A**, Calcofluor-induced fluorescence of the parental strain, Rm 1021, (b) and of mutant strains Rm 1142 (a) and Rm 1045 (c) when illuminated with ultraviolet light. The *exo*B mutant, Rm 7094, (d) and the *exo*H mutant, Rm 7154, (e) are shown for comparison. **B**, Motility of the parental strain, Rm 1021, (b) and of mutants Rm 1142 (a) and Rm 1045 (c) as indicated by the diameter of colonies grown in 0.3% agar.

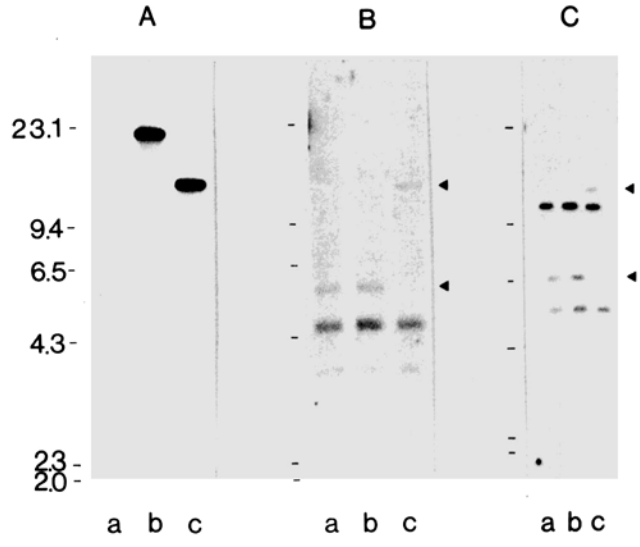

Fig. 5. Southern blot analyses of the transposon insertions in the mutant strains. Hybridizations were performed using pKC7 as a probe for the neomycin-resistant gene of Tn5 (**A**) and using pCD523 (**B**) and pRK290.112 (**C**) as probes for the *Rhizobium meliloti ndv* gene region. Total DNA from Rm 1021 (a), Rm 1045 (b), and Rm 1142 (c) was digested with *Eco*RI and used to prepare Southern blots. Blots **A** and **B** were identical; blot **C** was prepared in a separate experiment. Fragments discussed in the text are indicated with arrows. Size markers were from *Hin*dIII-digested lambda DNA.

to the transposon was cloned in λEMBL4. Restriction map and Southern blot analyses of the clone λD241 indicated the presence of approximately 3.3 kb of *Rhizobium* DNA as well as Tn5 and Mu DNA (Fig. 6A). A subclone, pBL1, containing a 2.8-kb *Eco*RI fragment of *Rhizobium* DNA was used to probe a slot blot prepared with RNA isolated from stationary-phase cultures of strains Rm 1045, Rm 1142 (*ndv*B), and Rm 7094 (*exo*B), as well as from the parental strain, Rm 1021 (Fig. 7A). RNA from all four strains hybridized to the probe, though uninfected alfalfa root RNA did not (data not shown). The hybridization signal from each strain was compared by densitometry and equalized based on the extent of hybridization to a ribosomal DNA probe. RNA from strain Rm 1045 hybridized at about 80% of the intensity of the parental strain. In contrast, RNA from strains Rm 7094 and Rm 1142 hybridized more intensely than did the parental strain, 130 and 200%, respectively.

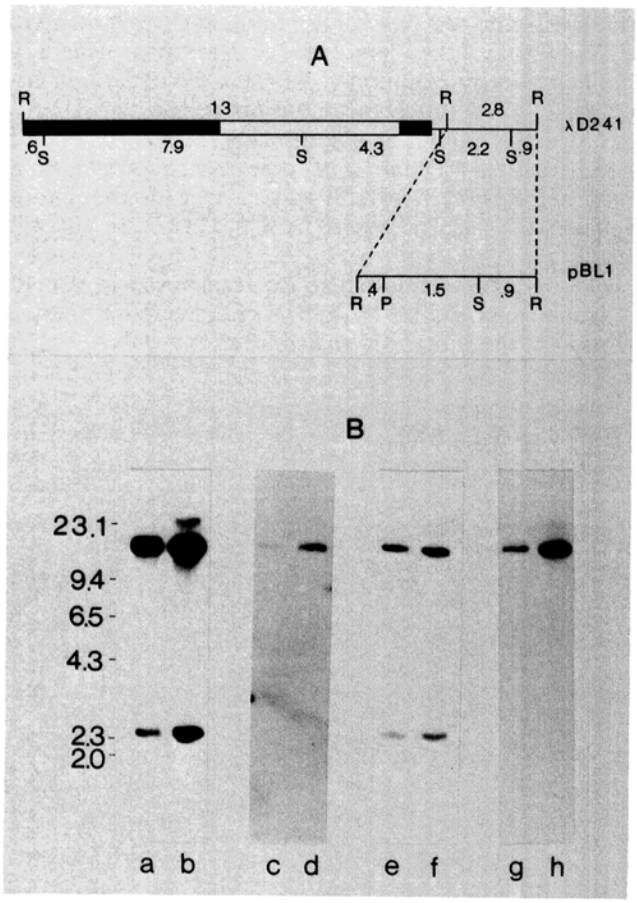

Fig. 6. Organization of the DNA sequences adjacent to the transposon in Rm 1045. **A**, An *Eco*RI (R), *Sal*I (S) restriction map of the insert of the Rm 1045 genomic clone λD241 indicates the presence of Mu DNA (shaded bar), Tn5 DNA (open bar), and *Rhizobium* DNA (line). The *Eco*RI (R), *Pst*I (P), *Sal*I (S) restriction map of the insert of the subclone, pBL1, is also shown. Fragment sizes are indicated in kilobases (kb). **B**, Four identical Southern blots of *Eco*RI-digested genomic DNA from Rm 1021 (a, c, e, and g) and from Rm 1045 (b, d, f, and h) were probed with the pBL1 insert (a and b); the *Eco*RI-*Pst*I 0.4-kb fragment of pBL1 (c and d); the *Pst*I-*Sal*I 1.5-kb fragment of pBL1 (e and f); and the *Sal*I-*Eco*RI 0.9-kb fragment of pBL1 (g and h). Marker DNA fragments are indicated in kb at left.

DNA adjacent to the transposon in Rm 1045 is duplicated in the genome. Because one of the *Eco*RI sites in the subclone pBL1 is derived from the cloning vector, λEMBL4, the size of the complete *Rhizobium Eco*RI fragment containing pBL1 sequences was determined. Surprisingly, two *Eco*RI restriction fragments in genomic digests of Rm 1045 and the parental strain, Rm 1021, hybridized with the pBL1 insert (Fig. 6B). The two *Eco*RI fragments were 2.4- and 16-kb. Because the pBL1 insert is 2.8-kb, it could not have been derived from the 2.4-kb *Eco*RI genomic fragment and must be from the 16-kb *Eco*RI genomic fragment. To confirm that a second homolog of the pBL1 sequence is present in the *R. meliloti* genome, Southern blots of Rm 1045 and Rm 1021 DNA digested with *Bam*HI, *Hin*dIII, or *Kpn*I were also probed with pBL1. Though the pBL1 insert does not contain any of these restriction sites, in each case two restriction fragments were detected (data not shown).

The region of pBL1 responsible for the hybridization at a second site in the genome was determined by probing genomic digests with three restriction fragments isolated from a *Sal*I-*Pst*I digest of the pBL1 insert. As shown in Figure 6B, only the 1.5-kb *Pst*I-*Sal*I restriction fragment hybridized with the 2.4-kb genomic *Eco*RI fragment, though all three regions of the pBL1 insert hybridized to the 16-kb genomic *Eco*RI fragment.

Competition versus extracellular complementation between noninvasive *R. meliloti* mutants. *Rhizobium* surface molecules are known to be important for nodule invasion and may also participate in nodule meristem induction. This suggested that a mixture of mutant strains with different cell surface defects might result in a partial or full rescue of the Fix^+ nodule phenotype. To test this hypothesis, we performed pairwise mixed inoculation of alfalfa seedlings with our two mutant strains and with an *exo*B strain. Seedlings inoculated with the parental strain or a mixture of a mutant strain and the parental strain were Fix^+; however, seedlings inoculated with individual or paired mutant strains were all Fix^-.

To test the ability of the bacteria in the mixed inocula to invade the nodules they induced, the number of bacteria

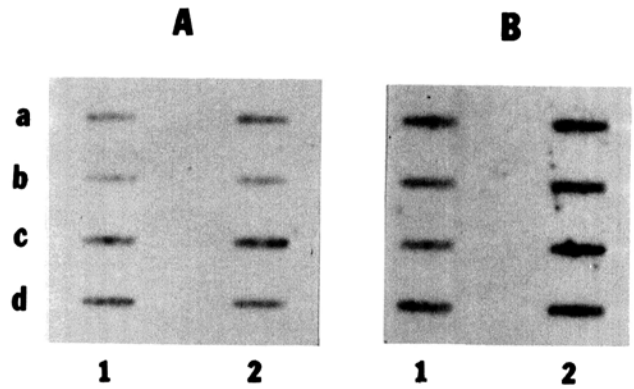

Fig. 7. RNA from cultures of free-living Rm 1021 (a), Rm 1045 (b), Rm 1142 (c), and Rm 7094 (d) was hybridized with pBL1 (**A**). Twice as much RNA was loaded in lane 2 as in lane 1. This slot blot was then stripped and reprobed with the ribosomal DNA probe, pBC2.30 (**B**).

within surface-sterilized nodules was determined. Most nodules induced by mixed mutant inoculation contained fewer than 10 bacteria per nodule as did nodules induced by the individual strains. In up to one third of the nodules induced by mixed inocula involving Rm 1045, 10^3 bacteria per nodule were found. This amount is similar to the number of bacteria found within nodules induced by Rm 1045 alone and represents two orders of magnitude fewer bacteria than found in Fix^+ control nodules. Using the phenotypic differences of Rm 1045, Rm 1142, and Rm 7094, we determined that in those mixed mutant inoculum nodules which contained bacteria, Rm 1045 comprised 99% of the bacteria found within the nodule. In contrast, Rm 1045 comprised only 1% of the bacteria found within nodules induced by a mixed inoculum of Rm 1045 and the parental strain, Rm 1021.

We also tested the ability of mixed mutant inocula to rescue the rate of nodulation. Surprisingly, these results suggest competition rather than extracellular complementation between strains. Individually, Rm 7094 nodulated alfalfa seedlings at a slightly faster rate than did Rm 1045; however, nodulation by a mixed inoculum of Rm 7094 and Rm 1045 occurred at a rate equivalent to that of Rm 1045 (Fig. 3B). Mutant Rm 1142 appeared to be unable to influence nodulation by either Rm 7094 or Rm 1045 (data not shown).

DISCUSSION

The immunoassay used in this study has proven to be a rapid preliminary step in sorting *R. meliloti* symbiotic mutants. Three subsets of mutants were defined by this assay. The predicted nodule phenotype of the two mutants in one of these subsets was confirmed by bacteriological and microscopic methods. Further microscopic studies are needed to confirm the apparent correlation between the immunological and microscopic phenotypes of the two other subsets of mutants.

This study focused on two mutants defective in the infection process. The mutation in one strain, Rm 1142, appears to be in the *ndv*B locus previously described by Dylan *et al.* (1986). This locus is probably required for the production of β-glucan because it is homologous to an *Agrobacterium* locus, *chv*B, required for synthesis of β-(1→2) glucan (Dylan *et al.* 1986; Puvanesarajah *et al.* 1985). The Fix^-, empty nodule, and amotile phenotypes of Rm 1142 have also been previously described for known *Rhizobium* β-glucan mutants (Geremia *et al.* 1987). The delayed nodulation phenotype of Rm 1142 has not been previously described. Interestingly, an *Agrobacterium* transconjugant containing the *R. phaseoli* Dangeard symbiotic plasmid will nodulate *Phaseolus* only if the *chv*AB loci are unmutated (Van Veen *et al.* 1987). It is not clear why in this *Agrobacterium* transconjugant there is an absolute requirement for the *chv*AB loci for nodulation, whereas we have found in *R. meliloti* that the loss of the *ndv*B locus reduces but does not eliminate nodulation. One possibility is that the functions of the *ndv* gene products in *R. meliloti* may be partially duplicated by other gene products not present in *Agrobacterium*.

The mutation in strain Rm 1045 appears to be pleiotropic, affecting leucine biosynthesis and several steps in nodule development. Nodulation by this strain is delayed. About two thirds of the nodules induced by Rm 1045 are devoid of bacteria. The remaining nodules are infected to varying degrees, but those bacteria that do become intracellular apparently do not fix nitrogen. Free-living Rm 1045 is unlike known *exo* or *ndv* mutants. It does not autoagglutinate in culture, unlike the lipopolysaccharide mutants described by Noel *et al.* (1987). Rm 1045 is most like the leucine auxotrophs described by Truchet *et al.* (1980). The loci defined by Truchet's mutants were never genetically characterized and are no longer available (G. Truchet, personal communication). Thus, it is impossible to know if Rm 1045 represents a leaky version of a previously described mutant or an entirely new mutant.

Analyses of neomycin-resistant transductants derived from Rm 1045 indicated that both the delayed nodulation and Fix^- phenotypes of this strain are linked to the transposon. It is likely that the defects in nodule invasion and leucine biosynthesis are also linked to the transposon, although this was not specifically tested.

The presence of Mu DNA adjacent to the transposon in Rm 1045 only slightly reduced the transduction frequency from this strain as compared to Rm 1142 or published frequencies (Finan *et al.* 1984). This suggests that a specific Mu sequence may be responsible for previously described problems with transduction (Finan *et al.* 1984) or conjugation (Buikema *et al.* 1983) from *Rhizobium* strains containing Mu. In our case, Rm 1045 may lack that particular Mu sequence.

We found that RNA from free-living Rm 1021 hybridized with a probe which extended to within about 500 base pairs of the insertion site in Rm 1045. At least part of the DNA probe appears to be within the mutant locus, because Rm 1045 cells contained less homologous RNA than that found in the parental strain or in *ndv*B or *exo*B mutant strains. There may be one or more reasons for the reduction rather than loss of all Rm 1045 RNA homologous to our probe. One possibility is that the 2.8-kb pBL1 DNA probe also contains a transcription unit which was not affected by the insertion. A second possibility is that a copy of at least part of the locus is independently transcribed in free-living cells. In support of these possibilities, preliminary results of northern analysis indicate the presence of two RNA species that hybridize with pBL1 (data not shown). Because the other mutants tested appear to have more RNA homologous to the pBL1 probe than does the parental strain, it will be important to consider the regulatory relationships between the locus defined by the Rm 1045 mutation and the *ndv* and *exo* loci.

Evidence from this study suggesting that at least part of the locus defined by the Rm 1045 mutation is duplicated may also explain why Rm 1045 successfully invades up to a third of the nodules it induces. It has been shown that a mutation in a single copy of functionally homologous genes may result in a reduction in symbiotic efficiency (Györgypal *et al.* 1988; Masepohl *et al.* 1988; Morett *et al.* 1988). The mutation in Rm 1045 results in a reduction in its ability to nodulate and to invade the nodules formed.

This mutation apparently also eliminates the ability of the bacteria to fix nitrogen. Presumably, a double mutation in which both the homologs of this locus are inactivated would result in a nonleaky, noninvasive *R. meliloti* strain.

It appears that the locus defined by the Rm 1045 mutation as well as the *exo* and *ndv* loci are transcribed in free-living cells, so it is interesting to consider their influence on nodulation. This influence is most evident in the case of the *ndv*B mutation in strain Rm 1142; however, a delay in nodulation is also apparent when the Rm 1045 mutant is the inoculating strain. Interestingly, the *exo*B mutant, Rm 7094, appears almost unaltered in nodulation ability except that it competes poorly with most other Nod^+ strains (see also Klein *et al.* 1988). In the mixed strain inoculations described here, the presence of Rm 1045 appeared to inhibit nodulation by the *exo*B mutant. Because Rm 1045 cells are themselves defective in nodulation, we propose that *exo*B mutant cells are defective at an earlier step in nodulation. For instance, *exo*B mutant cells may have a reduced binding affinity for root hairs while Rm 1045 cells may attach normally but have a reduced ability to signal the plant to initiate a nodule meristem. Another indication that the *exo*B gene product(s) function during an early step in nodule formation is the inability of *exo*B mutants to induce root hair curling in some *R. meliloti* strains (Finan *et al.* 1985).

The locus defined by Rm 1045 is on a megaplasmid (Buikema *et al.* 1983), but the location of its homolog remains to be determined. Given the apparent involvement of the locus in leucine metabolism, we anticipate that the homolog is chromosomal. Gene duplication is unusual in bacteria. The two genes known to be duplicated in at least some *Rhizobium* are *nodD* (Honma and Ausubel 1987; Györgypal *et al.* 1988) and *nifH* (Quinto *et al.* 1985). Both genes have key roles in symbiotic nitrogen fixation. The function of the gene(s) at the locus defined by Rm 1045 is unknown. One interesting possibility is that the gene(s) encodes a protein required for the biosynthesis of both leucine and a diffusible symbiotic signal molecule. Characterization of the product(s) of the gene(s) at the Rm 1045 locus may help to clarify this possibility.

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