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Analysis of *Rhizobium meliloti* Nodulation Mutant WL131: Novel Insertion Sequence IS*Rm3* in *nodG* and Altered *nodH* Protein Product

JOY OGAWA, HEIDI L. BRIERLEY, AND SHARON R. LONG*

Department of Biological Sciences, Stanford University, Stanford, California 94305-5020

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Nodulation (*nod*) genes are required for invasion of legumes by *Rhizobium* bacteria. Mutant WL131 is a derivative of 102F51 that has a severe Nod⁻ phenotype on alfalfa. Upon examination of the extended DNA region containing host-specific nodulation genes *nodFEG* and *nodH*, we found that the *nodG* gene of WL131 bears a novel insertion sequence, IS*Rm3*. Complementation studies implied, however, that the phenotype on alfalfa correlated with the *nodH* locus. We found that *nodH* in WL131 encodes an altered gene product. Correlation of the WL131 defect with *nodH* was also supported by phenotypic behavior. Each mutation affected nodulation more severely on alfalfa (*Medicago sativa*) than on sweet clover (*Melilotus albus*). However, we found that the degree of requirement for *nodH* in nodulation varied with the conditions under which the plant was grown.

The nitrogen-fixing symbiotic bacterium *Rhizobium meliloti* infects and stimulates root nodule formation on a group of host plants that include alfalfa (*Medicago sativa*), sweet clover (*Melilotus albus*), and fenugreek (*Trigonella foenum-graecum*). *R. meliloti* carries a group of nodulation (*nod*) genes which are required for the bacterium to establish nodules on its host plants (reviewed by Long [20, 21]). Some of these genes, such as *nodABC*, are found in other *Rhizobium* species and strains that infect diverse hosts and are apparently conserved in function. These genes are known as common *nod* genes. A second group of genes appears to control or affect bacterial selection of the host. Some of these host specificity genes, such as *nodFE*, may have homologs in other bacteria, but they function in a host-specific way. Other genes, including *nodH* and *nodPQ*, appear to be unique to *R. meliloti* and are hypothesized to encode enzymes that modify symbiotis signals to be specific for alfalfa (9, 29).

The gene cluster that includes *nodH* and *nodQ* is carried on a large symbiosis plasmid in *R. meliloti*. The genes for this region have been identified in our laboratory and two others by a variety of approaches: complementation of Nod⁻ mutations or deletions, phenotype of Tn*5* mutants, nucleic acid sequence determination, and protein product characterization (3–5, 9, 10, 13, 16, 28, 29, 31). Our initial cloning of this gene cluster in strain Rm1021 (derived from wild-type strain SU47) was carried out through complementation of the Nod⁻ phenotype of mutant WL131, derived from a different wild-type *R. meliloti*, 102F51 (26); this produced the wild-type SU47 region carried in recombinant plasmid pRmJT5 (31). The gene organization of wild-type 102F51 had not itself been studied. We carried out molecular characterization of the *nod* gene region in WL131; in the process, we discovered a new insertion sequence in *R. meliloti* and determined that the Nod⁻ phenotype of WL131 is due to a defective *nodH* gene product. We also found that the phenotypes of WL131 and *nodH* mutants are influenced by the plant growth substrate.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and plasmids. Plasmids used in this study are shown in Fig. 1 or described below. *R. meliloti* Rm1021 is a streptomycin-resistant derivative of SU47 (22), and *R. meliloti* 102F51 is a wild-type strain (Nitragin Co.); both are Nod⁺ Fix⁺ on alfalfa (*Medicago sativa*) and sweet clover (*Melilotus albus*). *R. meliloti* RCR2011 is equivalent to strain SU47 (2). *R. meliloti* WL131 is a Nod⁻ derivative of strain 102F51 which was isolated following *n*-nitrosoguanidine mutagenesis (26). JF210 and JT912 are mutants of Rm1021 (31); both contain a Tn*5* insertion in *nodH* (10). Bacteria were maintained on LB (23) or TY (1) rich medium and M9-sucrose minimal medium (23). When appropriate, media were supplemented with ampicillin (50 μg/ml), neomycin (50 or 100 μg/ml), streptomycin (500 μg/ml) or tetracycline (10 μg/ml).

Cloning of the host-specific *nod* gene region from WL131 and 102F51. *Pst*I-digested total bacterial DNAs from WL131 and 102F51 were size fractionated by electrophoresis through a 0.7% agarose gel, and bands corresponding to 9.0 kb for WL131 and 7.6 kb for 102F51 were isolated. These fragments were ligated with *Pst*I-digested, dephosphorylated pUC18 (24) DNA, transformed into *Escherichia coli* DH5α, and plated on LB containing ampicillin and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml. White transformants were screened by colony hybridization by probing with ^{32}P-labeled pRmS17 (10) insert DNA. A clone bearing the host-specific *nod* gene region of WL131 was designated pRmW8, and one bearing that of 102F51 was designated pRmF5.

Other plasmid constructions. pRmOG1 was constructed by cloning a 2.0-kb *Bam*HI fragment from pRmF49 (10) into broad-host-range expression vector pTE3 (8). pRmOG3 was constructed by cloning a 4.2-kb *Bam*HI fragment from pRmS5 (31) into pTE3. pRmOH1 and pRmOH2 were constructed by cloning a 2.1-kb *Bam*HI-*Bgl*II fragment from pRmS5 into pTE3.

pRmF5BS5 was generated by isolating and circularizing a 4.7-kb *Bam*HI fragment from pRmF5. Similarly, pRm W8BS4 was made by isolating and circularizing a 4.7-kb *Bam*HI fragment from pRmW8.

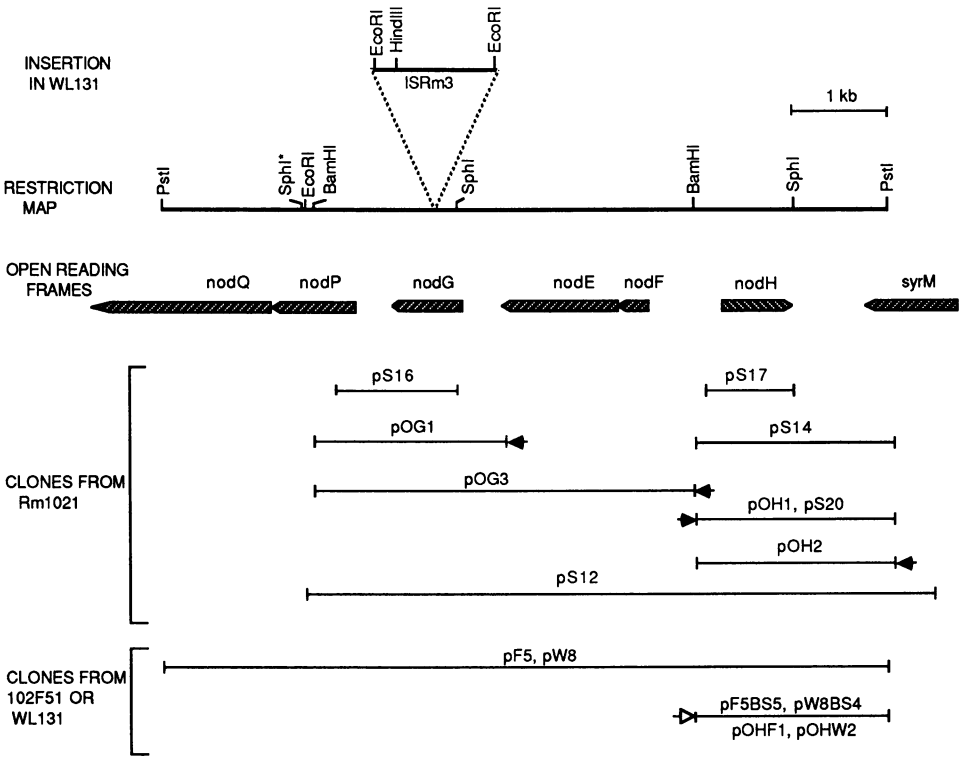

FIG. 1. Map of the host-specific *nod* region of *R. meliloti* Rm1021 (SU47 Str^r) and 102F51, location of the insertion sequence IS*Rm3* in mutant WL131, locations of open reading frames as determined for Rm1021, and clones used in this study. Restriction sites shown are present in both Rm1021 and 102F51, except for one *Sph*I* site present only in 102F51. For convenience, the Rm designation (e.g., pRmOG1) was omitted from plasmid names. For some of the clones used for complementation and for clones used to analyze *nodH* protein products, expression was controlled by the *Salmonella typhimurium trp* promoter (—▷) of pAD10 or pTE3 (8) or the *lac* promoter (—▶) of pUC18 or pLAFR3 (24, 31). The orientation of the arrow indicates the direction of transcription controlled by the *trp* or *lac* promoter.

pRmOHF1 was constructed by inserting a 2.0-kb *Eco*RI-*Hin*dIII fragment from pRmF5BS5 into pLAFR3 (31). Similarly, pRmOHW2 was constructed by inserting a 2.0-kb *Eco*RI-*Hin*dIII fragment from pRmW8BS4 into pLAFR3.

pRmS14 (30a) consists of a 2.1-kb *Cla*I-*Bgl*II fragment containing the Rm1021 *nodH* gene cloned into pRK290-based vector pWB5a (11). pRmS16 (30a) consists of a 1.3-kb *Sst*I-*Sph*I fragment containing most of the Rm1021 *nodG* gene cloned into pUC18. pRmS17 (10) is a pUC18-based clone containing the Rm1021 *nodH* gene. pRmS20 (10) contains the Rm1021 *nodH* gene cloned into pUC8-based expression vector pAD10. pRmS12 (31) is a broad-host-range clone of the *nodFEG-nodH* region contained on an *Eco*RI-*Xba*I fragment.

Southern blots. Isolation of total bacterial DNA, agarose gel electrophoresis, Southern blotting, and hybridization were carried out as previously described (31).

Bacterial genetic techniques. Triparental conjugations to transfer pRK290-based recombinant plasmids were carried out by using helper plasmid pRK2013 by the method of Ditta et al. (6).

Protein products. Coupled transcription-translation reactions and separation and visualization of protein products were carried out as described by Fisher et al. (10).

Nodulation assays. For testing complementation of WL131, alfalfa seeds (AS-13; Ferry Morse Seeds, Mountain View, Calif.) were sterilized, planted on agar slants, and incubated as described by Jacobs et al. (14) by using the Jensen recipe described below. Plant tubes were inoculated with bacteria suspended in 10 mM $MgSO_4$ from M9-sucrose plates at a density of 10^6 to 10^7 bacteria per plant.

For testing the effects of various growth conditions, alfalfa (*Medicago sativa*) seeds were disinfected with ethanol, followed by sodium hypochlorite, as described above. Sweet clover (*Melilotus albus*) was a gift of T. LaRue and was propagated to produce seed in our greenhouses. Seeds were scarified with concentrated sulfuric acid (15 min) and disinfected with mercuric chloride (0.1%, 15 min). After disinfection, seeds were washed in sterile water and allowed to imbibe until they were swollen. The seeds were then transferred to inverted agar plates to sprout. Two recipes for nodulation medium were used. No significant difference between the two recipes was observed. One recipe, that of Jensen (15), included (per liter) 1 g of $CaHPO_4$, 0.2 g of K_2HPO_4, 0.1 g of $MgSO_4$, 0.2 g of NaCl, 0.07 g of $FeCl_3$, and 1× minor salts (19, 25). A second, buffered, variation (BN) included (per liter) 0.27 g of $CaSO_4$, 0.37 g of K_2HPO_4, 0.12 g of KH_2PO_4, 0.12 g of $MgSO_4$, 0.06 g of NaCl, 0.03 g of $FeCl_3$, 0.39 g of morpholineethanesulfonic acid, and 1× minor salts; this was adjusted to pH 7 with KOH. These media were solidified with 12 g of agar per liter for slants, with 10 ml per test tube (18 by 150 mm), or they were used in liquid form with vermiculite, with 10 ml of medium added to one-third of a tube of dry vermiculite. One or two sprouted seeds were transferred to each autoclaved nodulation tube and inoculated 1 to 2 days later. Rhizobia were grown in M9-sucrose, pelleted, and suspended in 10 mM $MgSO_4$ to an optical density at 600 nm of 0.05 to 0.1; 0.5 ml

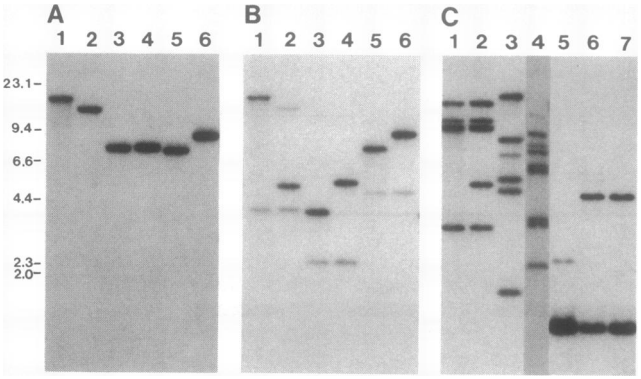

FIG. 2. Southern blot analysis of mutant WL131 and wild-type *R. meliloti*. (A) Total DNAs from 102F51 (lanes 1, 3, and 5) and WL131 (lanes 2, 4, and 6) digested with *Hin*dIII (lanes 1 and 2), *Bam*HI (lanes 3 and 4), and *Pst*I (lanes 5 and 6) were hybridized to ^{32}P-labeled insert DNA from pRmS17. (B) The filter shown in panel A, stripped and then probed with a radiolabeled pRmS16 insert. Note that the insert for pRmS16 contains part of the open reading frame of *nodP* (Fig. 1), which exists in a second unlinked copy in *R. meliloti* (28), and this gave rise to lightly hybridizing bands that were unaffected by rearrangements in the *nod* gene region. (C) *Bam*HI-digested 102F51 DNA (lane 1), *Bam*HI-digested WL131 DNA (lane 2), and *Pst*I-digested 102F51 DNA (lane 3), corresponding to lanes 3, 4, and 5 in panels A and B; *Bam*HI-digested 1021 DNA (lane 4); and *Eco*RI-digested Rm1021 (lane 5), 102F51 (lane 6), and WL131 (lane 7) DNAs were probed with the internal *Eco*RI fragment of IS*Rm3*. Molecular size standards are indicated on the left in kilobase pairs.

was added to each nodulation tube. Control tubes were mock inoculated with 10 mM $MgSO_4$. Some tubes were wrapped with aluminum foil from the base to 3 cm from the top. Plants grown on agar slants without foil were scored for nodules every few days. Others were checked only at the end of the experiment.

RESULTS

Physical map of the 102F51 and WL131 *nod* gene regions. Mutant WL131 is severely Nod$^-$ (on average, fewer than 5% of plants were nodulated; usually, none were) when added to *Medicago sativa* cultivar AS-13 on agar slopes in glass tubes. In previous work, we took advantage of this tight phenotype by conjugating a pLAFR1 bank of wild-type Rm1021 (SU47 Str^r) DNA into WL131. We recovered clones that complemented the Nod$^-$ phenotype, yielding plasmid pRmJT5 (31), which contains 20.5 kb of Rm1021 DNA. Subsequent analysis of the Tn*5* mutations and nucleic acid sequence of this clone indicated the existence of a number of genes, including the tandemly arranged genes *nodFEG*, and divergent from these, *nodH* (Fig. 1; 10). However, WL131 was derived following *n*-nitrosoguanidine mutagenesis of a different wild-type strain, 102F51 (26). We compared the physical map of WL131 with the maps of its parent and strain Rm1021, from which pRmJT5 had been cloned. Figure 2 shows the hybridization of probes subcloned from the pRmJT5 region to DNA of the mutant and wild-type strains. The physical map of mutant WL131 differs from that of wild-type 102F51 in the region that includes *nodG*. Specifically, when a small subclone representing *nodH* was used as a probe, we saw alterations in fragment mobility for restriction digests that left the region between *nodH* and *nodG* uncut. In contrast, the mutant and wild-type DNAs showed identical patterns of hybridization to the *nodH* probe when *Bam*HI, which cuts between *nodH* and *nodF*, was used to digest the genomic DNAs (Fig. 2A, lanes 3 and 4). When we probed identical digests with a *nodG* subclone, distinct restriction pattern changes were observed for each enzyme (Fig. 2B, compare lanes 1 and 2, 3 and 4, and 5 and 6).

The corresponding *nod* gene regions of wild-type 102F51 and mutant WL131 were individually cloned as pRmF5 and pRmW8 in pUC18. The cloned segment from WL131 differed from the wild-type segment in that it carried a physical insertion of about 1.3 kb in the region representing the *nodG* open reading frame. We asked whether this insertion might represent a transposable insertion sequence; in that case, it would be expected to exist in multiple copies. The restriction map of pRmW8 indicated that most of the inserted DNA within *nodG* could be removed as an *Eco*RI fragment; this DNA was used to probe the genome of mutant WL131, its parent 102F51, and also strain Rm1021. We observed several fragments hybridizing with the probe in all strains (Fig. 2C and data not shown).The additional bands seen in WL131 (compare, for example, parental strain DNA in Fig. 2C, lane 1, with WL131 DNA in lane 2) correspond to the altered fragments that hybridized to the *nodG* probe (compare Fig. 2B, lane 4, and C, lane 2). This implies that the insertion has transposed, and we designated this insertion sequence IS*Rm3*. IS*Rm3* is present in strain Rm1021, as well as 102F51 (Fig. 2C, lanes 4 and 5). Two other insertion sequences, IS*Rm1* (27, 31a) and IS*Rm2* (7), have previously been found in *R. meliloti*. Although IS*Rm3* is similar in size to IS*Rm1*, its restriction pattern is different. We also conclude that IS*Rm3* is distinct from IS*Rm1* on the basis of a report that IS*Rm1*-homologous sequences are not present in strain 102F51 (27). The restriction pattern, size, and copy number of IS*Rm2*, found in Rm41 and present in other *R. meliloti* strains, including Rm1021, all show that IS*Rm2* is different from IS*Rm3*. Unlike the other *R. meliloti* insertion sequences, most IS*Rm3* copies bear *Eco*RI sites such that digestion with *Eco*RI yields an internal 1.3-kb fragment (Fig. 2C, lanes 5 to 7). On the basis of hybridization analysis of DNAs digested with enzymes that do not cut within the cloned copy of IS*Rm3*, we estimated that strain Rm1021 contains eight or nine copies of IS*Rm3* while 102F51 contains four to six (Fig. 2C, lane 4 and lanes 1 and 3, respectively, and data not shown). Wheatcroft and Laberge (32) have found and sequenced a new insertion sequence in *R. meliloti* 102F70. Their insertion sequence, which they have also designated IS*Rm3*, has the same restriction map and copy number in strain SU47 as ours, and by hybridization with our clones, they have determined that the sequences are the same.

Complementation of WL131. To determine whether the *nodG* mutation was responsible for the Nod$^-$ phenotype of WL131, we cloned several segments of the region into broad-host-range vectors and tested them for complementation (Table 1). pRmS12, containing *nodH* and *nodFEG*, restored nodulation (31; Table 1), but clones pRmOG1 and pRmOG3, which include, respectively, *nodG* and *nodFEG* (expressed from the *trp* promoter), did not. We found that each of four clones that contained the *nodH* gene (three from Rm1021, one from 102F51) restored nodulation to WL131. Complementation did not depend on the presence or orientation of exogenous vector promoters, indicating that the partial or entire promoter sequences present on the cloned inserts provided sufficient *nodH* product for complementation. As expected, when the cloned *nodH* gene region from

TABLE 1. Complementation of mutant WL131

R. meliloti strain	Plasmid (description)	Nodulation on alfalfa[a]
102F51	None	+
WL131	None	−
WL131	pRmS12 (*nodFE nodG nodH*)	+
WL131	pRmOG1 (*trp* promoter-*nodG*)	−
WL131	pRmOG3 (*trp* promoter-*nodFE nodG*)	−
WL131	pRmOH1 (*trp* promoter-*nodH*)	+
WL131	pRmOH2 (*nodH* opposite *trp* promoter)	+
WL131	pRmS14 (*nodH*)	+
WL131	pRmOHW2 (*lac* promoter-*nodH* from WL131)	−
WL131	pRmOHF1 (*lac* promoter-*nodH* from 102F51)	+
102F51	pRmOHW2 (*lac* promoter-*nodH* from WL131)	+

[a] Duplicates of each construction were tested on a total of at least 20 plants. Numbers of plants nodulated and numbers of nodules per plant were scored at 3- to 4-day intervals. A plus sign signifies wild-type nodulation proficiency or better (at least 75% of plants nodulated by 20 days). A minus sign signifies that 0 to 10% of plants developed a single white nodule by 20 days.

mutant WL131 was placed in WL131, it failed to complement the lesion.

***nodH* gene product.** The complementation data indicated that the lesion responsible for the Nod$^-$ phenotype of WL131 lay in *nodH*, although it was not detectable as a major physical rearrangement. To determine whether an altered *nodH* gene product might be the basis for the Nod$^-$ phenotype of WL131, we compared the protein products of the WL131 and 102F51 *nodH* genes. Coupled transcription-translation reactions using wild-type and mutant DNAs as templates revealed that the 102F51 *nodH* gene encoded a 29-kDa protein comparable to that encoded by the Rm1021 *nodH* gene (10; Fig. 3, lanes 3, 5, and 6, upper arrowhead). However, WL131 DNA encoded a smaller protein of approximately 22 kDa (Fig. 3, lanes 4 and 7, lower arrowhead). We obtained this result with both *Escherichia coli* and *R. meliloti* S-30 extracts. Transcription could be driven either by the exogenous *lac* promoter or by the intrinsic *nodH* promoter. Thus, the WL131 *nodH* gene product appears to be truncated, and considering the complementation data, it is likely that this protein defect is the basis for the WL131 symbiotic defect.

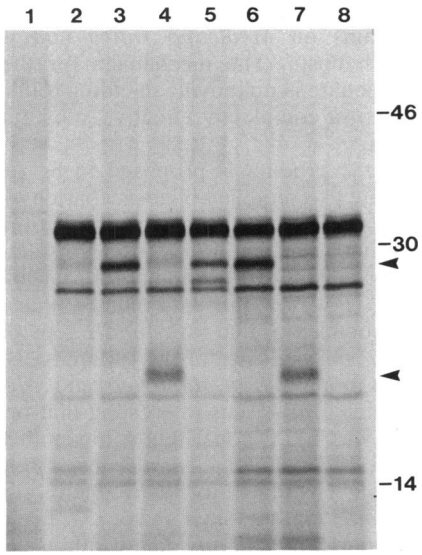

FIG. 3. In vitro expression of *nodH* protein products from *R. meliloti* 102F51 and mutant WL131. Coupled transcription-translation was carried out as described by Fisher et al. (10). The autoradiograph of ^{35}S-labeled proteins separated on an SDS–13.5% polyacrylamide gel displays proteins encoded by vector pUC18 (lanes 2 and 8), pRmF5BS5 (lanes 3 and 6), pRmW8BS4 (lanes 4 and 7), and pRmS20 (lane 5). No template DNA was included in the reaction mixture for lane 1. S-30 extracts were from *E. coli* HB101 (lanes 1 to 5) or *R. meliloti* RCR2011 (lanes 6 to 8). A 0.5 mM concentration of cyclic AMP was included in all of the reaction mixtures. Migration of protein standards is indicated in kilodaltons. For an explanation of the arrowheads, see the text.

Phenotype of WL131 on two hosts. If the *nodH* lesion in WL131 is responsible for the defective-nodulation phenotype as shown by complementation, then other phenotypic properties should be parallel. Horvath et al. (13) reported that *nodH* mutant derivatives of Rm41 were Nod$^-$ on alfalfa (*Medicago sativa*) but Nod$^+$ on sweet clover (*Melilotus albus*). However, when we tested our *nodH*::Tn*5* strains on *Melilotus albus* (using growth on agar slopes as in the work by Horvath et al.), we did not typically see high levels of nodulation; rather, the nodulation level was usually low. We noted that Seegers and LaRue (30) reported at least some nodulation, scored qualitatively, for WL131 on *Melilotus albus*. One difference in the conditions used was that Seegers and LaRue grew their plants in vermiculite rather than on agar. We carried out a systematic study of the effects of growth conditions on *nodH* mutant, WL131, and wild-type strains inoculated onto *Medicago sativa* and *Melilotus albus* as hosts.

Wild-type strain Rm1021 showed little variation in its ability to nodulate the two plant species under the different growth conditions. However, we discovered that the numbers of nodules formed and percentages of plants nodulated (Fig. 4) by *nodH* mutants and WL131 were markedly affected by growing *Melilotus albus* in vermiculite. A similar effect of vermiculite growth was seen with *Medicago sativa*, although the overall level of *Medicago* nodulation by mutants was lower than that of *Melilotus* nodulation. *Melilotus* plants grown on agar slants shaded from light with foil had only a slight increase in nodulation by WL131; thus, the effect of vermiculite may not simply be protection from light. As noted above, the behavior of WL131 and that of *nodH*::Tn*5* mutants were very similar on *Medicago sativa*. However, we observed considerable variation in the degree of nodulation of *Melilotus albus* with *nodH*::Tn*5* mutants; the behavior of WL131 was more consistent. Since some trials were not completely concurrent, the variation may have been in part due to unknown environmental effects. Horvath et al. also observed variability, from 20% nodulation to over 80% nodulation, in *nodH*::Tn*5* mutants on *Melilotus albus* (12a).

DISCUSSION

Random mutagenesis, followed by screening for the symbiotic phenotype, is a standard approach to identifying nodulation genes (21). In this report, we have shown that in strain 102F51, as in other cases (7, 27), transposition of a native insertion sequence can be a complication in such studies. It is striking that a number of symbiotic defects have occurred because of insertion sequence transposition during either Tn*5* or *n*-nitrosoguanidine mutagenesis. Dusha et al. (7) found that IS*Rm2* inserted into *nodH* (*hsnD*) and *fixX* after Tn*5* mutagenesis of Rm41. Ruvkun et al. (27) and Watson and Wheatcroft (31a) reported IS*Rm1* insertions in

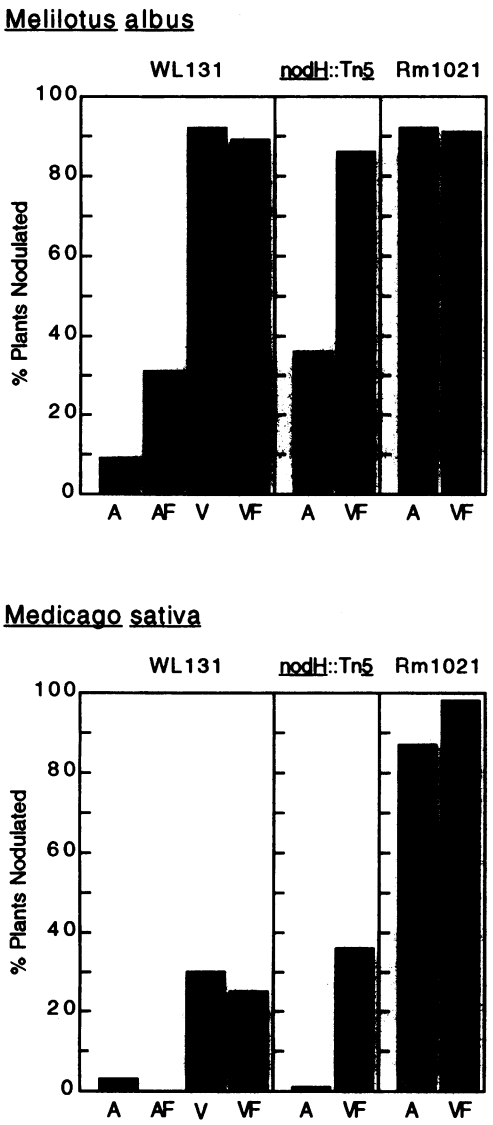

FIG. 4. Effects of plant growth conditions on nodulation by wild-type and mutant *R. meliloti*. *Melilotus albus* or *Medicago sativa* seedlings were grown in test tubes either on agar slants or in vermiculite; the bottoms of the tubes were either wrapped with aluminum foil or left unwrapped (see Materials and Methods). Plants were inoculated with *R. meliloti* WL131, *nodH*::Tn*5* mutant JT210 or JO912, or Rm1021 and inspected for nodules after 4 weeks. Each bar represents a total of 60 to 120 plants and at least two separate trials. Where no bar is shown for *Medicago sativa*, no nodulation was observed. A, agar; V, vermiculite; F, foil.

locations subsequently mapped as *fixC* (12) and *nodC*. We have previously observed that mutant WL113, also isolated by Paau et al. (26) from 102F51, carries a rearrangement in the common *nod* gene region (31), and here we have identified a novel insertion sequence, IS*Rm3*, in the coding region for *nodG* in mutant WL131. We found that WL131 carries a closely linked *nodH* mutation in addition to the IS*Rm3* insertion. It is not known whether this is a strict coincidence or an example of closely linked *n*-nitrosoguanidine-caused events.

Reports of studies in three laboratories (including ours) have stated that the *nodH* gene is critical for nodulation of *Medicago sativa* by *R. meliloti*. The requirement for *nodG* is slight: in strain Rm41, a *nodG*::Tn*5* shows somewhat delayed nodulation, but in Rm1021 it is almost indistinguishable from the wild type. Our results have led us to the conclusion that despite the presence of IS*Rm3* in *nodG*, the mutant phenotype of WL131 on alfalfa in fact arises from a mutation in *nodH*. (i) WL131 was restored to completely normal nodulation by clones including only *nodH* (Table 1 and data not shown). (ii) We showed that the WL131 *nodH* gene segment encodes an altered protein. (iii) The phenotypes of WL131 and *nodH*::Tn*5* mutants are comparable on alfalfa, including the responsiveness of the phenotype to environmental conditions. We noted, however, that on *Melilotus albus* grown on agar the WL131 mutant was more consistently Nod$^-$ than were *nodH*::Tn*5* strains, which showed more variability. It is possible that a minor effect of the *nodG* mutation or some difference in the 102F51 background becomes more apparent when it is combined with the effects of the *nodH* mutation and the restrictive environmental conditions.

The uniqueness of *nodH* to *R. meliloti* has led to its designation as a host specificity gene (13). Also, the presence of *nodH* in a strain affects the host specificity of supernatant factors produced by that strain (9). However, our observations indicate that the relationship of *nodH* effects to bacterial host range may be somewhat complicated. *Melilotus albus* is in the *R. meliloti* cross-inoculation group; Horvath et al. (13) found some nodulation of *Melilotus albus* by *nodH*::Tn*5* mutants, but they also observed that this combination of strain and host sometimes gave very low nodulation (12a). We observed substantial variation in the level of *nodH*::Tn*5* nodulation on *Melilotus albus*, and in optimizing the response we found that the requirement for *nodH* function in *Melilotus albus* was not as stringent when plants were grown in vermiculite rather than on agar slopes. The same effect was seen, somewhat less dramatically, with *nodH*::Tn*5* strains on *Medicago sativa* and with strain WL131 on both hosts. The mechanism for the effect of growth in vermiculite is unknown; shielding roots from light with foil was not a completely effective substitute for vermiculite growth.

The *nodH* gene product is proposed to be required for transfer of sulfate to a modified oligosaccharide which, in its sulfated form (NodRm-1), is a specific nodulation factor for *Medicago sativa* (9, 17, 18). The nonsulfated form, NodRm-2, is proposed to be a signal with a different host specificity (18). However, our data indicate that the role of *nodH* is more complex or that if the *nodH* gene product is a sulfotransferase, the presence of the sulfate on NodRm-1 is not absolutely required for *Medicago* and *Melilotus* response to the signal. It is thus interesting that on *Melilotus albus*, WL131 was more consistently lower in nodulation than the *nodH*::Tn*5* mutants. If *nodG* has a role in signal modification, as is postulated for *nodH*, then a signal from a double *nodG-nodH* mutant, such as WL131, may cause less of a response on *Melilotus albus* than would a signal from a strain carrying either mutation alone. The *Melilotus-Medicago* and agar-vermiculite differences may be useful for determining the threshold of plant response to chemically different versions of NodRm factors.

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