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Genetics

Rhizobium meliloti has three functional copies of the *nodD* symbiotic regulatory gene

(nodulation gene/plant–bacterial interaction/host specificity)

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ABSTRACT **We have identified two *Rhizobium meliloti* genes (*nodD*$_2$ and *nodD*$_3$) that are highly homologous and closely linked to the regulatory gene *nodD* (*nodD*$_1$). *R. meliloti* strains containing mutations in the three *nodD* genes in all possible combinations were constructed and their nodulation phenotypes were assayed on *Medicago sativa* (alfalfa) and *Melilotus alba* (sweet clover). A triple *nodD*$_1$-*nodD*$_2$-*nodD*$_3$ mutant exhibited a Nod$^-$ phenotype on alfalfa and sweet clover, indicating that *nodD* is an essential nodulation gene in *R. meliloti*. A *nodD*$_2$ mutant exhibited no discernable defect in nodulation and *nodD*$_3$ mutants exhibited a delayed nodulation phenotype of 2–3 days when inoculated onto either host. Alfalfa nodules elicited by a *nodD*$_1$ mutant appeared 5–6 days after wild-type nodules, and sweet clover nodules elicited by a *nodD*$_1$ mutant appeared 2–3 days after wild-type nodules. *nodD*$_1$-*nodD*$_2$ double mutants formed nodules with the same delay as single *nodD*$_1$ mutants on both hosts. *nodD*$_2$-*nodD*$_3$ double mutants elicited sweet clover nodules at the same rate as single *nodD*$_3$ mutants, but this same double mutant was slightly more delayed in alfalfa nodule formation than the *nodD*$_3$ mutant. The *nodD*$_1$-*nodD*$_3$ mutant exhibited an extremely delayed nodulation phenotype on alfalfa and elicited no nodules on sweet clover. These experiments indicate that *nodD*$_1$ and *nodD*$_3$ have equivalent roles in nodulating sweet clover but that *nodD*$_1$ plays a more important role than *nodD*$_3$ in eliciting nodules on alfalfa. The *nodD*$_2$ gene appears to have some effect on alfalfa nodulation and none on sweet clover. Our results indicate that *R. meliloti* has three functional *nodD* genes that modulate the nodulation process in a host-specific manner.**

In *Rhizobium meliloti*, two clusters of nodulation (*nod*) genes involved in the early stages of symbiotic nodule formation are located on a large symbiotic plasmid (pRmeSU47a) and are closely linked to a cluster of nitrogen fixation (*nif*) genes (1–4). The so-called "common" nodulation genes (*nodA, nodB, nodC, nodD*) are conserved structurally and functionally among several *Rhizobium* and *Bradyrhizobium* species (5–9). In contrast, the host-specific nodulation genes are involved in determining the range of plant hosts that a particular *Rhizobium* species will nodulate. For example, the *R. meliloti* host-specific nodulation genes (*nodE, nodF, nodG, nodH*) allow it to form nodules on plants of the genera *Medicago, Melilotus*, and *Trigonella* but not on *Trifoliium*, which is a host of *Rhizobium trifolii* (2, 4).

In *Rhizobium leguminosarum, R. trifolii*, and *R. meliloti*, the *nodABC* genes are induced only in the presence of specific flavone exudates from the host root (11–16). This induction was shown to be dependent on the *nodD* gene in *R. meliloti* and *R. leguminosarum* (11, 13, 17). In the case of *R. leguminosarum*, the host specificity genes (*nodFE*) are also regulated by *nodD* (18) and it seems likely that the same is true for other *Rhizobium* species as well. Mutations in the *nodABC* operon block nodulation in all *Rhizobium* and *Bradyrhizobium* species tested so far. Similarly, *R. leguminosarum* and *R. trifolii* strains carrying mutations in *nodD* are Nod$^-$. In contrast, *R. meliloti nodD* mutants still elicit nodules on alfalfa, suggesting that *R. meliloti* has more than one functional *nodD* gene (5–7, 19).

In this report, we describe, two *R. meliloti nodD* ("*nodD*$_1$") homologous genes ("*nodD*$_2$" and "*nodD*$_3$") that are closely linked to *nodD*$_1$. Nodulation experiments with *nodD*$_1$, *nodD*$_2$, and *nodD*$_3$ mutants on alfalfa and sweet clover indicate that all three *nodD* genes are functional and suggest that the different *nodD* genes have host-specific roles in the nodulation process.

MATERIALS AND METHODS

Bacterial Strains, Phages, and Plasmids. Bacterial strains, phages, and plasmids used in these experiments are listed in Table 1.

DNA Biochemistry. Isolation of total DNA from *R. meliloti* (24), agarose gel electrophoresis, and radiolabeling of DNA were performed as described (25). Southern blotting and DNA hybridization using GeneScreen or GeneScreen*Plus* were carried out according to the manufacturer's instructions or by substituting 0.4 M NaOH/0.6 M NaCl for 1.5 M NaCl/0.15 M sodium citrate (26) as transfer buffer. T4 DNA ligase was purchased from New England Biolabs or International Biotechnologies (New Haven, CT) and restriction enzymes were purchased from Boehringer Mannheim or New England Biolabs.

Cloning of *nodD*$_2$ and *nodD*$_3$. DNA from *R. meliloti* strain Rm3600 (derivative of Rm1021), which is deleted for *nodD*$_1$ and *nodD*$_3$, was used to construct a size-selected *Eco*RI gene library consisting of 6.5- to 7-kb fragments cloned in plasmid πR (S. Canning and B. Seed, personal communication). A recombinant plasmid (pNodD$_2$) carrying the 6.8-kb *Eco*RI fragment was identified by colony screening using GeneScreen Colony/PlaqueScreen and the 3.5-kb *Eco*RI–*Bam*HI fragment containing the *R. meliloti nodABCD* genes as a hybridization probe. The 15.5-kb *Eco*RI fragment containing *nodD*$_3$ (as well as the *hsn* genes) had previously been cloned into plasmid pACYC184 (N. Olszewski and F.M.A., unpublished).

Construction of *nodD*$_2$, *nodD*$_3$, *nodD*$_1$-*nodD*$_2$, *nodD*$_1$-*nodD*$_3$, *nodD*$_2$-*nodD*$_3$, and *nodD*$_1$-*nodD*$_2$-*nodD*$_3$ Mutants. A *R. meliloti nodD*$_2$ deletion/insertion mutation was constructed as follows. A 0.5-kb *Xba* I–*Cla* I restriction fragment (see Fig. 1) containing part of *nodD*$_2$ was deleted from plasmid pNodD$_2$. Two *Cla* I sites were present in plasmid pNodD$_2$; however, since the *Cla* I site that overlaps with one *Pvu* I site (see Fig. 1) was methylated by *dam* methylase, only one *Cla* I site was susceptible to digestion with *Cla* I. A 3.3-kb *Bam*HI fragment

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Table 1. Bacterial strains, plasmids, and phages

	Relevant markers	Source or ref.
R. meliloti strain		
Rm1021	*str*^{r} derivative of SU47	This laboratory
AK631	Compact colony isolate of Rm41, wild type	A. Kondorosi
Rm3600	Rm1021 deletion mutant	This laboratory
Rm2011	SU47, wild type	J. Denarie
GMI963	Rm2011 deletion mutant	3
GMI255	Rm2011 deletion mutant	3
TJ9B8	Rm1021 (*nodD*$_1$::Tn*5*)	17
RmD2	Rm1021 (*nodD*$_2$::tm)	This study
RmD3-1	Rm1021 (*nodD*$_3$::sp/g-1)	This study
RmD3-2	Rm1021 (*nodD*$_3$::sp/g-2)	This study
RmD1D2	Rm1021 (*nodD*$_1$::Tn*5*, *nodD*$_2$::tm)	This study
RmD1D3-1	Rm1021 (*nodD*$_1$::Tn*5*, *nodD*$_3$::sp/g-1)	This study
RmD1D3-2	Rm1021 (*nodD*$_1$::Tn*5*, *nodD*$_3$::sp/g-2)	This study
RmD2D3-1	Rm1021 (*nodD*$_2$::tm, *nodD*$_3$::sp/g-1)	This study
RmD2D3-2	Rm1021 (*nodD*$_2$::tm, *nodD*$_3$::sp/g-2)	This study
RmD1D2D3-1	Rm1021 (*nodD*$_1$::Tn*5*, *nodD*$_2$::tm, *nodD*$_3$::sp/g-1)	This study
RmD1D2D3-2	Rm1021 (*nodD*$_1$::Tn*5*, *nodD*$_2$::tm, *nodD*$_3$-2)	This study
Plasmid		
pNodD$_2$	*nodD*$_2$ in πR	This study
pNodD$_3$	15.5-kb *Eco*RI fragment (*nodD*$_3$) in pACYC184	N. Olszewski
pWB5A	pRK290 derivative, *tet-r*	W. J. Buikema
pAgRR4	Tm gene in 3.3-kb *Bam*HI fragment	W. J. Buikema
pRK607	Tn*5*-233 in pRK2013	20
pHP45omega	Sm/Sp gene in pBR322 derivative	21
pGMI467	30-kb region of pSym, contains *nodD*$_2$	22
Phage		
pNodD$_1$	785 bp of *nodD*$_1$ (6) in M13mp10	This study
M12	*R. meliloti* transducing phage	23

R. meliloti were grown in TY medium (18). When appropriate, media were supplemented with streptomycin (Sm) (250–500 μg/ml), neomycin (40–100 μg/ml), spectinomycin (Sp) (10–100 μg/ml), gentamycin (5–25 μg/ml), trimethoprim (Tm) (300 μg/ml), tetracycline (5 μg/ml), or ampicillin (50 μg/ml). Ampicillin/tetracycline selections for π plasmids used ampicillin (25 μg/ml) and tetracycline (7.5 μg/ml). kb, Kilobase; bp, base pairs.

carrying the trimethoprim gene from R751 (23) was inserted following treatment of the fragments with DNA polymerase to create blunt ends. The mutated *nodD*$_2$ gene was introduced into the wild-type Rm1021 genome by marker exchange (27).

A *R. meliloti nodD*$_3$ mutation was constructed by using Tn*5* mutagenesis of pNodD$_3$ in *Escherichia coli* followed by marker exchange in *R. meliloti* (27). Two different insertions (*nodD*$_3$::sp/g-1 and *nodD*$_3$::sp/g-2) were isolated that mapped within the 2.2-kb *Cla* I fragment carrying *nodD*$_3$. Southern hybridization analysis indicated that both of these Tn*5* insertions were located very close to or within *nodD*$_3$ (data not shown). The two *nodD*$_3$ alleles were introduced into the Rm1021 genome by marker exchange. Because the *nodD*$_1$ mutant already carried a Tn*5* insertion, the Tn*5* in the *nodD*$_3$ gene was replaced by recombination with Tn*5*-233 (gentamycin/spectinomycin) (20), resulting in a *nodD*$_3$ mutation marked by gentamycin and spectinomycin resistance.

All possible combinations of *nodD* mutants (RmD1D2, RmD1D3, RmD2D3, RmD1D2D3) were made by using phage M12-mediated transduction (28). To verify these constructions, four transductants from each experiment were screened by Southern blotting and hybridization using pNodD$_1$, ColE1::Tn*5*, pAgRR4 (trimethoprim), and pHP45 (spectinomycin) as hybridization probes (data not shown).

Nodulation Assays. Plant tests with alfalfa and sweet clover were done as described in the legend to Fig. 3. To optimize nodulation conditions, the amount of wild-type inoculum (Rm1021) was varied over a range of 5×10^2 to 10^8 cells per plant. When 5×10^2 to 4×10^6 wild-type bacteria were inoculated onto alfalfa, nodules began to appear after 5 days. By 13 days, >90% of the plants had one or more nodules. Sweet clover nodules appeared earliest on plants inoculated with 5×10^2 to 10^4 wild-type bacteria.

In an initial experiment, the eight strains carrying the two different *nodD*$_3$ alleles (*nodD*$_3$::sp/g-1 and *nodD*$_3$::sp/g-2) were tested along with wild-type Rm1021 on alfalfa and sweet clover plants. The *nodD*$_3$-1 mutation showed a more severe phenotype, whereas the *nodD*$_3$-2 mutation showed a partially defective phenotype. The RmD1D2D3-1 mutant was completely Nod$^-$ on both hosts, and the RmD1D2D3-2 mutant still nodulated. Therefore, the *nodD*$_3$-1 mutation was used in the experiments that follow.

RESULTS

***R. meliloti* pSym Contains Two Genes Homologous to *nodD*.** The *R. meliloti nodD* gene (*nodD*$_1$) is directly upstream of *nodABC* in strains Rm1021 and Rm41 (two independent wild-type isolates) and is located on 8.7- and 8.5-kb *Eco*RI restriction fragments, respectively (Fig. 1). A ^{32}P-labeled plasmid (pNodD$_1$) carrying the coding sequences of *nodD*$_1$ (6) was used to probe a Southern blot of *Eco*RI-digested genomic DNA from Rm1021 and Rm41. Two hybridization bands, in addition to the one carrying *nodD*$_1$, were observed in each strain. In Rm1021, these were 15.5 and 6.8 kb, whereas the additional hybridization bands in Rm41 were 12 and 14 kb (Fig. 2).

To determine whether the *nodD*$_1$-homologous sequences in Rm1021 were located in the *nod/nif* region of pRmeSU47a, a *nodD*$_1$ probe was hybridized to a Southern blot containing *Eco*RI-digested DNA from three *R. meliloti* strains containing deletions in the *nod/nif* region (Rm3600, RmGMI255, RmGMI963; see Fig. 1). In addition, the *nodD*$_1$ probe was hybridized to plasmid pGMI467 DNA, which contains a 30-kb insert of *R meliloti* pRmeSU47a DNA originating ≈80 kb upstream of *nodD*$_1$ (Fig. 1). The results (shown in Fig. 2 and illustrated in Fig. 1) showed that the 6.8- and 15.5-kb *Eco*RI *nodD* homologous fragments in Rm1021 map within 80 kb of *nodD*$_1$. We designated the *nodD*$_1$ homologous regions in the 6.8-kb and 15.5-kb *Eco*RI fragments *nodD*$_2$ and *nodD*$_3$, respectively. In additional hybridization experiments (data not shown), *nodD*$_2$ was mapped to a 1.5-kb *Eco*RI–*Hin*dIII segment within the central 6.8-kb *Eco*RI fragment (Fig. 1); similarly, *nodD*$_3$ was mapped 5 kb downstream of *nodH* within a 2.2-kb *Cla* I fragment.

Nodulation Phenotypes of Single, Double, and Triple *nodD* Mutants. To assay the symbiotic phenotypes of the different *nodD* mutants, alfalfa and sweet clover plants were grown and inoculated (as described in the legend to Fig. 3) with Rm1021 (wild type), RmTJ9B8 (*nodD*$_1$), RmD2, RmD3-1, RmD1D2, RmD1D3-1, RmD2D3-1, and RmD1D2D3-1. Genotypes of strains are listed in Table 1. Several independent nodulation experiments were carried out and similar results were obtained in each; in total, 3000 plants were inoculated and analyzed. The results of one experiment are shown in Fig. 3.

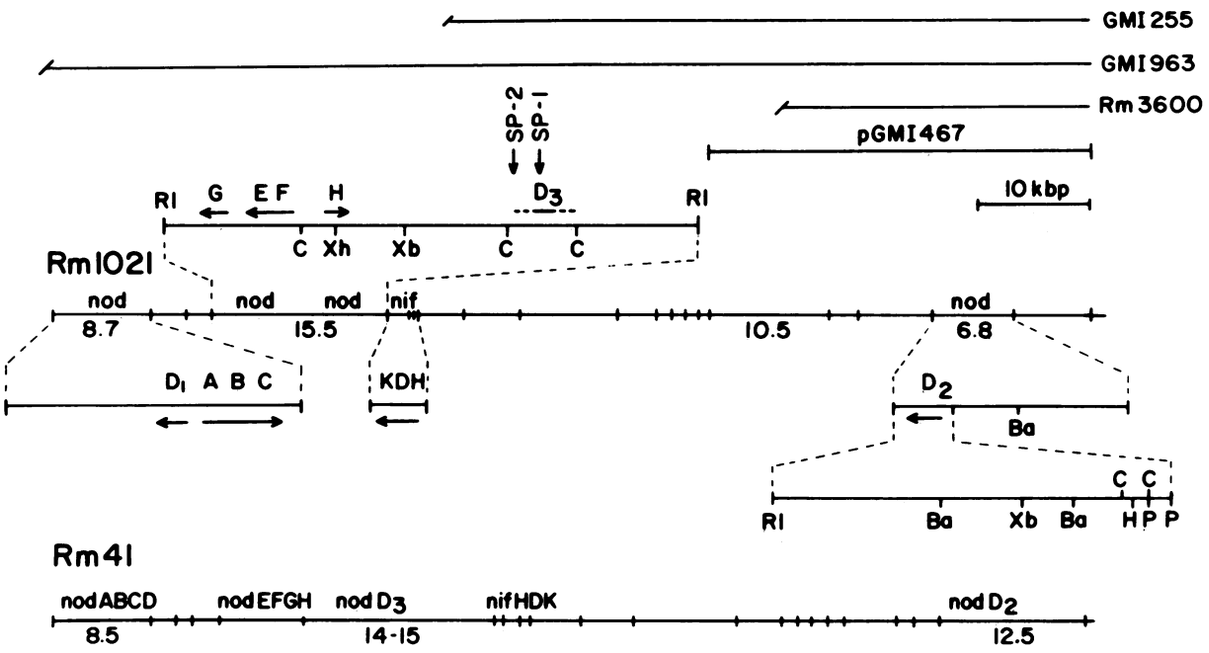

FIG. 1. Physical-genetic map of the *nod/nif* region of the symbiotic plasmids of *R. meliloti* Rm1021 and Rm41 (20). Short vertical hatch marks indicate *Eco*RI restriction sites. Other sites marked are Ba (*Bam*HI), C (*Cla* I), RI (*Eco*RI), H (*Hin*dIII), P (*Pvu* I), Xb (*Xba* I), and Xh (*Xho* I). The horizontal lines represent the region present in the Rm1021 and Rm2011 deletion mutants. pGMI467 is a pLAFR1 clone containing $nodD_2$. The numbers below each genome map denote the lengths of selected *Eco*RI restriction fragments in kb.

Alfalfa and sweet clover nodules elicited by wild-type *R. meliloti* (Rm1021) first appeared 5–6 days after inoculation. By 13 days after inoculation, >90% of the plants had one or more nodules. The triple $nodD_1$-$nodD_2$-$nodD_3$ mutant exhibited a Nod^- phenotype when inoculated onto alfalfa and sweet clover, indicating that *nodD* codes for an essential nodulation function. The $nodD_2$ mutant elicited nodules at the same rate as wild type. In contrast, inoculation with strains carrying a mutation in either the $nodD_1$ or the $nodD_3$ gene resulted in nodule formation that was delayed in comparison to wild type. When alfalfa was inoculated with the $nodD_1$ mutant or double mutant $nodD_1$-$nodD_2$, the appearance of nodules was delayed 5–6 days compared to plants inoculated with wild-type *R. meliloti* (Fig. 3*A*). The $nodD_3$ mutant elicited alfalfa nodules at a rate that was intermediate to wild-type and $nodD_1$-induced nodules, with a delay of 2–3 days. The $nodD_2$-$nodD_3$ double mutant elicited nodules with a slight delay relative to the single $nodD_3$ mutant. The $nodD_1$-$nodD_3$ double mutant nodulated alfalfa plants but at a very delayed rate. Bumps and swellings began appearing on the roots of plants inoculated with this mutant 12 days after inoculation and nodules appeared 2–10 days later. By 28 days after inoculation, about 50% of the plants and bumps on their roots, and after 35 days, 40% of the plants had one or more nodules.

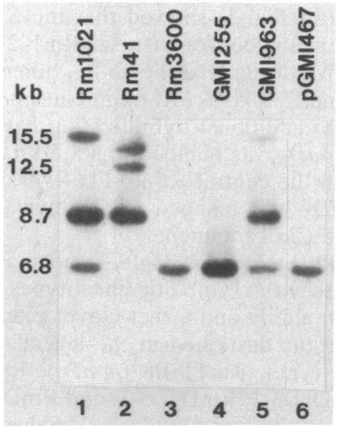

FIG. 2. Southern hybridization of a *R. meliloti* $nodD_1$-specific probe ($pNodD_1$, see Table 1) to *Eco*RI-digested DNA from *R. meliloti* 1021 (lane 1), Rm41 (lane 2), Rm3600 (lane 3), GMI255 (lane 4), GMI963 (lane 5), and plasmid pGMI467 (lane 6).

Interestingly, different results were obtained when the same mutants were used to inoculate sweet clover. The four mutants, $nodD_1$, $nodD_1$-$nodD_2$, $nodD_3$, and $nodD_2$-$nodD_3$, all exhibited the same 2- to 3-day delay in nodulation. In contrast to alfalfa, no nodules were formed on sweet clover roots after inoculation with double $nodD_1$-$nodD_3$ mutant, although a "browning response" was observed and 10% of the plants had root bumps after 5 weeks.

DISCUSSION

In several *Rhizobium* species, four common nodulation genes (*nodA, nodB, nodC, nodD*) are required for the induction of nodule meristems (5–8). Recently, several laboratories have shown that the *nodD* product is required for activation of the *nodABC* operon in the presence of root exudates or specific flavones or flavanones (11, 13). One possibility is that the *nodD* product interacts with specific root exudate molecules.

As shown in this paper, in contrast to *R. leguminosarum* and *R. trifolii*, *R. meliloti* has three copies of *nodD* that are located within an 80-kb region of the pRmeSU47a megaplasmid. We have designated the originally described *nodD* gene as $nodD_1$ and the other two copies as $nodD_2$ and $nodD_3$. Southern blot and sequence analysis (of the $nodD_2$ gene; M.A.H., unpublished data) showed that the *R. meliloti* $nodD_2$ and $nodD_3$ genes are highly homologous to $nodD_1$. The $nodD_2$ gene from Rm1021 corresponds to $nodD_2$ identified in *R. meliloti* strain 41 with respect to DNA sequence and genome location (ref. 29; M.A.H., unpublished data).

Additional functional *nodD* genes would explain the observation that a single *R. meliloti* $nodD_1$ mutant has no defective nodulation phenotype on alfalfa (17), whereas *R. trifolii* and *R. leguminosarum nodD* mutants fail to elicit any

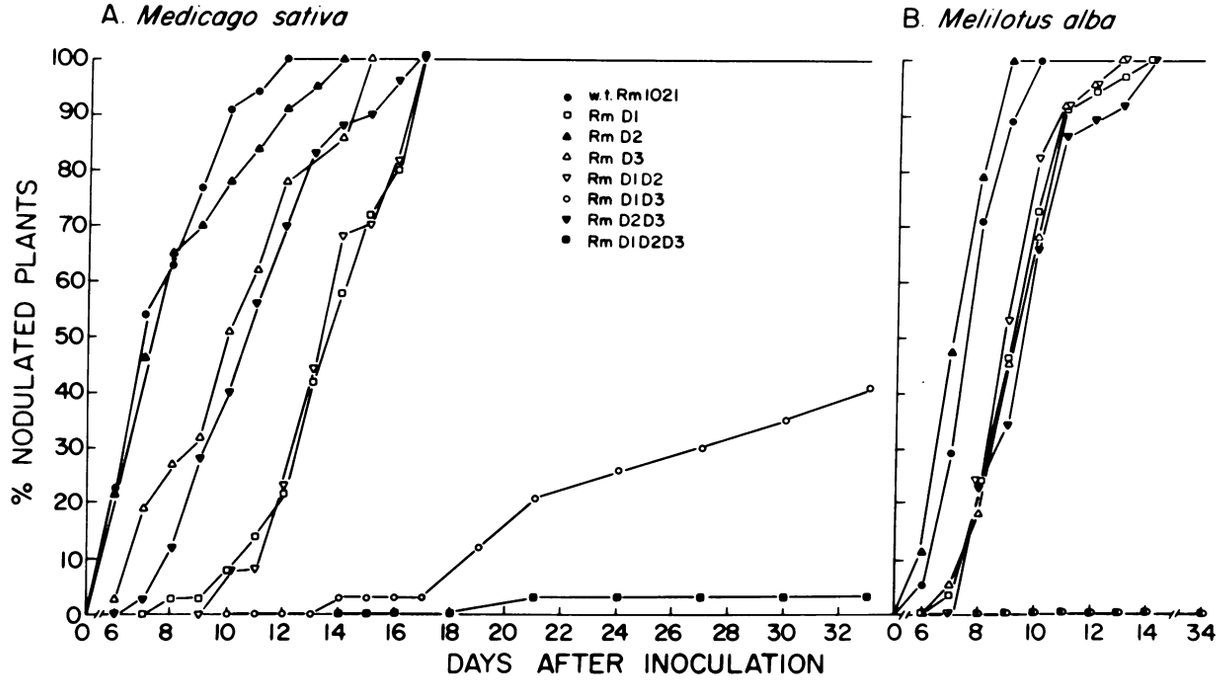

FIG. 3. Nodulation kinetics of wild-type *R. meliloti* 1021, RmD1, RmD2, RmD3-1, RmD1D2, RmD1D3-1, RmD2D3-1, and RmD1D2D3-1 mutants inoculated onto *Medicago sativa* (alfalfa) (*A*) and *Melilotus alba* (sweet clover) (*B*). ●, Rm1021, wild-type *R. meliloti*; □, RmD1; ▲, RmD2; △, RmD3-1; ▽, RmD1D2; ○, RmD1D3-1; ▼, RmD2D3-1; ■, RmD1D2D3-1. Plant tests were carried out as described with the exceptions noted below (24). Seeds were sterilized by immersion in concentrated H_2SO_4 for 10 min, rinsed, washed for several hours, and germinated overnight on 0.8% water agar. One-day-old seedlings were transferred to nitrogen-free plant medium in tubes. One to 2 days later, the seedlings were inoculated with 10^3 to 10^4 bacteria per plant. Twenty-five to 40 plants were inoculated with each bacterial strain and plant tubes were randomized in racks. Time of appearance and number of nodules were scored every day from day 5 until day 16 and every 2 days thereafter. *Medicago sativa* (variety Iroquois) seeds were obtained from Agway (Waltham, MA). *Melilotus alba* seeds were a generous gift of B. Kneen and T. La Rue (Boyce Thompson Institute, Cornell University, Ithaca, NY).

nodules on their respective host plants (5, 7). As described in this paper, we tested this possibility by constructing single, double, and triple mutants in the different *nodD* genes and assayed their phenotypes on alfalfa and sweet clover. Our results showed that all three *nodD* genes are functional and that at least two *nodD* genes are required for optimal nodulation. The triple *nodD* mutant exhibited a Nod^- phenotype on both hosts, demonstrating that *nodD* is an essential nodulation gene in *R. meliloti* as it is in *R. trifolii* and *R. leguminosarum*. All single and double *nodD* mutants, with the exception of the $nodD_1$-$nodD_3$ mutant, elicited nodules on both host plants. The $nodD_1$-$nodD_3$ mutant exhibited a severe nodulation phenotype. Alfalfa plants inoculated with this mutant formed nodules at an extremely delayed rate relative to plants inoculated with wild type. No nodules appeared on sweet clover inoculated with the $nodD_1$-$nodD_3$ mutant. These data indicate that $nodD_2$ is functional in alfalfa but not sweet clover nodulation.

Our data also suggest that the *R. meliloti nodD* genes may play different roles in the establishment of the symbiosis with two alternative hosts *Medicago sativa* (alfalfa) and *Melilotus alba* (sweet clover). Although the $nodD_1$ and $nodD_3$ mutants nodulated at the same rate on sweet clover, the $nodD_3$ mutant nodulated at a faster rate than the $nodD_1$ mutant on alfalfa. In addition, the $nodD_2$ gene appears to have some minimal function in alfalfa nodulation but none in sweet clover nodulation. It appears that in alfalfa nodulation, the $nodD_1$ gene is more important than the $nodD_3$ gene; the $nodD_2$ gene has a minimal role. In sweet clover nodulation, the $nodD_1$ and the $nodD_3$ genes appear to be equivalent, and the $nodD_2$ gene has no function.

There are at least three possible explanations for the different phenotypes of the *nodD* mutants. (*i*) The $nodD_1$, $nodD_2$, and $nodD_3$ products may be functionally interchangeable, but they may be expressed at different levels. (*ii*) They may differ in their ability to activate expression of the other nodulation genes. (*iii*) They interact with different root exudate factors (flavones) or with varying affinities for the same flavone. Correspondingly, one would expect that different host plants would produce different flavones and/or different amounts of the same flavones. A variety of combinations of these explanations is also possible.

Direct evidence that supports the production of host-specific root exudate factors has been obtained by Hermann Spaink and co-workers (30). They compared the ability of *nodD* genes from different *Rhizobium* species to activate the *nodA* promoter of *R. leguminosarum* following addition of flavones or clover (host of *R. trifolii*) root exudate. Their results showed that the induction level of the *nodA* promoter was dependent on the origin of the *nodD* gene as well as the inducing substance. They concluded that, although the various *nodD* gene products were functionally interchangeable, they were not identical. It has been shown by Horvath and co-workers (10) that the *nodD* genes may encode determinants of host specificity by interacting with different plant factors. If *R. meliloti* $NodD_1$ and $NodD_2$ interact with different flavones, one would expect that sweet clover produces less or no "$NodD_2$-specific" flavone compared to alfalfa.

Why does *R. meliloti* have three *nodD* genes? Under laboratory conditions at least two *nodD* genes are required for optimal nodulation of *R. meliloti*. It seems likely that the mutants that are delayed for nodulation in the test tube would be severely impaired in the field.

R. meliloti forms an effective symbiosis on plants of at least three genera: *Medicago*, *Melilotus*, and *Trigonella*. The different *nodD* genes of *R. meliloti* may have evolved to optimize the interaction with specific flavones in the root

exudates of different host plants. Therefore, multiple *nodD* genes may define one level of host specificity in the interaction of *R. meliloti* with its legume hosts.

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1. Kondorosi, E. & Kondorosi, A. (1986) *Trends Biochem. Sci.* **11,** 296–299.
2. Horvath, B., Kondorosi, E., John, M., Schmidt, J., Torok, I., Gyorgypal, Z., Barabas, I., Wieneke, U., Schell, J. & Kondorosi, A. (1986) *Cell* **46,** 335–343.
3. Truchet, G., Debelle, F., Vasse, J., Terzaghi, B., Garnerone, A., Rosenberg, C., Batut, J., Maillet, F. & Denarie, J. (1985) *J. Bacteriol.* **164,** 1200–1210.
4. Debelle, F., Rosenberg, C., Vasse, J., Maillet, F., Martinez, E., Denarie, J. & Truchet, G. (1986) *J. Bacteriol.* **168,** 1075–1086.
5. Djordjevic, M. A., Schofield, P. R. & Rolfe, B. D. (1985) *Mol. Gen. Genet.* **200,** 463–471.
6. Egelhoff, T. T., Fisher, R. F., Jacobs, T. W., Mulligan, J. T. & Long, S. R. (1985) *DNA* **4,** 241–248.
7. Downie, J. A., Knight, D. D., Johnston, A. W. B. & Rosson, L. (1985) *Mol. Gen. Genet.* **198,** 255–262.
8. Kondorosi, E., Benfalvi, Z. & Kondorosi, A. (1984) *Mol. Gen. Genet.* **193,** 445–452.
9. Marvel, D., Kuldau, G., Hirsch, A., Richards, E., Torrey, J. & Ausubel, F. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 5841–5845.
10. Horvath, B., Bachem, C., Schell, J. & Kondorosi, A. (1987) *EMBO J.* **6,** 841–848.
11. Rossen, L., Shearman, C., Johnston, A. & Downie, J. (1985) *EMBO J.* **4,** 3369–3373.
12. Innes, R., Kuempel, P., Plazinski, J., Canter-Cremers, H., Rolfe, B. & Djordjevic, M. (1985) *Mol. Gen. Genet.* **201,** 426–432.
13. Mulligan, J. T. & Long, S. R. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 6609–6613.
14. Redmond, J., Batley, J., Djordjevic, M., Innes, R., Kuempel, P. & Rolfe, B. (1986) *Nature (London)* **323,** 632–635.
15. Peters, N., Frost, J. & Long, S. (1986) *Science* **233,** 977–980.
16. Firmin, J. L., Wilson, K. E., Rossen, L. & Johnston, A. W. B. (1986) *Nature (London)* **324,** 90–92.
17. Zaat, S. A. J., Wijffelman, C. A., Spaink, H. P., van Brussel, A. A. N., Okker, R. J. H. & Lugtenberg, B. J. J. (1987) *J. Bacteriol.* **169,** 198–204.
18. Shearman, C., Rossen, L., Johnston, A. & Downie, J. (1986) *EMBO J.* **5,** 647–652.
19. Jacobs, T. W., Egelhoff, T. T. & Long, S. R. (1985) *J. Bacteriol.* **162,** 469–476.
20. de Vos, G., Walker, G. C. & Signer, E. R. (1986) *Mol. Gen. Genet.* **204,** 485–491.
21. Frey, J. & Kirsch, H. (1985) *Gene* **36,** 143–150.
22. Renalier, J., Batut, J., Ghai, J., Terzaghi, B., Denarie, J., Lewin, A., Garnerone, A., David, M., Gheriardi, M., Huguet, T. & Boistard, P. (1987) *J. Bacteriol.* **169,** 2231–2238.
23. Rella, M., Mercenier, A. & Haas, D. (1985) *Gene* **33,** 293–303.
24. Meade, H., Long, S., Ruvkun, G., Brown, S. & Ausubel, F. (1982) *J. Bacteriol.* **49,** 114–122.
25. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
26. Reed, K. & Mann, D. (1985) *Nucleic Acids Res.* **20,** 7207–7221.
27. Ruvkun, G. B. & Ausubel, F. M. (1981) *Nature (London)* **289,** 85–88.
28. Finan, T. M., Hartwieg, E., Lemieux, K., Bergman, K., Walker, G. C. & Signer, E. R. (1984) *J. Bacteriol.* **159,** 120–124.
29. Gottfert, M., Horvath, B., Kondorosi, E., Putnocky, P., Rodriguez-Quinones, F. & Kondorosi, A. (1986) *J. Mol. Biol.* **191,** 411–420.
30. Spaink, H. P., Wijffelman, C. A., Pees, I., Okker, R. J. H. & Lugtenberg, B. J. J. (1987) *Nature (London)* **328,** 337–340.