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Rhizobium meliloti nodD Genes Mediate Host-Specific Activation of *nodABC*

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To differentiate among the roles of the three *nodD* genes of *Rhizobium meliloti* 1021, we studied the activation of a *nodC-lacZ* fusion by each of the three *nodD* genes in response to root exudates from several *R. meliloti* host plants and in response to the flavone luteolin. We found (i) that the $nodD_1$ and $nodD_2$ products ($NodD_1$ and $NodD_2$) responded differently to root exudates from a variety of hosts, (ii) that $NodD_1$ but not $NodD_2$ responded to luteolin, (iii) that $NodD_2$ functioned synergistically with $NodD_1$ or $NodD_3$, (iv) that $NodD_2$ interfered with $NodD_1$-mediated activation of *nodC-lacZ* in response to luteolin, and (v) that a region adjacent to and upstream of $nodD_2$ was required for $NodD_2$-mediated activation of *nodC-lacZ*. We also studied the ability of each of the three *R. meliloti nodD* genes to complement *nodD* mutations in *R. trifolii* and *Rhizobium* sp. strain NGR234. We found (i) that $nodD_1$ complemented an *R. trifolii nodD* mutation but not a *Rhizobium* sp. strain NGR234 $nodD_1$ mutation and (ii) that *R. meliloti* $nodD_2$ or $nodD_3$ plus *R. meliloti syrM* complemented the *nodD* mutations in both *R. trifolii* and *Rhizobium* sp. strain NGR234. Finally, we determined the nucleotide sequence of the *R. meliloti* $nodD_2$ gene and found that *R. meliloti* $NodD_1$ and $NodD_2$ are highly homologous except in the C-terminal region. Our results support the hypothesis that *R. meliloti* utilizes the three copies of *nodD* to optimize the interaction with each of its legume hosts.

In *Rhizobium meliloti*, nodulation 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 (for a review, see reference 28). The *R. meliloti* nodulation genes can be classified into the following three groups: the so-called common nodulation genes (*nodA*, *nodB*, and *nodC*) are structurally and functionally conserved among several *Rhizobium* and *Bradyrhizobium* species (8, 12, 29). In contrast, the host-specific nodulation genes (*nodE*, *nodF*, *nodG*, and *nodH*) allow *R. meliloti* to form nodules on plants of the genera *Medicago*, *Melilotus*, and *Trigonella*, but not on *Trifolium*, which is the host of *R. trifolii* (4, 25). The third category of *nod* genes is represented by *nodD*, whose product (NodD) is a positive regulator that activates both the common and host-specific nodulation genes in the presence of inducing compounds exuded by host roots (34, 41, 46). The inducing compounds are flavones, flavonones, or other closely related compounds that vary from one plant species to another. For example, the most active inducing compound of the *R. meliloti nodABC* genes in alfalfa seed exudate is the flavone luteolin (36), whereas dihydroxyflavone was identified as the most active inducer of the *R. trifolii nod* genes in white clover root exudate (40). A conserved sequence, the *nod* box, to which NodD binds in vitro (15, 22, 27), is present upstream of all inducible *nod* operons and appears to function as a *cis*-acting regulatory element of *nod* gene expression (14, 16, 42, 47).

R. trifolii and *R. leguminosarum* each have one copy of the *nodD* gene, and null mutations in these *nodD* genes result in a Nod^- phenotype (8, 10). In contrast, *R. meliloti* has three functional copies of *nodD* (20, 23, 35). We reported previously that *R. meliloti* strains containing different combinations of mutations in the three *nodD* genes exhibited different nodulation phenotypes on different host plants, suggesting that the *nodD* genes have a host-specific role in the nodulation process (23). This latter conclusion is consistent with data obtained by Spaink et al. (48) and by Horvath et al. (24), who found that the *nodD* genes of different *Rhizobium* species differed in their responses to flavonoid inducers in a species-specific way. The experiments reported in this paper provide additional support for the hypothesis that the three *R. meliloti nodD* genes are involved in mediating host-specific activation of the *nodABC* genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. Rhizobia were grown on either TY medium (0.5% tryptone, 0.3% yeast extract, 3.4 mM $CaCl_2$) or MES minimal medium at pH 6.8 for *R. meliloti* and pH 6.5 for *R. trifolii*. MES minimal medium contained 10 mg of MES (morpholineethanesulfonic acid) per ml, 14.4 mM K_2HPO_4, 18 mM KH_2PO_4, 1 mM $MgSO_4$, 0.136 mM $CaCl_2$, 24 μM $FeCl_3$, 40 μM disodium EDTA, 3.4 mM NaCl, 2.0 mM NH_4Cl, 0.05% sodium glutamate, 0.2% sucrose, 0.1% Gamborg trace elements (19), 1 μg of thiamine hydrochloride per ml, 1 μg of nicotinic acid per ml, 1 μg of pyridoxine hydrochloride per ml, 0.02 μg of biotin per ml, and 2 μg of pantothenate per ml. Tetracycline was added at 3 to 5 μg/ml as required.

Plasmid transfer. Plasmids were transferred from *Escherichia coli* to *Rhizobium* spp. by triparental matings as described previously (43), followed by plating on either TY medium containing streptomycin (200 μg/ml) and tetracycline (5 μg/ml) or MES minimal medium containing tetracycline (3 to 5 μg/ml).

Construction of *R. meliloti nodD* multiple mutants. In a previous report (23), we described the construction of a set of *R. meliloti nodD* mutants which contained all possible combinations of null mutations in each of the three *nodD*

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Relevant characteristics	Source or reference
R. meliloti		
1021	Sm^r derivative of SU47	30
JM57	1021 pRmeSU47a *nodC-lacZ*	34
RmD1D2-1	1021 $nodD_1$::Tn*5*(*9B8*) $nodD_2$::*tm*	23
RmD1D3-1	1021 $nodD_1$::Tn*5*(*9B8*) $nodD_3$::*sp/g-1*	23
RmD1D2D3-1	1021 $nodD_1$::Tn*5*(*9B8*) $nodD_2$::*tm* $nodD_3$::*sp/g-1*	23
RmD1D2-3	1021 $nodD_1$::Tn*5*(*9B7*) $nodD_2$::*tm*	This study
RmD1D3-3	1021 $nodD_1$::Tn*5*(*9B7*) $nodD_3$::*sp/g-1*	This study
RmD1D2D3-3	1021 $nodD_1$::Tn*5*(*9B7*) $nodD_2$::*tm* $nodD_3$::*sp/g-1*	This study
RmD1-4	1021 $nodD_1$::Tn*5*(*9B7*) *nodC-lacZ*	This study
RmD2-4	1021 $nodD_2$::*tm nodC-lacZ*	This study
RmD3-4	1021 $nodD_3$::*sp/g-1 nodC-lacZ*	This study
RmD1D2-4	1021 $nodD_1$::Tn*5*(*9B7*) $nodD_2$::*tm nodC-lacZ*	This study
RmD1D3-4	1021 $nodD_1$::Tn*5*(*9B7*) $nodD_3$::*sp/g-1 nodC-lacZ*	This study
RmD2D3-4	1021 $nodD_2$::*tm*, $nodD_3$::*sp/g-1 nodC-lacZ*	This study
RmD1D2D3-4	1021 $nodD_1$::Tn*5*(*9B7*) $nodD_2$::*tm* $nodD_3$::*sp/g-1 nodC-lacZ*	This study
Rmnod291-3	1021 *nod-291*::Tn*5*	This study
Rmnod291-4	1021 *nod-291*::Tn*5 nodC-lacZ*	This study
Plasmids		
pUC13	Cloning vector	51
pACYC184	Cloning vector	3
pUC18(*lacZ*)	*lacZ* probe	52
pHP45Ω	Source of Ω fragment	38
pHM5	ColE1::Tn*5*; Tn*5* probe	30
M13mp18	Sequencing vector	52
M13mp19	Sequencing vector	52
Bluescript	Cloning vector	Stratagene
pRK290	Broad-host-range IncP plasmid, Tet^r	7
pWB5A	IncP, Tet^r; pRK290 + polylinker in *Eco*RI site	W. Buikema
$pNodD_1$	785 bp of $nodD_1$ in M13mp10	23
$pNodD_2$	6.8-kb *Eco*RI fragment ($nodD_2$) in plasmid πR	23
pMHD2	6.8-kb *Eco*RI fragment ($nodD_2$) from $pNodD_2$ in pUC13	This study
pMH901	2.7-kb *Pvu*II-*Hin*dIII fragment ($nodD_1$) in pWB5A	This study
$pNodD_2$(1.5)	1.5-kb *Eco*RI-*Hin*dIII fragment ($nodD_2$) from $pNodD_2$ in pUC13	This study
pMH93	6.8-kb *Eco*RI fragment ($nodD_2$) from $pNodD_2$ in pRK290	This study
pMH93-207	pMH93 with 0.6-kb deletion	This study
pMH93-209	pMH93 with 1.3-kb deletion	This study
pMH93-224	pMH93 with 1.6-kb deletion	This study
pMH93-236	pMH93 with 0.6-kb deletion (deletes $nodD_2$)	This study
pMH93-291	pMH93 with Tn*5* insertion (5) 2.5 kb upstream of $nodD_2$	This study
pMH93-292	pMH93-291 with most of Tn*5* deleted	This study
pMH93-352	pMH93 with 2.4-kb deletion + Ω fragment	This study
pMH93-373	pMH93 with Ω fragment at the *Xho*I site	This study
$pNodD_3$	15.5-kb fragment (*syrM* $nodD_3$) in pACYC184	23
pMH624	10.2-kb *Xho*I-*Eco*RI fragment (*syrM* $nodD_3$) from $pNodD_3$ in pUC13	This study
pMH682	9.0-kb *Hin*dIII fragment (*syrM* $nodD_3$) from pMH624 in pWB5A	This study
pMH903	3.5-kb *Pst*I fragment ($nodD_3$) from $pNodD_3$ in pWB5A	This study
pMH904	4.2-kb *Hin*dIII-*Cla*I fragment (*syrM*) from pMH624 in pWB5A	This study

genes. In the current study, we transduced a *nodC-lacZ* fusion into each of these *nodD* mutants and surprisingly found that all strains that carried the $nodD_1$ mutation expressed the *nodC-lacZ* fusion constitutively. The $nodD_1$ mutation (allele no. *9B8*) used previously to construct the various *nodD* mutants was a Tn*5* insertion close to the N terminus of the $nodD_1$ gene (26). Because the $nodD_1$ and *nodA* genes are divergently transcribed and because their promoters probably overlap, the most likely explanation for the constitutive expression of *nodC* was that Tn*5* was providing promoter sequences from which transcription could be initiated; this has been reported previously for *R. meliloti* (44).

To overcome the constitutive expression of *nodABC* associated with the $nodD_1$ (*9B8*) allele, we reconstructed the double mutants $nodD_1$-$nodD_2$ and $nodD_1$-$nodD_3$ and the triple mutant $nodD_1$-$nodD_2$-$nodD_3$, with a different $nodD_1$ allele (*9B7*) from strain RmTJ9B7, which contained Tn*5* inserted in the C-terminal region of $nodD_1$ (26). This was accomplished by using bacteriophage M12-mediated transduction as described previously (13). The reconstructed *nodD* mutants were designated RmD1D2-3, RmD1D3-3, and RmD1D2D3-3, to distinguish them from the ones constructed previously, which had been designated RmD1D2-1, RmD1D3-1, and RmD1D2D3-1. The structures of the newly constructed mutants were verified by the Southern transfer and hybridization procedure (data not shown). We also tested the nodulation phenotypes of the reconstructed *nodD* mutants RmD1D3-3 and RmD1D2D3-3 on alfalfa and sweet clover as described previously (23) and found that they had the same phenotypes as the previously tested *nodD* mutants (23), even though the original set of *nodD* mutants containing the $nodD_1$ (*9B8*) allele transcribed the *nodABC* promoter constitutively (data not shown). This latter result was not

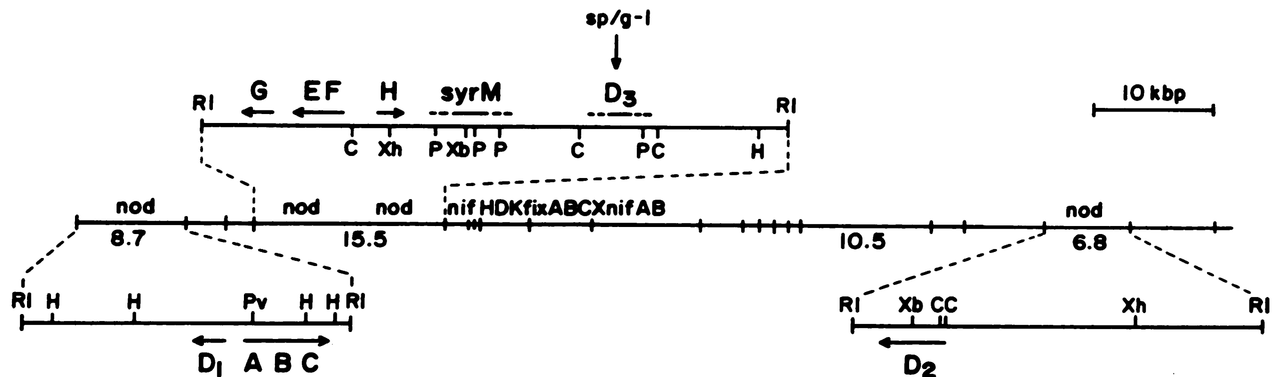

FIG. 1. Physical-genetic map of the *nod-nif* region of pRmeSU47a, the symbiotic plasmid of *R. meliloti* 1021. Short vertical hatch marks indicate *Eco*RI restriction sites. Other sites marked are C (*Cla*I), H (*Hin*dIII), P (*Pst*I), Pv (*Pvu*II), RI (*Eco*RI), Xb (*Xba*I), and Xh (*Xho*I). Horizontal arrows indicate the direction of transcription. The numbers below the genome map denote the lengths of selected *Eco*RI fragments in kilobase pairs. The large capital letters, A, B, C, G, E, F, H, D_1, D_2, and D_3, indicate nodulation genes. The 10-kb scale marker refers to the central, nonexpanded map.

unexpected, since the host-specific nodulation genes (*nodF*, *nodE*, *nodG*, and *nodH*), which are also regulated by the *nodD* genes, would still be subject to normal regulation in the strains that contained the $nodD_1$ (*9B8*) allele.

Construction of *nodD* plasmids. Plasmids carrying the *R. meliloti nodD* genes that were constructed for this study are listed in Table 1, along with relevant features. The restriction fragments used to construct these plasmids are diagrammed in Fig. 1. A series of deletion and deletion-insertion derivatives of pMH93 were constructed by using *Bal*31 nuclease (see Fig. 3). The 2.0-kilobase-pair (kb) Ω fragment (*Sma*I fragment from pPH45Ω), which carries genes encoding resistance to streptomycin and spectinomycin and has transcription and translation terminators from bacteriophage T4 at its ends (18, 38), was used to construct the deletion-insertion derivatives. To construct pMH93-292, pMH93-291 was digested with *Xho*I and then subjected to self-ligation. This removed most of the Tn*5* present in pMH93-291 and generated a small (0.5-kb) deletion between the Tn*5* insertion site and the *Xho*I site of pMH93-291. Additional details concerning the construction of the plasmids can be obtained from the authors.

Construction of *nodC-lacZ* fusion strains. *R. meliloti* phage M12 (13) was used to construct *R. meliloti* strains carrying a *nodC-lacZ* fusion with all possible combinations of *nodD* mutations (i.e., $nodD_1$, $nodD_2$, $nodD_3$, $nodD_1$-$nodD_2$, $nodD_1$-$nodD_3$, $nodD_2$-$nodD_3$, and $nodD_1$-$nodD_2$-$nodD_3$). The *nodC-lacZ* fusion was derived from strain JM57 (which carries a spectinomycin resistance gene downstream of a *nodC-lacZ* fusion). Transductants were plated on LB-M9 (1:1) medium (6). Concentrations of antibiotics used to select the transductants were 50 μg of spectinomycin per ml, 100 μg of neomycin per ml, 100 μg of trimethoprim per ml, and 25 μg of gentamicin per ml. The *nodD* mutations used in these constructions were derived from strains RmTJ9B7, RmD2, RmD3-1, RmD1-4, RmD3-4, and RmD2D3-1. All strain constructions were verified by Southern blotting and hybridization with pHM5 (ColE1::Tn*5*), pUC18(*lacZ*), $pNodD_1$ (carrying 785 base pairs [bp] of $nodD_1$ coding sequence), and pHP45Ω as probes (data not shown).

Root exudates and inducing compounds. Alfalfa seed exudate was prepared as described by Mulligan and Long (34). Alfalfa exudate was collected, centrifuged to remove debris, and filter sterilized. *Melilotus alba* and *Medicago truncatula* root exudates were prepared from 10 and 60 g of seeds, respectively, as described by Redmond et al. (40). *Melilotus alba* exudate was vacuum concentrated to dryness, and the residue was suspended in 6 ml of methanol. *Medicago truncatula* exudate was vacuum concentrated to 60 ml. Sterile exudates were stored at −20°C.

Induction of the *nodC-lacZ* fusion and β-galactosidase assays. Early-log-phase cells grown overnight in MES minimal medium (pH 6.8) were diluted to A_{600} = 0.1, and root exudate or luteolin was added. The alfalfa, *Melilotus alba*, and *Medicago trancatula* root exudates (extracts) were diluted 1:20, 1:500, and 1:10, respectively. These were the concentrations that maximally activated the *nodC-lacZ* fusion in strain JM57 carrying either pMH901 or pMH93 (data not shown). When required, luteolin was added to 10 μM. Bacteria were grown in the presence of the inducers for 4 h.

Assays for β-galactosidase activity were performed essentially as described by Miller (32). Four separate cultures of each strain were assayed for each treatment with exudate or luteolin. The wild-type strain Rm1021, which does not carry the *nodC-lacZ* fusion, was also grown as a control in the presence of each inducer, and the β-galactosidase activity of these cells (5 to 10 U) was subtracted from the activity obtained for each strain.

Nodulation assays. Alfalfa and sweet clover nodulation tests were carried out as described previously (23, 30) with the following exceptions. One to two days after planting, alfalfa or sweet clover seedlings were inoculated with *R. meliloti* cultures that had been grown overnight at 32°C until late log phase in TY medium, centrifuged, and suspended in 10 mM $MgSO_4$. To determine the optimal inoculum dose, we varied the amounts of wild-type (Rm1021) and $nodD_1$-$nodD_2$ mutant (strain RmD1D2-1) inoculum over a range of 50 to 8 × 10^7 cells per plant. Cell numbers were determined by viable counts. An inoculum dose of 10^3 to 10^4 cells per plant was not only optimal for nodulation of the wild-type strain but also optimal for distinguishing between the wild-type and delayed-nodulation phenotypes. Two experiments with 25 plants per strain were carried out for each condition tested, except that the experiments in which the *nodD* triple-mutant strain was complemented with each *nodD* gene on a plasmid were performed only once. For each set of experiments, tubes inoculated with different *R. meliloti* strains were randomized in racks. Plants were incubated in growth chambers at 20 to 24°C in a 16-h light, 8-h dark cycle. The time of appearance and number of nodules were scored every day

from day 5 until day 16 and every 2 days thereafter. *Medicago sativa* var. Iroquois seeds were obtained from Agway, Inc., Waltham, Mass. *Melilotus alba* seeds were a generous gift of B. Kneen and T. LaRue, Boyce Thompson Institute, Cornell University, Ithaca, N.Y.

Nodulation assays for siratro (*Macroptilium atropurpureum*) and white clover (*Trifolium repens*) were performed as described above with the following exceptions. The pH of the plant medium for white clover was 6.5. White clover seedlings were inoculated with 10^7 bacteria per plant, and siratro seedlings were inoculated with 10^7, 5×10^7, or 10^8 bacteria per plant, as indicated. Siratro and white clover plants were grown at 25 to 28°C and 19 to 21°C, respectively. The appearance of nodules was scored every 1 to 2 days from days 6 to 14 postinoculation and every 3 to 8 days thereafter. Twenty to twenty-five plants were inoculated with each strain, and each experiment was performed three times, except for experiments involving pMH93, which were carried out twice. Siratro and white clover seed was obtained from Wright Stephenson, Ltd., New South Wales, Australia, and John D. Lyon, Inc., Cambridge, Mass., respectively.

Isolation of bacteria from nodules. Nodules were surfaced sterilized by immersion in 50% commercial chlorine bleach for 1 to 2 min and then given several washes in sterile water. Nodules were squashed in 10 mM $MgSO_4$ by using a wooden applicator stick, and the liquid was plated on TY containing the appropriate antibiotics.

DNA sequencing. The *nodD*$_2$ sequence was generated as follows. The 1.5-kb *Eco*RI-*Hin*dIII fragment containing the *nodD*$_2$ structural gene was subcloned from plasmid pNodD$_2$ into pUC13, yielding pNodD$_2$(1.5). Deletion derivatives of pNodD$_2$(1.5) were generated with *Bal* 31 nuclease as described by Poncz et al. (37), and a series of deleted 1.5-kb *Eco*RI-*Hin*dIII fragments were subcloned into M13mp18 and M13mp19. Because the 1.5-kb *Eco*RI-*Hin*dIII fragment does not contain the entire *nodD*$_2$ promoter region, additional M13 clones were made by subcloning *Bam*HI-*Pvu*I (420-bp), *Cla*I-*Bam*HI (2,350-bp), and *Bam*HI-*Cla*I (300-bp) fragments from pNodD$_2$ into M13mp18 and M13mp19 (see Fig. 3). Two *Cla*I sites were present in plasmid pNodD$_2$; however, since the *Cla*I site which overlaps with one *Pvu*I site (see Fig. 3) is methylated by *dam* methylase, only the other *Cla*I site is susceptible to digestion with *Cla*I. Dideoxy sequencing reactions with Klenow polymerase and [^{35}S]dATP were carried out as described previously (1). Sequence data were analyzed on a VAX11/780 computer (Digital Equipment Corp.) as described by Earl et al. (11).

RESULTS

Experimental strategy. We and others have recently shown that *R. meliloti* contains three copies of the *nodD* gene, all of which are functional, and have hypothesized that these three *nodB* genes are involved in optimizing the recognition of particular legume hosts (21, 23). To provide additional support for this hypothesis, we determined the DNA sequence of the *R. meliloti nodD*$_2$ gene to determine its degree of divergence from the *nodD*$_1$ gene, tested the ability of each of the three *R. meliloti nodD* genes to respond to root exudates from three different *R. meliloti* hosts and to the flavone luteolin, and determined the ability of each of the three *R. meliloti nodD* genes to complement *nodD* mutations in *R. trifolii* and in the broad-host-range *Rhizobium* strain NGR234.

Nucleotide sequence of *nodD*$_2$. The DNA sequence of the *R. meliloti* 1021 *nodD*$_2$ gene is compared with the previously published sequence of *R. meliloti* 1021 *nodD*$_1$ (12) in Fig. 2. The *nodD*$_2$ gene codes for a predicted protein of 310 amino acids, compared with the 308 amino acids encoded by *nodD*$_1$. The homology between *nodD*$_1$ and *nodD*$_2$ is 85 and

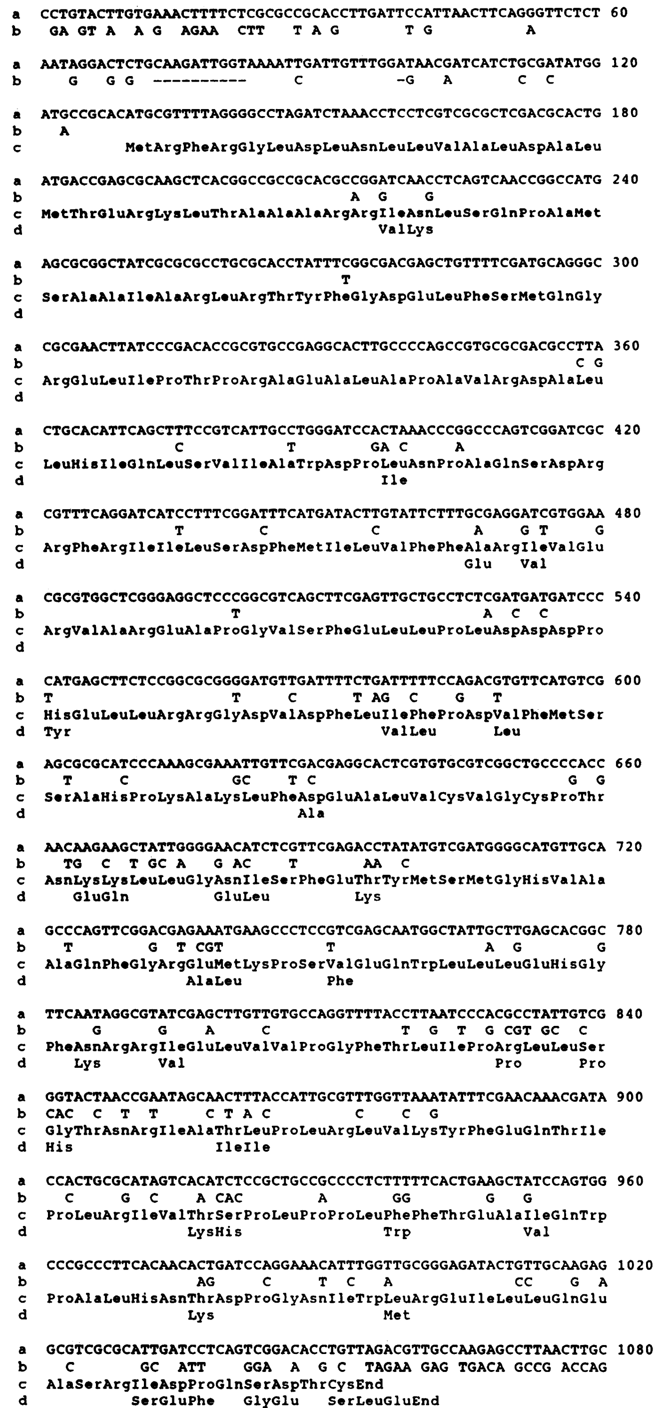

FIG. 2. Nucleotide and amino acid sequences of *R. meliloti* 1021 *nodD*$_1$ (12) and *nodD*$_2$ (this work). The nucleotide sequence of *nodD*$_1$ is shown on line a, and the deduced amino acid sequence is shown on line c. Only differences in bases and amino acids are shown for the *nodD*$_2$ sequences (lines b and d). Dashes indicate missing bases in the *nodD*$_2$ sequence. These data have been submitted to GenBank and have been assigned accession number M29367.

TABLE 2. Activation of *nodC-lacZ* fusion in response to different *nodD* genes and host root exudates

Strain	Plasmid (gene present)	Activation (β-galactosidase units with following inducer):				
		None	Luteolin	*Medicago sativa* exudate	*Melilotus alba* exudate	*Medicago truncatula* exudate
1. JM57	None	3	14	13	ND[a]	ND
2. RmD1-4	None	5	6	3	ND	ND
3. RmD2-4	None	2	89	34	ND	ND
4. RmD3-4	None	2	12	11	ND	ND
5. RmD1D2-4	None	4	4	2	ND	ND
6. RmD1D3-4	None	5	5	4	ND	ND
7. RmD2D3-4	None	2	76	27	ND	ND
8. RmD1D2D3-4	None	4	4	3	ND	ND
9. JM57	None	3	17	14	2	8
10. RmD2-4	None	3	85	23	ND	ND
11. Rmnod291-4	None	3	22	10	ND	ND
12. JM57	pMH901 (*nodD*$_1$)	2	430	270	43	64
13. JM57	pMH93 (*nodD*$_2$)	8	9	217	28	112
14. JM57	pMH903 (*nodD*$_3$)	5	34	27	5	17
15. JM57	pMH904 (*syrM*)	10	48	31	13	33
16. JM57	pMH682 (*nodD*$_3$ *syrM*)	1,036	1,398	1,131	1,154	1,268
17. RmD1D2D3-4	None	5	5	4	2	5
18. RmD1D2D3-4	pMH901 (*nodD*$_1$)	3	405	328	51	69
19. RmD1D2D3-4	pMH93 (*nodD*$_2$)	7	6	18	4	22
20. RmD1D2D3-4	pMH93-291 (*nodD*$_2$)	5	5	4	3	6
21. RmD1D2D3-4	pMH93-236 (*nodD*$_2$)	4	4	3	2	4
22. RmD1D2D3-4	pMH93-352 (*nodD*$_2$)	4	4	7	2	4
23. RmD1D2D3-4	pMH903 (*nodD*$_3$)	7	10	7	5	11
24. RmD1D2D3-4	pMH904 (*syrM*)	6	5	4	3	5
25. RmD1D2D3-4	pMH682 (*nodD*$_3$, *syrM*)	1,112	1,298	1,122	1,139	1,368

[a] ND, Not done.

87% at the DNA and amino acid levels, respectively. As has been observed previously in comparisons of *nodD* genes from different *Rhizobium* species (24, 45), we found that *R. meliloti* NodD$_1$ and NodD$_2$ are most highly conserved in the N-terminal halves and diverge in the C-terminal halves. In particular, the last six of eight amino acids of NodD$_1$ are different from those in NodD$_2$; NodD$_2$ also has two additional amino acids at the C-terminal end. As discussed below, these results are consistent with the hypothesis that the C-terminal regions of NodD proteins are involved in determining flavonoid specificity.

The 5'-noncoding regions of *R. meliloti* *nodD*$_1$ and *nodD*$_2$ show strong homology to each other, with the exception of a 10- to 12-bp deletion in the region upstream of *nodD*$_2$, adjacent to the *nod* box consensus sequence (42).

The *nodD*$_1$ and *nodD*$_2$ genes of *R. meliloti* Rm41 (an independent wild-type isolate) have been sequenced (20). Comparison between the DNA sequences of the *nodD*$_1$ and *nodD*$_2$ genes or strains Rm1021 and Rm41 revealed very few differences. There are only two base changes between *nodD*$_1$ of Rm1021 and Rm41: one each in the coding and upstream regions. There are 12 base differences between the DNA sequences of Rm41 and Rm1021 *nodD*$_2$; 5 of these base changes result in different amino acids.

Induction of *nodC-lacZ* in *nodD* mutants. To determine the relative contributions of each of the three *R. meliloti* *nodD* genes in activation of *nodABC*, we studied the expression of a *nodC-lacZ* fusion (integrated at the *nodC* locus) in *R. meliloti* strains which contained all possible combinations of mutations in each of the three *nodD* genes. (See Materials and Methods for details of strain construction and Table 1 for a listing of each strain with its genotype.)

The effect of alfalfa seed exudate or luteolin on *nodC-lacZ* expression is shown in Table 2, lines 1 to 8. The NodD$^+$ control, JM57, showed a four- to fivefold induction upon addition of luteolin or alfalfa seed exudate (line 1). The *nodC-lacZ* fusion was not induced by either luteolin or alfalfa seed exudate in a *nodD*$_1$ mutant (line 2). In contrast, in a *nodD*$_2$ mutant, the *nodC-lacZ* fusion was induced to significantly higher levels than in JM57, suggesting that the *nodD*$_2$ product may have a negative effect on *nodC-lacZ* induction (line 3). In a *nodD*$_3$ mutant, *nodC-lacZ* was induced to about the same levels as in the wild type. The double mutants *nodD*$_1$-*nodD*$_2$ and *nodD*$_1$-*nodD*$_3$ and the triple *nodD* mutant had a phenotype similar to the single *nodD*$_1$ mutant (lines 5, 6, and 8), and the *nodD*$_2$-*nodD*$_3$ double mutant had a phenotype similar to the single *nodD*$_2$ mutant (line 7).

In general, levels of *nodC-lacZ* induction were low in these strains, and it was difficult to draw conclusions based on small differences in expression between the different mutants.

Expression of *nodC-lacZ* in strains carrying multiple copies of each of the *nodD* genes. Initially, we sought to test the effect of several different seed exudates, in addition to alfalfa seed exudate and luteolin, on the expression of the *nodC-lacZ* fusion in the *nodD* mutants. However, because expression of the *nodC-lacZ* fusion was very low (less than 8 U of β-galactosidase [Table 2, line 9]) in the presence of *Melilotus alba* (sweet clover) or *Medicago truncatula* (barrel clover) exudates, we tested the ability of multiple copies of each *nodD* gene to activate *nodC-lacZ* expression. This was done by introducing multicopy plasmids (derivatives of pRK290 that most probably have a copy number of approximately 5 to 8 [7]) carrying one of the *nodD* genes into either a triple *nodD* mutant strain (RmD1D2D3-4) carrying the *nodC-lacZ*

fusion or a NodD$^+$ strain (JM57) carrying the *nodC-lacZ* fusion.

First, to verify that each of the cloned plasmid-borne *nodD* genes was functional, plasmids carrying each of the cloned *nodD* genes were tested for their ability to complement a *nodD* triple mutant (RmD1D2D3-3). The *nodD* triple mutant carrying either pMH901 (*nodD*$_1$) or pMH903 (*nodD*$_3$) elicited alfalfa or sweet clover nodules at the same rate as wild-type strains did (data not shown). However, only 80% of the alfalfa plants and 30% of the sweet clover developed one or more nodules when inoculated with the *nodD* triple mutant carrying pMH93-292 (*nodD*$_2$), and these nodules appeared with a significant delay. In addition to nodules, numerous rounding swellings which did not appear to be true nodules were present on all plants. We concluded from these experiments that both pMH901 (*nodD*$_1$) and pMH903 (*nodD*$_3$) carry fully functional *nodD* genes but that the *nodD*$_2$ gene carried on pMH93-292 is inherently less active in the nodulation assay than *nodD*$_1$ or *nodD*$_3$.

The results we obtained with the *nodD*$_2$-containing plasmid were similar to those previously obtained when alfalfa and sweet clover plants were inoculated with a *nodD*$_1$-*nodD*$_3$ double mutant. In the case of the double *nodD*$_1$-*nodD*$_3$ mutant, 50% of the alfalfa plants formed nodules, no nodules were observed on sweet clover plants, and swellings were observed on some, but not all, alfalfa and sweet clover plants (23). These previous results, combined with those obtained here, suggest that the function of *nodD*$_2$ can be enhanced when it is present in multiple copies.

Having determined that each of the cloned *nodD*$_1$, *nodD*$_2$, and *nodD*$_3$ genes were functional, we tested the ability of each of these cloned genes to activate *nodC-lacZ* expression in response to luteolin or to three different root exudates. The results are summarized in Table 2. Both strain JM57 and strain RmD1D2D3-4 carrying pMH901 (*nodD*$_1$) expressed β-galactosidase at high levels when induced with either luteolin or alfalfa seed exudates (lines 12 and 18). The same two strains expressed lower levels of β-galactosidase when induced with exudates from either *Melilotus alba* or *Medicago truncatula* (lines 12 and 18).

The *nodC-lacZ* fusion in strain JM57 carrying pMH93 (*nodD*$_2$) showed a two- to threefold increase in basal expression compared with strain JM57 and was activated to high levels when exposed to exudates of *Medicago sativa* or *Medicago truncatula* (Table 2, line 13). In fact, multiple copies of *nodD*$_2$ activated *nodC-lacZ* to higher levels than multiple copies of *nodD*$_1$ in response to *Medicago truncatula* seed exudate (compare lines 12 and 13). The opposite was true when the inducer was *Melilotus alba* exudate (lines 12 and 13). These results indicated that levels of the presumptive NodD$_2$-specific inducer are highest in *Medicago sativa* exudate, lower in *Medicago truncatula*, and lowest in *Melilotus alba*. The *nodD* triple-mutant strain with pMH93 (*nodD*$_2$) also responded to *Medicago sativa* and *Medicago truncatula* exudates, but to a significantly lesser extent (line 19). The difference in *nodC* expression observed between the wild-type host and a triple *nodD* mutant suggested that NodD$_2$ acts synergistically with either NodD$_1$ or NodD$_3$ in activation of the *nodC-lacZ* fusion.

Interestingly, NodD$_2$ did not respond to luteolin, although it apparently did respond to another compound(s) present in the seed exudates tested (Table 2, lines 13 and 19). Indeed, the presence of *nodD*$_2$ on a plasmid appeared to inhibit luteolin activation in the wild-type strain JM57 (compare lines 9 and 13), suggesting that NodD$_2$ interferes with NodD$_1$-luteolin-mediated activation. This result is in agreement with the results presented in lines 3 and 7, where a *nodD*$_2$ mutant was able to activate the *nodC-lacZ* fusion to levels higher than wild-type levels in the presence of either luteolin or alfalfa seed exudate.

NodD$_3$-mediated activation of *nodC-lacZ* appears to require another gene, *syrM*, that is tightly linked to *nodD*$_3$ (35). A plasmid carrying both *syrM* and *nodD*$_3$ (pMH682) caused high constitutive expression of *nodC-lacZ* in either a *nodD* triple mutant (Table 2, line 25) or a wild-type background (line 16). However, the presence of a plasmid carrying only *nodD*$_3$ (pMH903) in either the *nodD* triple mutant or the wild-type strain increased both the basal and induced levels of expression of the *nodC-lacZ* fusion only twofold (lines 14 and 23). Since both JM57 and the *nodD* triple-mutant strain are *syrM*$^+$, it appears that NodD$_3$ and SyrM can activate when present in *trans*, although the level of activation was marginal, presumably because SyrM is limiting. Addition of a plasmid carrying *syrM* (pMH904) to strain JM57 resulted in a three- to fivefold increase in *nodC-lacZ* expression (line 15), whereas addition of the same plasmid to the *nodD* triple-mutant strain (RmD1D2D3-4; line 24) had no effect on expression. Although our results with the *syrM*-containing plasmid suggest that NodD$_3$ requires *syrM* to function as an activator, we cannot exclude the possibility that SyrM has a general stimulatory effect on *nodD* genes or their protein products.

Identification of a region upstream of *nodD*$_2$ required for *nodD*$_2$-mediated activation. The *nodD*$_2$ gene is located on one end of the 6.8-kb *Eco*RI fragment cloned in plasmid pMH93 (Fig. 3). As described above, two lines of evidence indicated that pMH93 carries a functional *nodD*$_2$ gene. First, a plasmid derived from pMH93 (pMH93-292) that carries a fragment of Tn*5* and a small deletion 2.5 kb upstream of *nodD*$_2$ partially complemented a *nodD* triple mutant. Second, plasmid pMH93 activated a *nodC-lacZ* fusion in the presence of various root exudates (Table 2, line 19). On the other hand, the plasmid from which pMH93-292 was derived, pMH93-291, which carries an intact Tn*5* at the same position as the deleted Tn*5* in pMH93-292, did not activate the *nodC-lacZ* fusion (line 20). One explanation for these results is that the Tn*5* insertion in pMH93-291 and pMH93-292 is located in or has a polar effect upon a gene required for *nodD*$_2$ to function. It is likely that pMH93-292 was able to complement the *nodD* triple mutant because the triple mutant carries an intact copy of the gene mutated by Tn*5* on pMH93-292. If this reasoning is correct, it is unlikely that the Tn*5* insertion is polar upon *nodD*$_2$ itself, because pMH93-292 complemented the *nodD* triple mutant.

Further evidence that the Tn*5* insertion in pMH93-291 is not polar upon *nodD*$_2$ was obtained as follows. An *R. meliloti* strain was constructed (Rmnod291-4) carrying the Tn*5*-291 mutation and the *nodC-lacZ* fusion recombined into pRmeSU47a. Rmnod291-4 was then tested for its ability to respond to luteolin and alfalfa seed exudate (Table 2, lines 9 to 11). As shown in line 3, a *nodD*$_2$ strain expressed higher levels of *nodC-lacZ* than the wild type did, presumably because NodD$_2$ interferes with NodD$_1$-mediated activation (line 10). However, strain Rmnod291-4 expressed *nodC-lacZ* in a manner similar to the wild type (lines 9 and 11), suggesting that *nodD*$_2$ is still active in Rmnod291-4 and that the putative, newly identified gene upstream of *nodD*$_2$ is not required for the negative effect of NodD$_2$ on NodD$_1$ activation.

To further investigate the putative gene upstream of *nodD*$_2$, we constructed a set of deletion and deletion-insertion mutations which originated at the *Xho*I site in plasmid

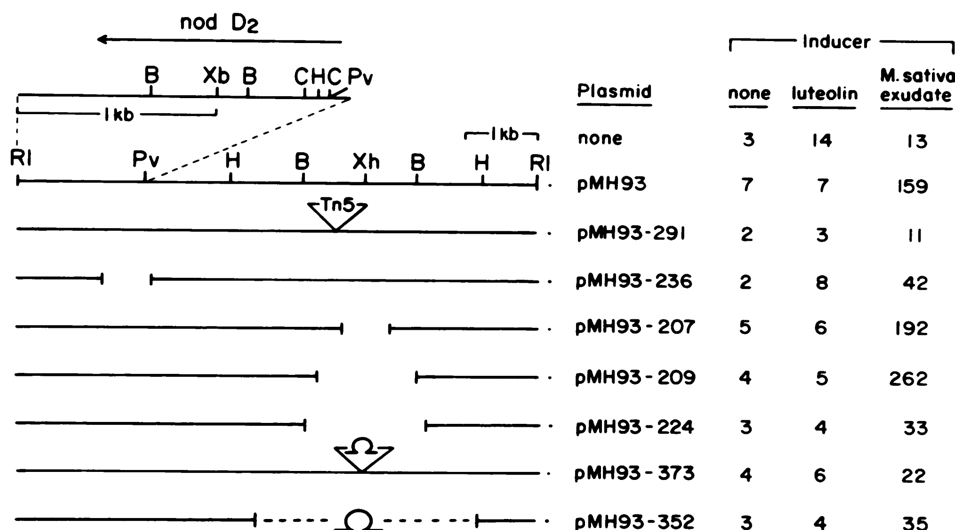

FIG. 3. Activation of *nodC-lacZ* by *R. meliloti* $nodD_2$-containing plasmids. (Left) Physical map of 6.8-kb *Eco*RI fragment of *R. meliloti* 1021 carrying $nodD_2$. pMH93 contains the entire 6.8-kb *Eco*RI fragment. The horizontal lines corresponding to each plasmid represent regions present in various deletion and/or insertion derivatives of pMH93. The triangle represents the 2.0-kb Ω fragment. Abbreviations: B, *Bam*HI; C, *Cla*I; RI, *Eco*RI; H, *Hin*dIII; Pv, *Pvu*I; Xb, *Xba*I; and Xh, *Xho*I. (Right) The table represents β-galactosidase units obtained with each plasmid in JM57 (*nodC-lacZ*) with the specified inducer.

pMH93, 3.0 kb upstream of $nodD_2$ (Fig. 3). The deletion-insertion mutations contained the so-called Ω fragment, which carries genes encoding resistance to streptomycin and spectinomycin and has transcriptional and translational stop signals from bacteriophage T4.

Each of the mutant derivatives of pMH93 was tested in the wild-type strain JM57 for its ability to activate the *nodC-lacZ* fusion in response to luteolin and to alfalfa seed exudate. Plasmids pMH93-207 and pMH93-209, which contain 0.6- and 1.3-kb deletions from the *Xho*I site, respectively, both activated the *nodC-lacZ* fusion (Fig. 3). In contrast, pMH373, which contains the Ω fragment inserted at the *Xho*I site, failed to activate the *nodC-lacZ* fusion (Fig. 3), suggesting that the gene required for $nodD_2$-mediated activation is located on either side of the *Xho*I site and is part of an operon. Plasmid pMH93-224, which contains a 1.6-kb deletion, and plasmid pMH93-352, which contains a 3.0-kb deletion and the Ω fragment, also failed to activate the *nodC-lacZ* fusion (Fig. 3).

To test whether the region upstream of $nodD_2$ is also required for activation of the *nodC-lacZ* fusion in response to exudates from plants other than alfalfa, we also tested selected mutant plasmids in the *nodD* triple-mutant strain with *Medicago truncatula* and *Melilotus alba* exudates. In the triple-mutant background, pMH93 activated *nodC-lacZ* in the presence of *Medicago truncatula* as well as alfalfa exudates (Table 2, line 19), whereas pMH93-352, which contains the Ω fragment inserted at the *Xho*I site, did not (line 22). As a control, a derivative of pMH93, pMH93-236, carrying an internal 0.6-kb deletion in $nodD_2$, was also tested and failed to activate the *nodC-lacZ* fusion in the triple *nodD* mutant background (line 21). It appears that activation of *nodC-lacZ* with either alfalfa or *Medicago truncatula* root exudate requires both $nodD_2$ and the region upstream of $nodD_2$.

All deletion derivatives of plasmid pMH93 (originating from the *Xho*I site) inhibited luteolin induction of *nodC* expression, as was seen with the wild-type plasmid pMH93 (Fig. 3).

Complementation of *R. trifolii* and *Rhizobium* sp. strain NGR234 *nodD* mutants with *R. meliloti nodD* genes. To provide additional evidence that the three *R. meliloti nodD* genes are functional but not equivalent, we tested the ability of the three *nodD* genes to complement mutations in the *nodD* genes of two other *Rhizobium* species: *R. trifolii*, which nodulates clovers, and *Rhizobium* sp. strain NGR234, which nodulates a wide variety of legumes (9, 33, 49, 50). *R. trifolii* ANU843 has a single *nodD* gene (8). Strain NGR234 has two regions in its genome which hybridize to a *nodD* probe; however, only one of these ($nodD_1$) appears to be functional, since NGR234 $nodD_1$ mutants have a Nod^- phenotype on all hosts tested (M. Nayudu, personal communication).

The *nodD* mutant strains ANU851 (*R. trifolii*) and ANU1255 (*Rhizobium* sp. strain NGR234), carrying each of the *R. meliloti nodD* genes, were inoculated on white clover seedlings (ANU851 derivatives) or siratro seedlings (ANU1255 derivatives) to assay for nodulation. *R. meliloti* $nodD_1$ complemented the *R. trifolii nodD* mutation, with nodulation delayed about 4 days compared with that of wild-type *R. trifolii*, but failed to complement the NGR234 *nodD* mutation (Table 3). The *R. meliloti* $nodD_2$ gene weakly complemented the *nodD* mutations in both *R. trifolii* and *Rhizobium* sp. strain NGR234; that is, 5 to 6 weeks postinoculation, 17% of white clover plants and 5 to 32% of the siratro plants (depending on inoculation dose) developed nodules. Finally, both *R. meliloti syrM* and $nodD_3$ were required for complementation of the *R. trifolii nodD* mutation; however, either *syrM* or $nodD_3$ alone was sufficient to partially complement the NGR234 *nodD* mutation. Bacteria were reisolated from nodules elicited by the *R. trifolii* and *Rhizobium* sp. strain NGR234 *nodD* mutants carrying the *R. meliloti nodD* genes to verify that the presence of nodules was not due to contamination of wild type bacteria.

The *R. trifolii nodD* mutant carrying *R. meliloti* $nodD_1$ elicited Fix^+ nodules on white clover. In contrast, although the *R. meliloti syrM* and $nodD_3$ genes together could com-

TABLE 3. Interspecies complementation of *nodD* mutations

Strain (genotype)	Plasmid (*R. meliloti* gene present)	Plant host	Phenotype		
			Nod	Delay (days)	Fix
R. trifolii					
ANU843 (wild type)	None	White clover	+[a]	0	+
ANU851 (*nodD*)	None	White clover	−	NR[b]	NR
ANU851 (*nodD*)	pMH901 (*$nodD_1$*)	White clover	+	4	+
ANU851 (*nodD*)	pMH93 (*$nodD_2$*)	White clover	±[c]	15–20	−
ANU851 (*nodD*)	pMH682 (*syrM $nodD_3$*)	White clover	+	4	−
ANU851 (*nodD*)	pMH903 (*$nodD_3$*)	White clover	−	NR	NR
ANU851 (*nodD*)	pMH904 (*syrM*)	White clover	−	NR	NR
Rhizobium sp. strain NGR234					
ANU240 (wild type)	None	Siratro	+	0	NT[d]
ANU1255 (*$nodD_1$*)	None	Siratro	−	NR	NT
ANU1255 (*$nodD_1$*)	pMH901 (*$nodD_1$*)	Siratro	−	NR	NT
ANU1255 (*$nodD_1$*)	pMH93 (*$nodD_2$*)	Siratro	±[e]	15–20	NT
ANU1255 (*$nodD_1$*)	pMH682 (*syrM $nodD_3$*)	Siratro	+	0	NT
ANU1255 (*$nodD_1$*)	pMH903 (*$nodD_3$*)	Siratro	+	5	NT
ANU1255 (*$nodD_1$*)	pMH904 (*syrM*)	Siratro	+	1–2	NT

[a] +, All plants contain one or more nodules.
[b] NR, Not relevant.
[c] 17% of plants contain nodules 4 weeks postinoculation (see text for details).
[d] NT, Not tested.
[e] 5 to 32% of plants contain nodules 6 weeks postinoculation (see text for details).

plement the Nod^- phenotype of the *R. trifolii nodD* mutant, the nodules were Fix^-.

DISCUSSION

All three *R. meliloti nodD* genes are functional. In a previous report, we demonstrated that *R. meliloti* has three functional *nodD* genes by showing that each of the *nodD* genes alone was sufficient to elicit nodules on alfalfa or sweet clover but that a triple *nodD* mutant had a Nod^- phenotype (23). In the current set of experiments, we confirmed that each of the *R. meliloti nodD* genes is functional by showing that each was capable of activating a *nodC-lacZ* fusion in the presence of the appropriate root exudate or the presence of the flavone luteolin. Moreover, we showed that each of the cloned *R. meliloti nodD* genes was capable of genetically complementing *nodD* mutations in either *R. trifolii* or *Rhizobium* sp. strain NGR234. Gyorgypal et al. (21) and Mulligan and Long (35) have also demonstrated that the *R. meliloti nodD* genes are functional but not equivalent.

The *R. meliloti* $nodD_1$ and $nodD_2$ genes respond to different inducers. A major conclusion from the previous study (23) was that although each of the three *R. meliloti nodD* genes is functional, they are not equivalent in nodulation. That is, *R. meliloti* mutant strains carrying different combinations of mutations in the three *nodD* genes exhibited different nodulation phenotypes on two alternative hosts, alfalfa and sweet clover. This result suggested that the three *nodD* genes play a host-specific role in nodulation (23). In the current set of experiments, we sought to obtain additional evidence that the *nodD* genes were involved in determining host specificity by testing the hypothesis that the *nodD* genes mediate differential induction of *nod* gene expression in response to different inducing compounds in root exudates.

In support of the hypothesis that each *nodD* gene responds most efficiently to a different spectrum of flavonoids, we demonstrated that *R. meliloti* $NodD_1$ responded very efficiently to luteolin in activating a *nodC-lacZ* fusion, whereas $NodD_2$ failed to respond to luteolin at all but did respond to an inducer(s) present in alfalfa and *Medicago truncatula* seed exudates. Examination of the data in Table 2 suggests that the $NodD_2$-specific inducer(s) is present at lower levels in sweet clover exudate than in alfalfa or *Medicago truncatula* exudate.

The activation data in Table 2 are generally consistent with the conclusions drawn from the nodulation phenotypes of *R. meliloti nodD* mutants observed previously. For example, the fact that $nodD_1$ appeared to be more important than $nodD_2$ in nodulation of both alfalfa and sweet clover (23) is consistent with the fact that $nodD_1$ activated *nodC-lacZ* more effectively than $nodD_2$ did in the presence of either alfalfa or sweet clover exudates. The fact that a $nodD_1$-$nodD_3$ mutant weakly nodulated alfalfa but not sweet clover is consistent with the low level of $nodD_2$-mediated activation in the presence of sweet clover exudates. Interestingly, however, a multicopy plasmid carrying $nodD_2$ was able to complement a *R. meliloti nodD* triple mutant, eliciting nodules on 30% of the sweet clover plants inoculated. It seems likely that the additional copies of the $nodD_2$ gene compensated for the low levels of $NodD_2$-specific inducer present in *Melilotus alba* exudates. The data in Table 2 show that a plasmid carrying $nodD_3$ along with *syrM* caused high constitutive *nodC* expression, which did not increase further upon addition of seed exudates. Based on these results, it seems likely that $NodD_3$ does not respond to an inducer. However, it is also possible that increasing the copy number of both *syrM* and $nodD_3$ alleviates the requirement for an effector. Examples of effector-independent activation by positive regulators upon increasing gene dosage have been reported for the *xylS* gene of *Pseudomonas putida* (31) and the *malT* gene of *Escherichia coli* (39).

On the other hand, we also found that the levels of *nodC* expression in the *nodD* mutants did not always correlate perfectly with the nodulation phenotypes previously observed on alfalfa. For example, although the *R. meliloti* $nodD_1$ mutant nodulated alfalfa after a relatively short delay of 5 to 6 days, it activated *nodC* at the same level as did the $nodD_1$-$nodD_2$-$nodD_3$ mutant in response to alfalfa seed exudate. An explanation of this apparent discrepancy is that the composition of flavonoids in the alfalfa seed exudate is not the same as the root exudate of germinating seedlings. Also,

we did not measure activation of the host-specific *nod* genes, and this may be a critical factor in determining the nodulation phenotype.

Complementation of *R. trifolii* and *Rhizobium* sp. strain NGR234 *nodD* mutations. Additional support for the conclusion that *$nodD_1$* and *$nodD_2$* products are not equivalent in their ability to activate *nod* gene expression comes from the data in Table 3, which demonstrate that *R. meliloti* *$nodD_1$* complemented an *R. trifolii nodD* mutation for nodulation of white clover but not a *Rhizobium* sp. strain NGR234 *nodD* mutation for nodulation of siratro, whereas *R. meliloti* *$nodD_2$* weakly complemented the *nodD* mutations in both species. One explanation for these results is that $NodD_1$ responds to an inducer(s) produced by white clover exudate but not to an inducer(s) produced by siratro. This explanation is consistent with the results of Spaink et al. (48), who showed that the *$nodD_1$* gene of *R. meliloti* responded to white clover root exudate and activated the *R. leguminosarum nodA* promoter, and Horvath et al. (24), who showed that the *R. meliloti nodD* gene could not activate the NGR234 (MP1K3030) *nodA* promoter in the presence of siratro root exudate. Complementation of a *R. trifolii nodD* mutation by the *R. meliloti* *$nodD_1$* gene has been previously reported by two different laboratories (17, 48).

R. meliloti syrM plus *$nodD_3$* were also able to complement the *R. trifolii nodD* mutation; however, the nodules formed were Fix^-. It is likely that *syrM* plus *$nodD_3$* complement the *R. trifolii nodD* mutant because they are able to activate the *R. trifolii nod* genes without inducers, and it is possible that this unregulated expression of *nod* genes has a detrimental effect on nitrogen fixation in a heterologous host. Support for this conclusion is provided by the work of Burn et al. (2), who isolated *R. leguminosarum nodD* mutants that were able to activate *nod* gene expression to high levels in the absence of inducers. Interestingly, these *R. leguminosarum nodD* mutants elicited Fix^- nodules on pea plants, suggesting that high, unregulated *nod* gene expression is deleterious during late stages of nodule development.

The *R. meliloti* *$nodD_2$* gene was able to weakly complement the *R. trifolii nodD* mutation. Nodules elicited by this strain [ANU851(pMH93)] appeared much later than nodules elicited by either ANU851 carrying *R. meliloti* *$nodD_1$* or ANU851 carrying *syrM* and *$nodD_3$*. Although nodules elicited by ANU851(pMH93) were Fix^-, by the time the ANU851(pMH93)-elicited nodules appeared, the plants were so starved for nitrogen that they may have been unable to sustain nitrogen fixation.

A plasmid carrying both *syrM* and *$nodD_3$* was able to complement the NGR234 *nodD* mutation; however, in contrast to what was observed with *R. trifolii*, either *syrM* or *$nodD_3$* alone was sufficient to partially complement NGR234 *nodD*. We were somewhat surprised by this result, because in *R. meliloti* both *syrM* and *$nodD_3$* are required to activate the expression of the *nod* genes. Presumably, the *R. meliloti* *$nodD_3$* gene can activate the *nod* genes of strain ANU1255; this activation may or may not acquire inducers from siratro root exudate. By far the most curious result was that *syrM* alone complemented the NGR234 *nodD* mutation. Although SyrM alone may be able to activate the *nod* genes of NGR234, it is also possible that SyrM, along with the remaining *nodD* gene of NGR234, *$nodD_2$*, is able to activate *nod* gene expression. Although mutations in *$nodD_1$* of NGR234 render the strain Nod^- on nine hosts tested (Nayudu, personal communication), it is not known whether this second *nodD* gene (*$nodD_2$*) is functional.

DNA sequence of the *R. meliloti* *$nodD_2$* gene. There are a number of possible reasons why *R. meliloti* *$nodD_2$*, but not *$nodD_1$*, complemented the *Rhizobium* sp. strain NGR234 *nodD* mutation: higher expression of *$nodD_2$* than *$nodD_1$* in ANU1255, the ability of $NodD_2$ to respond more efficiently to siratro root exudate than $NodD_1$ does, or the ability of $NodD_2$ to activate the ANU1255 *nod* genes better than $NodD_1$ does. *R. meliloti* $NodD_1$ and $NodD_2$ are highly homologous except in the C-terminal halves. Six of the last eight amino acids of *R. meliloti* $NodD_1$ and $NodD_2$ are different, and $NodD_2$ has two additional amino acids. Since we have shown that $NodD_1$ and $NodD_2$ respond to different inducers, it is possible that the last few amino acids are important for flavonoid specificity.

Interaction between $NodD_2$ and $NodD_1$ or $NodD_3$. Comparison of the levels of *nodC-lacZ* expression in a *nodD* triple mutant versus a wild-type strain, each carrying a *$nodD_2$* plasmid, suggests that $NodD_2$ functions more effectively as an activator in the presence of either the *$nodD_1$* or *$nodD_3$* gene. From our experiments, we cannot determine whether *$nodD_1$* or *$nodD_3$* or both are responsible for this synergism. One possible explanation for this phenomenon would be that NodD is functional as a multimer in activation of transcription and that $NodD_2$-mediated activation is enhanced by the heterodimer $NodD_1$-$NodD_2$ or $NodD_3$-$NodD_2$.

Interestingly, although $NodD_2$ apparently requires $NodD_1$ or $NodD_3$ to efficiently activate the *nodC-lacZ* fusion, $NodD_2$ interfered with $NodD_1$-mediated luteolin activation. Strains which were *$nodD_1^+$ $nodD_2$ $nodD_3^+$* expressed *nodC* at levels higher than wild-type levels, and introduction of a plasmid carrying *$nodD_2$* into a wild-type strain also inhibited *nodC* expression. It is possible that $NodD_2$ interferes with luteolin activation by competing with $NodD_1$ for binding sites at the *nodA* promoter or by acting as a poison subunit in a $NodD_1$ multimer. Such interactions between the *nodD* gene products have also been observed by Mulligan and Long (35), who found that $NodD_2$ function required $NodD_1$ and that $NodD_2$ and $NodD_3$ reduce *nodABC* induction by $NodD_1$ and luteolin. Unlike Mulligan and Long, we did not observe an effect of *$nodD_3$* on $NodD_1$-luteolin activation; however, our experiments and those of Mulligan and Long used different *$nodD_3$* alleles.

Identification of a new nodulation gene. We have tentatively identified a region upstream of *$nodD_2$* which is required for *$nodD_2$*-mediated activation. This region may encode gene products which are required for $NodD_2$ function, or the region may affect *$nodD_2$* gene expression. At present, we cannot distinguish between the two possibilities that deletions and/or insertion(s) in this region either (i) affect a gene which is required for *$nodD_2$*-mediated activation or (ii) are polar on *$nodD_2$* expression. Our results do not indicate a precise position of the putative gene important for *$nodD_2$* function. It could reside on either side of the *Xho*I site in the 6-kb region upstream of *$nodD_2$*. Sequence analysis and transcript mapping of this region will help in identifying a gene(s) which is important for $NodD_2$ function. It is intriguing that both *$nodD_3$* and *$nodD_2$* appear to require additional linked regions to activate *NodC* expression. Comparisons between *$nodD_3$-syrM* and *$nodD_2$* and its upstream region may provide important clues about the functions of these regulatory genes.

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