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Interference between *Rhizobium meliloti* and *Rhizobium trifolii* Nodulation Genes: Genetic Basis of *R. meliloti* Dominance

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Transfer of an IncP plasmid carrying the *Rhizobium meliloti nodFE*, *nodG*, and *nodH* genes to *Rhizobium trifolii* enabled *R. trifolii* to nodulate alfalfa (*Medicago sativa*), the normal host of *R. meliloti*. Using transposon Tn*5*-linked mutations and in vitro-constructed deletions of the *R. meliloti nodFE*, *nodG*, and *nodH* genes, we showed that *R. meliloti nodH* was required for *R. trifolii* to elicit both root hair curling and nodule initiation on alfalfa and that *nodH*, *nodFE*, and *nodG* were required for *R. trifolii* to elicit infection threads in alfalfa root hairs. Interestingly, the transfer of the *R. meliloti nodFE*, *nodG*, and *nodH* genes to *R. trifolii* prevented *R. trifolii* from infecting and nodulating its normal host, white clover (*Trifolium repens*). Experiments with the mutated *R. meliloti nodH*, *nodF*, *nodE*, and *nodG* genes demonstrated that *nodH*, *nodF*, *nodE*, and possibly *nodG* have an additive effect in blocking infection and nodulation of clover.

Bacteria of the genus *Rhizobium* elicit nitrogen-fixing nodules on the roots of leguminous host plants. In general, a particular *Rhizobium* species elicits nodules on a limited number of host legumes (25). For example, *Rhizobium meliloti* nodulates *Medicago*, *Melilotus*, and *Trigonella* species; *R. trifolii* nodulates *Trifolium* species; and *R. leguminosarum* nodulates *Pisum* and *Vicia* species. In all of these symbioses, nodulation is a complex process involving the following steps: recognition between the symbionts, attachment of *Rhizobium* to root hairs, root hair curling, infection thread formation within root hairs, initiation of a nodule meristem, and nodule organogenesis (12, 25, 42).

In *R. meliloti*, genes involved in the early stages of the infection process (nodulation genes [*nod* genes]) are located on a megaplasmid called pSymA (2, 23, 32, 40, 41). The *nodA*, *nodB*, and *nodC* genes are referred to as common *nod* genes; these genes are conserved both structurally and functionally between *Rhizobium* and *Bradyrhizobium* species (10); the nodulation-defective phenotype of an *R. meliloti* mutant carrying a mutation in any one of these three genes can be complemented by the homologous cloned nodulation gene from another *Rhizobium* species (7, 23). Transfer of the common nodulation genes between species has no effect on host range. On the basis of these observations, it has been concluded that the common nodulation genes code for essential, evolutionarily conserved steps in the nodulation process that are common to all *Rhizobium*-legume symbioses (for reviews, see references 10 and 25).

In contrast to the common nodulation gene, *R. meliloti* also has a set of genes (*nodFE*, *nodG*, and *nodH*; *nodFEGH* are also referred to as *hsnABCD*, where *hsn* stands for host specificity of nodulation) that appear to be involved in defining the *R. meliloti* host range (7, 20, 38). Thus, whereas transfer of *R. meliloti nodABC* to *R. trifolii* had no effect on the *R. trifolii* host range, transfer of *nodFE* plus *nodG* plus *nodH* allowed *R. trifolii* to nodulate alfalfa (7, 30). The *nodH* gene appears to be unique to *R. meliloti*, and although *nodF* and *nodE* are conserved at the nucleotide sequence level among several *Rhizobium* species (8, 14, 20), mutations in these genes cannot be complemented by the homologous genes from other *Rhizobium* species (7, 20, 23). An additional line of evidence that *nodH* and *nodFE* are involved in determination of host specificity is that certain *R. meliloti nodH* or *nodFE* mutants elicit marked root hair curling on heterologous hosts such as clover and vetch (7, 20), whereas wild-type *R. meliloti* has no effect on these hosts.

In addition to the common and host-specific nodulation genes, *R. meliloti* has three functional copies of a regulatory gene, *nodD* (16, 17), that are responsible for activating the expression of the other *nod* genes in the presence of flavone root exudates (29). On the basis of the criteria stated above, the *R. meliloti* $nodD_1$ gene was originally designated as a common nodulation gene (10). On the other hand, recent evidence from different *Rhizobium* species indicates that the *nodD* genes play a role in host range determination by modulating gene expression as a function of the composition of the legume host root exudate (17, 19, 36).

In this report we provide additional data on the role of the *R. meliloti nodH*, *nodFE*, and *nodG* genes in host range determination by studying the changes in symbiotic behavior associated with the transfer of these genes to *R. trifolii*.

MATERIALS AND METHODS

Microbiological techniques. Bacterial strains, plasmids, and bacteriophages are described in Table 1 and Fig. 1.

Conditions used for bacterial growth, conjugation, and transduction have been described previously (7, 40). Selection for *Escherichia coli* C2110 and GMI3540 was carried out in 10 and 25 μg of nalidixic acid per ml, respectively. Other antibiotic concentrations are given in references 7 and 40.

The transfer of Tn*5* insertions from *R. meliloti* pSymA to pGMI515 was performed as follows. *E. coli* GMI35 40(pGMI515) and *R. meliloti* 2011(pSymA::Tn*5*) were grown to mid-log phase in TY medium and mixed in equal volumes, and 2.5 ml of the mixed culture was collected on nitrocellulose filter membrane (diameter, 2.5 cm) and incubated over-

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TABLE 1. Bacteria, bacteriophages, and plasmids used in this study

Strain, phage, or plasmid	Relevant characteristics	Source or reference
E. coli		
MC1061	*ara leu lac gal hsdR rpsL*	5
C2110	*polA1* Nal^r	22
GMI3540	*met supE supF hsdR hsdM recA56 nal-300*	P. Boistard
R. meliloti		
RCR2011 = SU47	Wild-type Nod$^+$ Fix$^+$ on *M. sativa*	29
1021	Sm^r derivative of RCR2011	25
GMI5376	*nodH2212*::Tn*5*; Sm^r Nm^r Bl^r	7
GMI5381	*nodE2309*::Tn*5*; Sm^r Nm^r Bl^r	7
GMI5117	*nodE2311*::Tn*5*; Sm^r Nm^r Bl^r	7
GMI5179	*nodF2407*::Tn*5*; Sm^r Nm^r Bl^r	7
GMI5388	Region II*115*::Tn*5*; Sm^r Nm^r Bl^r	7
GMI5616	Δ(*nodF*)*5*; Nm^r Bl^r	This study
GMI5617	Δ(*nodE*)*3*; Nm^r Bl^r	This study
GMI5618	Δ(*nodFEH*)*11*; Nm^r Bl^r	This study
GMI5619	Δ(*nodH*)*8*; Nm^r Bl^r	This study
GMI5621	Δ(*nodG*)*7*; Nm^r Bl^r	This study
GMI5622	Δ(*nodFE*)*4*; Nm^r Bl^r	This study
GMI5623	Δ(*nodFEGH*)*9*; Nm^r Bl^r	This study
GMI5629	Δ(*nodFEG*)*2*; Nm^r Bl^r	This study
R. trifolii		
ANU843	Wild-type Nod$^+$ Fix$^+$ on *T. repens*	11
IncP1 plasmids		
RP4	Tc^r Ap^r Km^r Tra$^+$	6
pPH1JI	Gm^r Tra$^+$	3
pGMI515	*R. meliloti hsn* insert in RP4; Tc^r Ap^r	35
ColE1 plasmids		
pGMI174	6-kilobase insert in pBR322 containing *R. meliloti hsn* region; Tc^r	8
pRK2013	Helper plasmid for mobilization of pRK290 derivatives; *tra*(RK2) Km^r	9
Bacteriophage		
N3	Transducing phage of *R. meliloti*	27

night at 34°C on a TY agar plate. A 0.2-ml portion of a mid-log-phase culture of *E. coli* GMI3540 was then spread carefully on the membrane; the plate was incubated again for 6 h at 34°C. *E. coli* GMI3540(pGMI515::Tn*5*) transconjugants were selected on LB medium containing tetracycline, kanamycin, and nalidixic acid; plasmid DNA was isolated and analyzed by restriction analysis, and the plasmids were transferred by conjugation, as described previously (7, 40), to *R. trifolii* ANU843.

DNA biochemistry. Restriction endonucleases, *Bal* 31 nuclease, and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals and New England BioLabs, Inc. Transformation of *E. coli*, plasmid isolation, digestion by restriction enzymes, ligations, and agarose gel electrophoresis were carried out by standard procedures (26). Isolation of total DNA from *R. meliloti* was performed as described previously (1). A nick translation kit from Boehringer Mannheim was used for radiolabeling DNA. Southern blotting and DNA hybridization using GeneScreen Plus were carried out as specified by the manufacturer (New England Nuclear Corp.).

Construction of deletions in the *nodHFEG* region. The deletions were first constructed in pGMI174, a pBR322 derivative that carries a 6-kilobase-pair *Eco*RI-*Pst*I DNA fragment containing the *R. meliloti nodH*, *nodFE*, and *nodG* genes (8). A combination of restriction endonucleases and *Bal* 31 nuclease was used to create the deletions as described below. A 1.5-kilobase-pair *Xho*I-*Hin*dIII fragment from

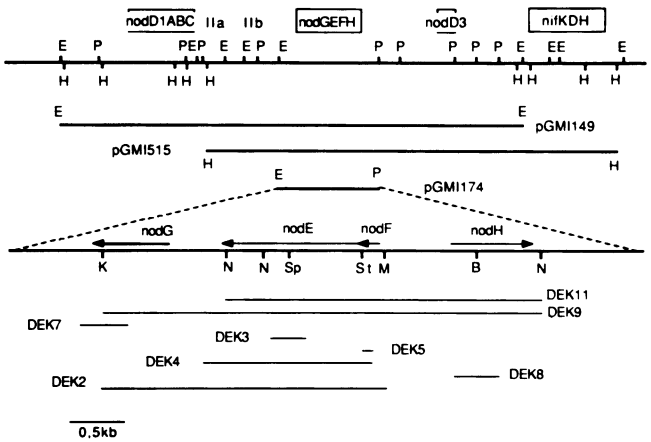

FIG. 1. Map of the nodulation region of pSymA(2011) including plasmids and deletions in the *nod* region. At the top of the figure is a restriction map of the nodulation region of pSymA(2011). Below, pGMI149, pGMI515, and pGMI174 are represented. An enlarged physical map of pGMI174 is shown below, with arrows indicating the positions and the directions of transcription of the *nodF*, *nodE*, *nodG*, and *nodH* genes. The horizontal lines below the physical map of pGMI174 represent the regions deleted in the mutants; the DEK nomenclature refers to the deletions listed in Table 1; for example, DEK11 refers to Δ(*nodFEH*)*11*. Abbreviations: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; N, *Nco*I; K, *Kpn*I; Sp, *Spe*I; St, *Stu*I; M, *Mst*II; B, *Bst*EII.

pKC7 (26) containing the kanamycin and bleomycin resistance genes of Tn*5* (Km^r cassette) was then ligated to the deleted plasmids, *E. coli* MC1061 was transformed with the ligation mixture, and colonies containing the Km^r cassette were selected on LB agar containing tetracycline and kanamycin. Each construction was verified by restriction analysis of plasmid DNA.

Deletion Δ(*nodFEG*)*2* was constructed by first digesting pGMI174 with *Mst*II and *Kpn*I and then digesting it briefly with *Bal* 31 to generate blunt ends. Δ(*nodE*)*3* was constructed by digesting pGMI174 with *Spe*I and *Bal* 31 to generate a 1,550-base-pair deletion. Δ(*nodF*)*5* was constructed by digesting pGMI174 with *Stu*I and then *Bal* 31 to give a 50-base-pair deletion. Δ(*nodG*)*7* was constructed by digesting pGMI174 with *Kpn*I and then *Bal* 31 to generate a 420-base-pair deletion. Δ(*nodH*)*8* was constructed by digesting pGMI174 with *Bst*EII and *Bal* 31 to give a 380-base-pair deletion. Deletion *nod*(*FEGH*)*9* was constructed by digesting pGMI174 with *Nco*I and *Kpn*I and then briefly with *Bal* 31 to generate blunt ends. Δ(*nodFEH*)*11* was obtained by digesting pGMI174 with *Nco*I before treating with the Klenow fragment of polymerase I to generate blunt ends. The various deletions are shown in Fig. 1.

To transfer the deletions to *R. meliloti*, the deleted pGMI174 derivatives containing the Km^r cassette were digested with *Eco*RI and then ligated into the *Eco*RI site of pRK290. The resulting cointegrate plasmids were selected by transforming *E. coli* C2110 *polA* with the ligation mixture. The cointegrate plasmids were then transferred to *R. meliloti* 1021 by triparental mating with pRK2013 as the helper plasmid (9). The deletions tagged by the Km^r cassette were crossed into *R. meliloti* 1021 pSymA by marker exchange (33) with pPH1JI (3). All the constructions were verified by Southern hybridization. The deletions were eventually transferred to wild-type *R. meliloti* 2011 by transduction with bacteriophage N3 (27) and then to plasmid pGMI515 as described above for the transfer of Tn*5* insertions to pGMI515.

Plant assays. Nodulation assays and plant treatments for light or electron microscopy have been described previously (7, 28, 31). To isolate bacteria from clover nodules, we sterilized nodules for 5 min in calcium hypochlorite (33 mg/ml), rinsed them five times in sterile water (the sterility of the last rinse was checked by plating a sample on TY agar), and crushed them in 0.5 ml of sterile water; bacteria were isolated on TY plates.

RESULTS

Transfer of alfalfa nodulation ability to *R. trifolii* ANU843. We showed previously that transfer of the *R. meliloti* nodulation region carried on the recombinant plasmid pGMI149 (Fig. 1) to *R. trifolii* enabled *R. trifolii* to elicit nodules on alfalfa (7). In the current set of experiments, we sought to differentiate between the roles of the *R. meliloti* common and host-specific nodulation genes in this process. Specifically, to determine whether the *R. meliloti* host-specific nodulation region is sufficient to alter the host range of *R. trifolii*, we introduced pGMI515 (Fig. 1) into wild-type *R. trifolii* ANU843. pGMI515 carries *nodH*, *nodFE*, *nodG*, $nodD_3$, and the so-called region II (7), which affects the efficiency of infection and nodulation. pGMI515 does not carry *nodABC* or $nodD_1$.

The transconjugant ANU843(pGMI515) elicited root hair curling (Hac^+; Fig. 2A to C), infection threads (Inf^+; Fig. 2B and C), and ineffective nodules (Nod^+; Fig. 2D) on alfalfa seedlings. The infection threads generally appeared in small root hairs and did not stain with methylene blue (Fig. 2B); when observed in longer root hairs, the small, white threads did not reach to the base of the root hair (Fig. 2C).

The ineffective nodules elicited by ANU843(pGMI515) arose from inner cortical cells (Fig. 2D) and showed the typical histological organization of indeterminate nodules with an apical meristem, peripheral vascular bundles, and an outer endodermis (Fig. 2E). Transfer cells, associated with vascular bundles, were also observed (data not shown). The submeristematic and the central cells of these nodules were filled with amyloplasts but were completely devoid of infection threads or released bacteria (Fig. 2E). Interestingly, a significant amount of intercellular infection was located in the epidermal and in the outermost cortical cells of 10 developing nodules that were studied (Fig. 2F).

The ANU843(pGMI515)-elicited nodules contained both intercellular and intracellular bacteria (Fig. 3A). Intercellular bacteria were often surrounded by electron-dense particulate plant material and were sometimes appended to the outer surface of plant cell walls (Fig. 3B). Intracellular bacteria were embedded in a fibrillar matrix surrounded by the plant cell wall (Fig. 3A and C); it is likely that these bacteria originated from intercellular penetration of bacteria and local cell wall dissolution (Fig. 3A). Whatever their location, no elongated bacteroid forms were observed, and the bacteria seemed senescent, as indicated by their abnormal morphology and electron-dense cytoplasm (Fig. 3B and C).

***R. trifolii* ANU843(pGMI515) is unable to nodulate clover.** It has been reported that the introduction of a heterologous symbiotic plasmid into a *Rhizobium* strain can affect nodulation of its normal host (4, 22). To test whether the transfer of pGMI515 to *R. trifolii* ANU843 had this effect, we inoculated ANU843(pGMI515) on white clover seedlings (*Trifolium repens* cv Ladino). Contrary to the control, ANU843(RP4), which was Hac^+ Inf^+ Nod^+ (Table 2; Fig. 4A), ANU843(pGMI515) was not able to curl clover root hairs (Hac^-), to induce infection threads (Inf^-) (Table 2; Fig. 4B), or to elicit nodule formation (Nod^-) (Table 2). These results indicate that the *R. meliloti* host range genes are epistatic to and/or dominant over the *R. trifolii* host range genes when both sets of genes are present in *R. trifolii*. The terminology "epistatic and/or dominant" is used here since it is not known whether genes homologous to all of the *R. meliloti* host range genes exist in *R. trifolii*.

Mutagenesis of *R. meliloti* host range genes. To assess the roles of the *R. meliloti nodH*, *nodF*, *nodE*, and *nodG* genes and of region II in the epistasis and/or dominance over the *R. trifolii* nodulation genes, previously obtained (7) Tn*5* insertion mutations in *nodH*, *nodF*, *nodE*, *nodG*, and in region II were transferred from *R. meliloti* pSymA into pGMI515 by homologous recombination (see Materials and Methods). In addition, to study the effect of inactivation of various combinations of *nodFEGH* genes, a new set of deletion-insertion mutations in *nodF*, *nodE*, *nodG*, *nodH*, *nodFE*, *nodFEG*, *nodFEH*, and *nodFEGH* (Fig. 1) was constructed in *E. coli* in a pBR322 derivative carrying the *R. meliloti hsn* region (see details in Materials and Methods). To transfer the deletion-insertion mutations to the *R. meliloti* genome, the deleted pBR322 derivatives were recloned in the broad-host-range vector pRK290 by using the kanamycin resistance cassette that had been inserted into each deletion. The deletion-insertion mutations were then conjugated into *R. meliloti* and crossed into *R. meliloti* pSymA by homogeno-

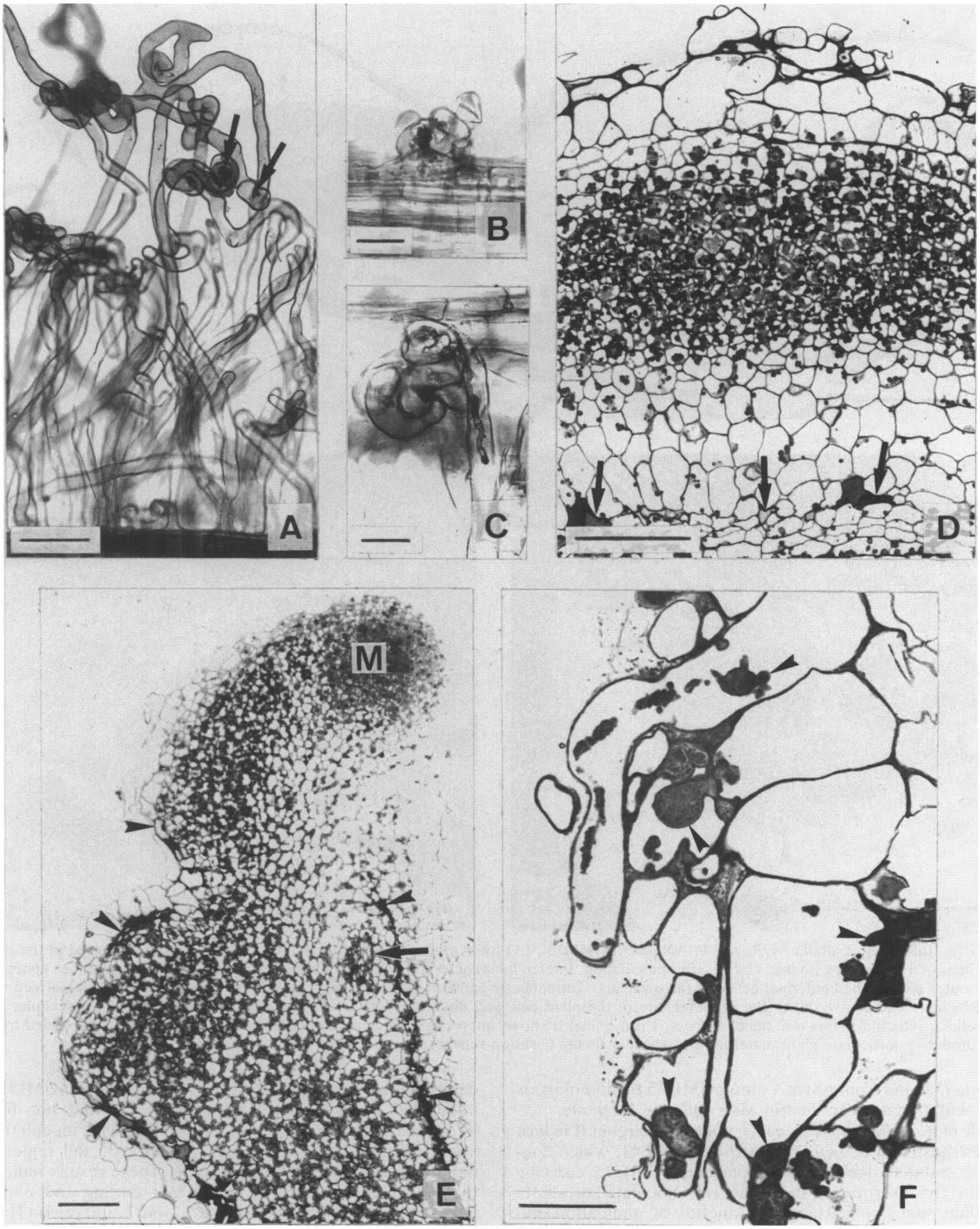

FIG. 2. Infection and nodulation of alfalfa by *R. trifolii* transconjugant ANU843(pGMI515). (A) Root hair curling (arrows) 10 days after inoculation (bar, 100 μm). (B and C) Infection occurring via a small root hair (B) or a mature root hair (C) 10 days after inoculation (bars, 50 μm). (D) Cortical origin in a developing nodule 12 days after inoculation. The arrows point to the endodermis (bar, 100 μm). (E) Mature nodule 3 weeks after inoculation. M, apical meristem; arrowheads, nodule endodermis; arrow, nodule vascular bundles. The central nodular cells are filled with amyloplasts (bar, 100 μm). (F) Intercellular infection (arrowheads) in the distal cells of a developing nodule fixed 12 days after inoculation (bar, 50 μm).

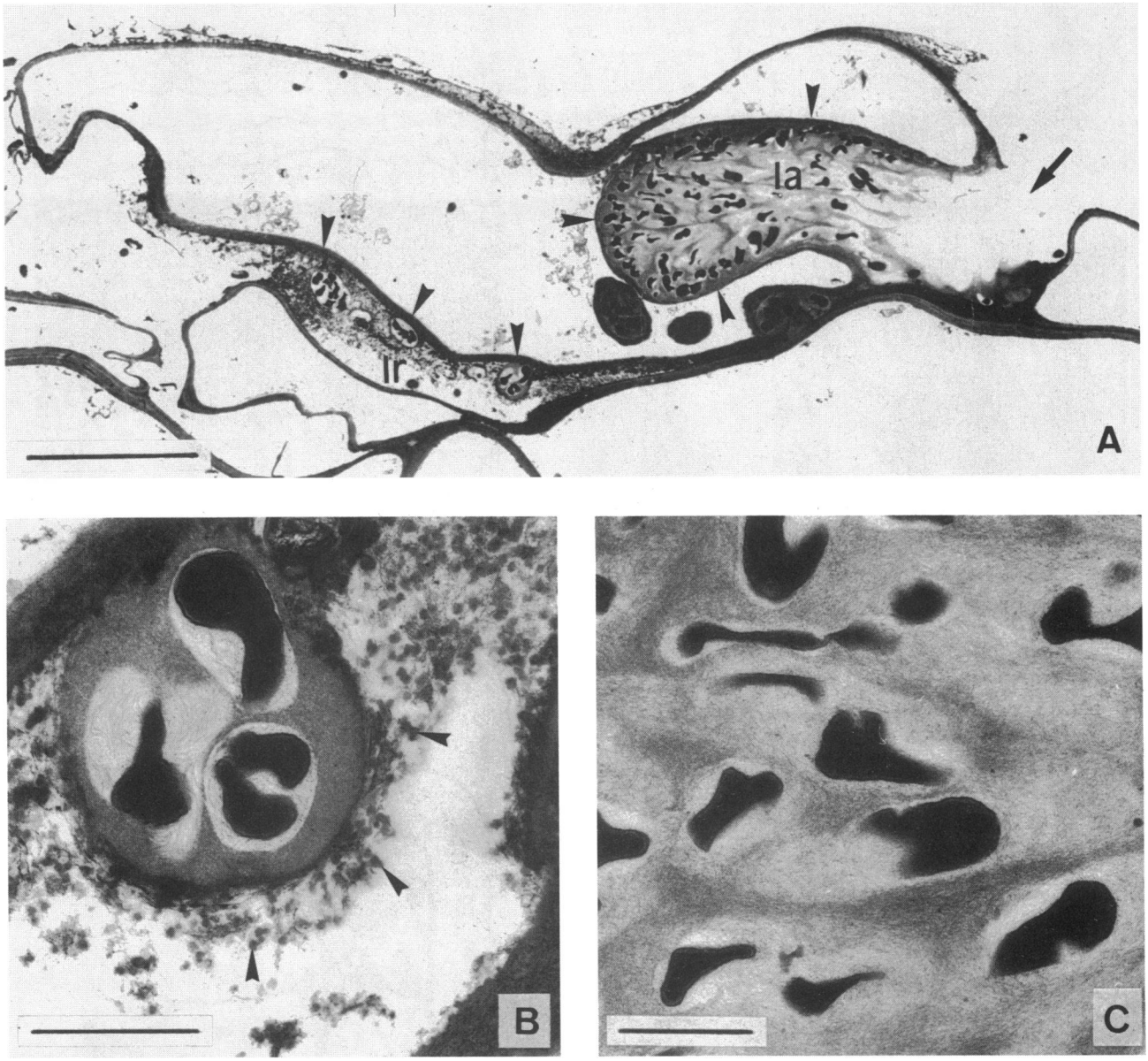

FIG. 3. Infection of alfalfa by *R. trifolii* ANU843(pGMI515). (A) Intercellular (Ir) and intracellular (Ia) infections observed in the most distal cells of a developing nodule 12 days after inoculation. Intercellular bacteria (Ir) are surrounded by a plant material sometimes appended to the outer part of the epidermal cell wall (arrowheads). Intracellular bacteria (Ia) are embedded in a matrix limited by the plant cell wall (arrowheads). The arrow shows the probable site of the plant cell wall dissolution. (Bar, 10 μm.) (B and C) Details of intercellular and intracellular infections shown in panel A; bars, 1 μm. Panel B shows intercellular bacteria embedded in a fibrillar matrix surrounded by an electron-dense particulate plant material (arrowheads). Panel C shows senescent intracellular bacteria in a fibrillar matrix.

tization (33) and from pSymA onto pGMI515 by homologous recombination as described in Materials and Methods.

Role of *R. meliloti nodFE*, *nodG*, *nodH*, and region II in host range transfer to *R. trifolii*. *R. trifolii* ANU843, which contained mutated derivatives of plasmid pGMI515 carrying various Tn*5* insertions or deletions, was inoculated on alfalfa seedlings; data concerning the kinetics of nodulation and microscopic observation of early symbiotic steps are presented in Table 2 and Fig. 5. All *nodH* mutants had a Hac^- Inf^- Nod^- phenotype, although some root hair deformations were observed. For *nodF*, *nodE*, *nodG*, or region II mutants, the appearance of root deformations (most probably ineffective nodules; however, these were not subjected to ultrastructural analysis) was delayed, and the number of these deformations was smaller than with ANU843(pGMI515). *nodF* and *nodE* mutants were Hac^+ but did not form infection threads (Inf^-) within 10 days following inoculation. *nodG* mutants had a Hac^s Inf^- phenotype, and region II mutants had a Hac^- Inf^- phenotype. These results indicate that *nodH* is required for alfalfa hair curling and nodule organogenesis and that *nodF*, *nodE*, *nodG*, and region II are involved in infection thread initiation and growth within the root hair.

Role of *R. meliloti nodFE*, *nodG*, *nodH*, and region II in epistasis and/or dominance over *R. trifolii* nodulation genes. To identify the genes carried by pGMI515 that were involved in the strong inhibitory effect of pGMI515 on clover infection and nodulation, *R. trifolii* ANU843(pGMI515) derivatives

TABLE 2. Symbiotic phenotypes of *R. trifolii* derivatives on alfalfa and clover

Strain	*nod*::Tn*5* or Δ(*nod*) mutation[a]	Alfalfa[b]			Clover[b]		
		Hac	Inf	Nod	Hac	Inf	Nod
ANU843(RP4)		−	−	−	+	+	+
ANU843(pGMI515)		+	+	+	−	−	−
GMI9139	*nodH2212*::Tn*5*	−	−	−	+	+/−	ND[c]
GMI9143	*nodE2309*::Tn*5*	+	+/−	ND	+	+/−	ND
GMI9147	*nodE2311*::Tn*5*	+	−	ND	+	+/−	ND
GMI9141	*nodF2407*::Tn*5*	+	−	ND	+	+/−	ND
GMI9144	Region II115::Tn*5*	−	−	ND	+	+/−	ND
GMI9571	Δ(*nodF*)*5*	+/−	−	ND	+	+/−	+
GMI9570	Δ(*nodE*)*3*	+	−	ND	s	+/−	+
GMI9569	Δ(*nodG*)*7*	s	−	ND	s	−	+
GMI9572	Δ(*nodH*)*8*	−	−	−	+	+/−	+
GMI9575	Δ(*nodFE*)*4*	+	−	ND	+/−	+/−	+
GMI9574	Δ(*nodFEH*)*11*	−	−	−	+	+	+
GMI9623	Δ(*nodFEG*)*2*	s	−	ND	+	+	+
GMI9576	Δ(*nodFEGH*)*9*	−	−	−	+	+	+

[a] *nod*::Tn*5* mutations and *nod* deletions (Δ) were present in plasmid pGMI515 introduced into *R. trifolii* ANU843.

[b] Abbreviations used to describe the infection phenotypes: Hac$^+$, marked root hair curling (shepherd's crook) with three mutant phenotypes: Hac$^-$, no marked hair curling; Hac$^{+/-}$, curling affecting very few root hairs; Hac^s, marked root hair curling but bright refractile spot only rarely present in the middle of the crook. Inf$^+$, infection thread formation within root hair with two mutant phenotypes: Inf$^-$, no infection thread; Inf$^{+/-}$, rare infection threads. Nod$^+$, nodule formation; Nod$^-$, no nodules; Hac and Inf phenotypes were recorded 10 days after inoculation, and the Nod phenotype was recorded 3 weeks after inoculation.

[c] ND, Root deformations present but not studied in detail by microscopy.

carrying various Tn*5* insertions and deletions in *nodH*, *nodF*, *nodE*, *nodG*, and region II were inoculated on clover seedlings, and the number of nodules elicited was recorded over a period of 3 weeks. Cytological studies were conducted to verify the structure of elicited nodules. In addition, bacteria were reisolated from nodules, and their plasmids were extracted and characterized by restriction analysis. All tested bacteria contained pGMI515 carrying the Tn*5* insertion or the deletion that had been introduced into them, indicating that the formation of nodules was not due to loss of the pGMI515 derivative.

Each of the Tn*5* insertion mutations had some effect on the inhibitory properties of pGMI515 (Table 2); however, none of these mutations allowed ANU843(pGMI515) to nodulate as efficiently as the ANU843(RP4) control (Fig. 6). Mutations in *nodH* and region II had less effect on the inhibitory phenotype than did mutations in *nodF*; i.e., both *nodH* and *nodF* mutations allowed nodules to form on clover, but considerably more nodules were formed when pGMI515 contained a Tn*5* insertion in *nodF* (Fig. 6). Two different *nodE* insertions gave somewhat different results; one insertion had a phenotype similar to the *nodH* insertion, and the other had a phenotype intermediate between the *nodH* and *nodF* insertions (Fig. 6). In any case, the fact that a Tn*5* insertion in *nodF* allowed more nodules to be formed on clover than did either insertion in *nodE* suggests that *nodF* has an effect separate from that of *nodE*; i.e., the observed phenotype of the *nodF*::Tn*5* mutant was not simply due to polarity of the Tn*5* insertion on the downstream *nodE* gene. All the Tn*5*-induced mutants tested had a Hac$^+$ Inf$^{+/-}$ phenotype. The infection threads formed were very short, and there were far fewer than with ANU843(RP4) (Fig. 4C). These results indicate that the inhibitory effect of pGMI515 is not due to a single gene; at least three genes, *nodF*, *nodE*, and *nodH*, are involved.

When ANU843(pGMI515) containing deletions in one or several of the *R. meliloti nodH*, *nodF*, *nodE*, or *nodG* genes was used to inoculate clover, the kinetic analysis of nodulation suggested that inactivation of both *nodH* and *nodFE* was required to restore the ability to nodulate clover as well as ANU843(RP4) did (Fig. 7). Deletions inactivating *nodH*, *nodF*, *nodE*, *nodG*, *nodFE*, and *nodFEG* genes restored only a partial ability to nodulate clover.

The response of clover to inoculation with ANU843 carrying the deleted versions of pGMI515 was also studied at the microscopic level (Table 2). Ten days after inoculation, ANU843(pGMI515) mutants with deletions covering at least *nodFE* and *nodH* were Hac$^+$ Inf$^+$ like the ANU843(RP4) control strain (Fig. 4D). ANU843(pGMI515) mutants deleted in *nodH*, *nodF*, *nodE*, and *nodFE* induced the formation of a reduced number of shepherd's crooks in comparison with ANU843(RP4), and the rare infection threads observed were most often seen on the top of a developing nodule (Fig. 4E). Finally, ANU843(pGMI515) mutants deleted in *nodG* had a Hac^s Inf$^-$ phenotype (Fig. 4F). These experiments suggest that a combined effect of *R. meliloti nodF*, *nodE*, and *nodH* is responsible for the blocking of clover nodulation observed when plasmid pGMI515 is introduced in *R. trifolii*.

DISCUSSION

A number of previously published results have suggested that the *R. meliloti nodFE*, *nodG*, and *nodH* genes are involved in the control of host range specificity. First, mutations in the *R. meliloti nodFEGH* genes could not be functionally complemented by cloned nodulation genes from other *Rhizobium* species (7, 20, 23). Second, *R. meliloti* mutants affected in these nodulation genes had a modified host range (7, 20); they were not only defective in infection and nodulation of alfalfa, but they could also induce marked root hair curling and infection thread initiation on heterologous hosts (*Vicia* or *Trifolium* species), which wild-type *R. meliloti* could not do. Third, transfer of both the *nodABCD*$_1$ and *nodFEGH* clusters to *R. trifolii* extended the *R. trifolii* host range to alfalfa, whereas transfer of the *nodABCD*$_1$ cluster alone had no effect on the *R. trifolii* host range (7, 30, 38).

In this report we provide direct evidence that *nodH*, *nodFE*, and *nodG* determine the host range of *R. meliloti*. First, in agreement with previously reported data (38), we

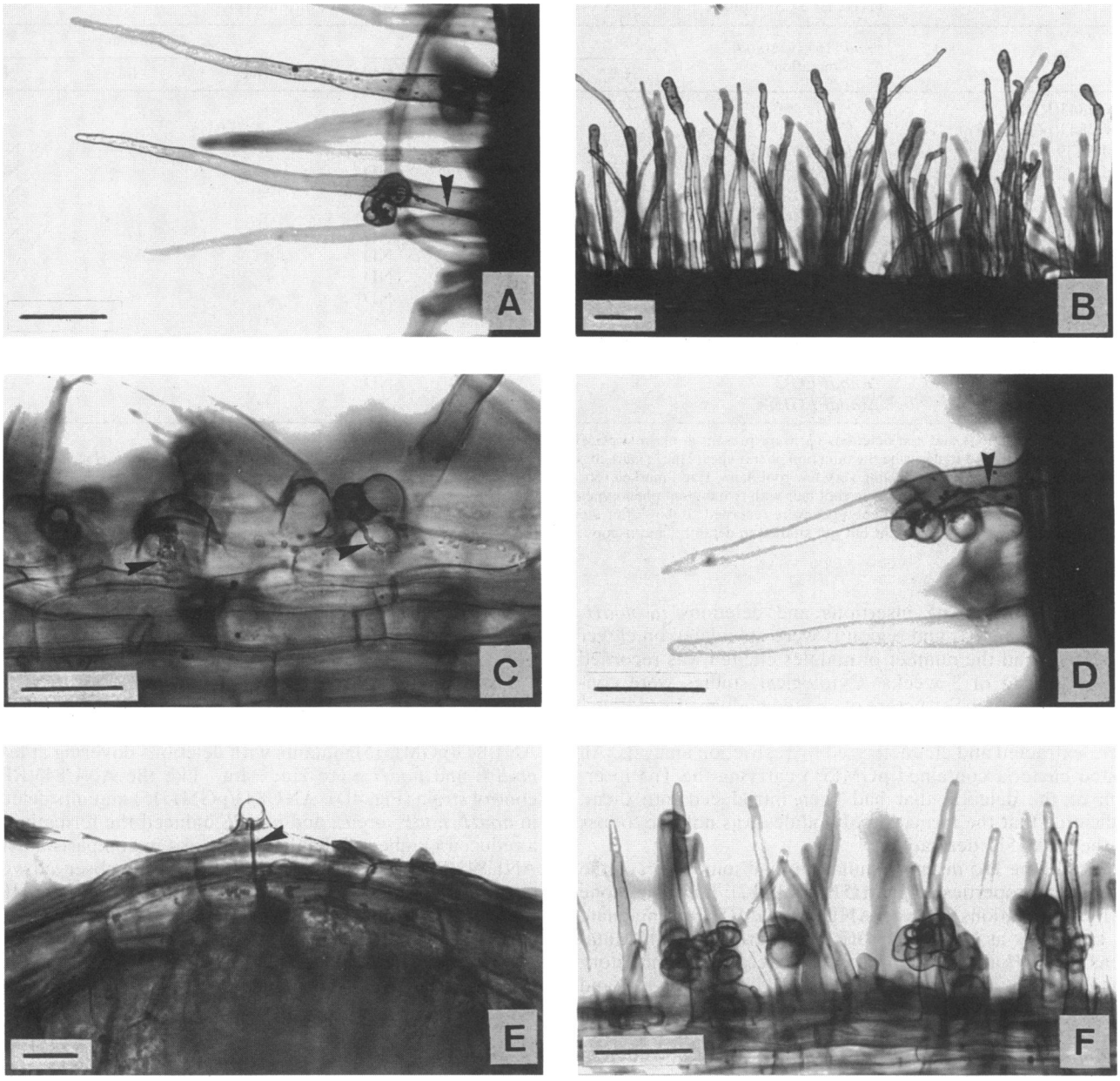

FIG. 4. Early symbiotic steps observed 10 days after inoculation of clover roots by *R. trifolii* ANU843(RP4), ANU843(pGMI515) or ANU843(pGMI515) derivatives with deletions in the *R. meliloti hsn* region carried by pGMI515. (A) Control ANU843(RP4). Normal infection (arrowhead) is occurring through a curled root hair. (B) ANU843(pGMI515). No hair curling or infection threads are observed. (C) ANU843(pGMI515) *nodH2212*::Tn*5*). Small infection threads (arrowheads) are observed in short root hairs. (D) ANU843[pGMI515 Δ(*nodFEH)11*]. Restoration of the wild-type infection (arrowhead; compare with panels A and B). (E) ANU843[pGMI515 Δ(*nodFE)4*]. Infection (arrowhead) through a root hair is observed on the top of a developing nodule. (F) ANU843[pGMI515 Δ(*nodG)7*]. Hac^s phenotype. In all micrographs, the bar represents 100 μm.

showed that transfer to *R. trifolii* of plasmid pGMI515, which contains the *R. meliloti nodFEGH* region but not the *nodABCD*$_1$ cluster, enabled *R. trifolii* to nodulate alfalfa. Second, using Tn*5*-induced mutations and in vitro-constructed deletions, we showed that *R. meliloti nodFE*, *nodG*, and *nodH* genes are necessary for the extension of the *R. trifolii* host range to alfalfa. Third, using cytological techniques, we showed that the *nodH* gene, when introduced into *R. trifolii* on pGMI515, is responsible for alfalfa root hair curling, whereas *nodFE* genes play a role in infection thread formation within root hairs. It is significant that the same functions had previously been assigned to *nodH* and *nodFE* in the homologous *R. meliloti*-alfalfa symbiosis (7, 20, 38).

We also found that *R. trifolii* carrying pGMI515 elicited the formation of short abortive infection threads and invaded alfalfa roots through an intercellular penetration mechanism that is different from the usual infection pathway via infection threads initiating within the root hairs that penetrate through the root cortex. Such an atypical mode of infection of alfalfa has already been described with *R. meliloti* mutants defective for exopolysaccharide synthesis (24) and with *Agrobacterium tumefaciens* transconjugants carrying the *R.*

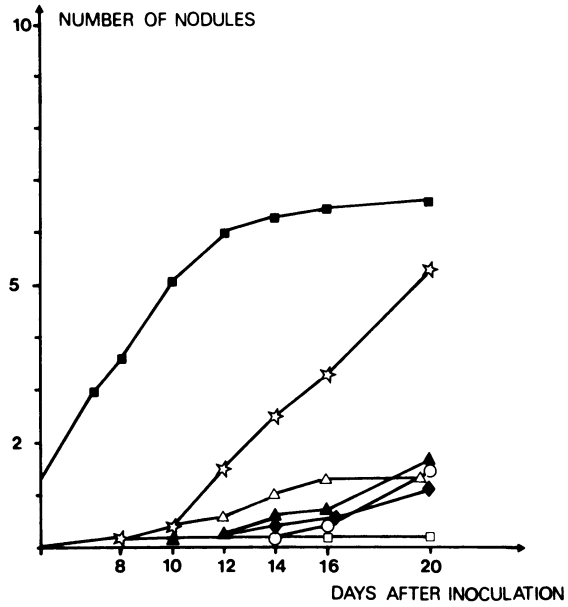

FIG. 5. Kinetics of nodule formation on alfalfa elicited by *R. meliloti* 2011, *R. trifolii* ANU843(pGMI515), and derivatives of *R. trifolii* ANU843(pGMI515) carrying Tn*5*-induced mutations in *nodFE*, *nodH*, and region II. Nodules were scored on *M. sativa* cv. gemini (10 tubes per treatment). The nodule number represents the average number per tube. Symbols: ■, *R. meliloti* 2011; ☆, ANU843(pGMI515); □, GMI9139(*nodH2212*::Tn*5*); ○, GMI9143 (*nodE2309*::Tn*5*); ▲, GMI9147(*nodE2311*::Tn*5*); △, GMI9144 (region II*115*::Tn*5*); ◆, GMI9141(*nodF2407*::Tn*5*).

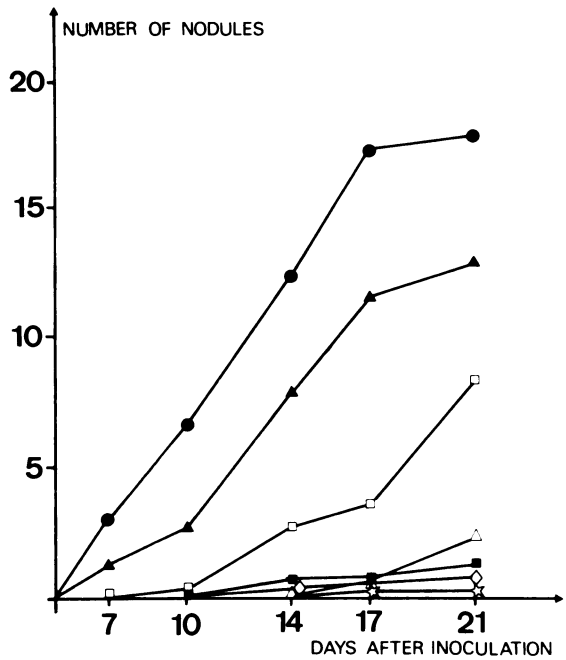

FIG. 6. Kinetics of nodule formation on clover elicited by *R. trifolii* ANU843(RP4), ANU843(pGMI515), and derivatives of ANU843(pGMI515) carrying Tn*5*-induced mutations in *nodFE*, *nodH*, and region II. Nodules were scored on *T. repens* cv. ladino (10 tubes per treatment). The nodule number represents the average number per tube. Symbols: ●, ANU843(RP4); ☆, ANU843(pGMI515); ▲, GMI9141(*nodF2407*::Tn*5*); □, GMI9147(*nodE2311*::Tn*5*); △, GMI9139(*nodH2212*::Tn*5*); ■, GMI9143(*nodE2309*::Tn*5*), ◆, GMI9144(region II115::Tn*5*).

meliloti pSymA nodulation region (41). All of these observations indicate that the induction of nodules does not require the development of a network of infection threads initiating within the root hairs. Despite the inability of *R. trifolii* ANU843(pGMI515) to initiate normal nodulation of alfalfa, the fact that nodules were elicited suggests that the *hsn* region determines not only the specificity of hair curling and infection thread initiation but also the specificity of nodule initiation.

Other genes carried by pGMI515, in particular those present in region II, appear to affect the efficiency of nodulation of alfalfa, since the transfer to *R. trifolii* of *R. meliloti nodFEGH* genes alone resulted in a very weak nodulation of alfalfa (30).

Several experiments involving the transfer of symbiotic plasmids or cloned nodulation genes between various *Rhizobium* species have been reported (4, 11, 18, 22). In general, a *Rhizobium* species that acquires nodulation genes from another *Rhizobium* species still nodulates its original host, although it may acquire the ability to nodulate a heterologous host as well. On the other hand, a type of physiological incompatibility between different pSym plasmids has been described. An *R. leguminosarum* strain carrying two pSym plasmids, one eliciting the nodulation of French bean and the other eliciting the nodulation of pea, was able to nodulate both hosts. In pure culture (ex planta) the two plasmids were stably maintained. However, most strains reisolated from the nodules of one host had lost the pSym, allowing the nodulation of the other host (4). This suggested that the expression of the nonhomologous pSym inhibits the efficiency of the interaction of the *Rhizobium* with the homologous host, resulting in a selective advantage for the *Rhizobium* strain having lost the nonhomologous pSym.

In the experiments reported here, we encountered an extreme case of negative interference between symbiotic genes. When pGMI515, which carries the *R. meliloti hsn* region, was conjugated into *R. trifolii*, the transconjugants, although acquiring the ability to nodulate alfalfa, lost the ability to infect and nodulate clover. Consistent with this observation, the introduction of cloned *R. trifolii* nodulation genes into *R. meliloti* did not extend the host range of *R. meliloti* (21). It appears that *R. meliloti* host range genes are dominant over and/or epistatic to those of *R. trifolii*, depending on whether *R. trifolii* has genes homologous (or analogous) to the *R. meliloti nodFE* and *nodH* genes. This epistasis and/or dominance was overlooked in previous studies in which the *R. meliloti hsn* region was introduced into *R. trifolii* in cloning vehicles derived from pRK290 (38; S. Sharma, personal communication); such vectors are known to be less stable in *Rhizobium* than RP4 (34, 39), and it is likely that the nodules formed by these *R. trifolii* transconjugants were formed by cells that had lost the pRK290-derived plasmid. It appears that demonstration of epistasis and/or dominance in our case was made possible by the use of the very stable self-transmissible plasmid RP4 as a cloning vector.

The host specificity of phytopathogenic bacteria has recently been shown to be determined by avirulence (*avr*) genes (15, 37). The introduction of a given *avr* clone can change the race specificity from virulent (compatible) to avirulent (incompatible) on the appropriate plant cultivars. The *avr* gene can be dominant in the presence of virulence alleles (15) or epistatic to nonallelic genes (37). Because the *R. meliloti* host range genes are dominant over and/or

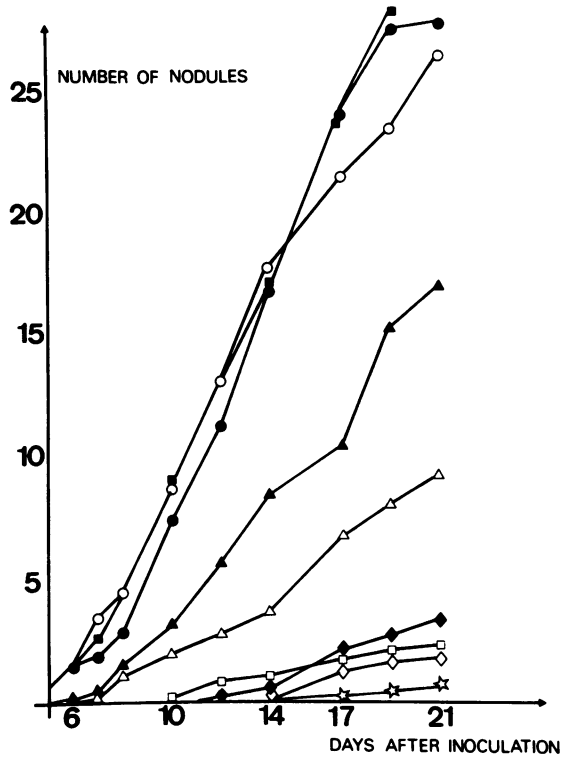

FIG. 7. Kinetics of nodule formation on clover by *R. trifolii* ANU843(RP4), ANU843(pGMI515), and derivatives of ANU843 (pGMI515) carrying deletions in the *hsn* region. Nodules were scored on *T. repens* cv. ladino (10 tubes per treatment). The nodule number represents the average number per tube. Symbols: ●, ANU843(RP4); ☆, ANU843(pGMI515); ■, GMI9574 Δ(*nodFEH*)*11*; ○, GMI9576 Δ(*nodFEGH*)*9*; ▲, GMI9575 Δ(*nodFE*)*4*; △, GMI9571 Δ(*nodF*)*5*; ◆, GMI9569 Δ(*nodG*)*7*; □, GMI9570 Δ(*nodE*)*3*; ◇, GMI9572 Δ(*nodH*)*8*.

epistatic to the *R. trifolii* host range genes, in a formal genetic sense, *R. meliloti* host range genes behave in *R. trifolii* like the *avr* genes of pathogenic bacteria. That is, inactivation of the *R. meliloti* host range genes restores the virulence of *R. trifolii* for its compatible host. The *nodFE* operon appears to be dominant over its allelic counterpart in *R. trifolii* (see above). *nodH*, according to DNA hybridization experiments, does not have an analog in *R. trifolii* (M. Djordjevic, personal communication); *nodH* is therefore most probably epistatic to nonallelic genes in *R. trifolii*. These formal analogies between the genetic control of host range in pathogenic and symbiotic bacteria might reflect some similarities at the molecular level.

The molecular basis for the epistasis and/or dominance by *R. meliloti* host range genes is not known. Innes et al. studied whether *R. trifolii* nodulation genes were properly expressed in the presence of pGMI515(21). Using *nod*::*lac* transcriptional fusions, they showed that the introduction of pGMI515 into *R. trifolii* did not change the transcription pattern of the *R. trifolii nodA* gene and did not significantly repress the transcription of *R. trifolii* genes involved in host specificity. *R. meliloti* host-specific genes may act instead at the translational level, or the epistasis and/or dominance may be explained by the activity of specific gene products rather than by changes in gene expression. For example, the *R. meliloti nodFE* and/or *nodH* genes may code for enzymes that alter a specific substrate required for clover nodulation into a product required for alfalfa nodulation. Because the inactivation of both *nodFE* and *nodH* is necessary to restore full nodulation of clover by ANU843(pGMI515), it is likely that *nodFE* and *nodH* products are not involved in a linear metabolic pathway; otherwise, the inactivation of either one should be sufficient to suppress the dominance.

It is doubtful that the copy number of pGMI515 plays a major role in the dominance effect. pGMI515 is derived from RP4, which is a low-copy-number plasmid (13, 39). In addition, even when *R. trifolii* nodulation genes are present on a high-copy-number plasmid in *R. meliloti*, the *R. meliloti* epistasis and/or dominance is observed (21). It is also unlikely that the epistasis and/or dominance is due to an excessive concentration of *nodFE* products that are very homologous between *R. trifolii* and *R. meliloti*; when *R. trifolii nodFE* genes are present on a multicopy plasmid in *R. trifolii*, clover nodulation is not affected (35).

In the light of these observations, we propose a simple model to explain the functioning of host range genes in *R. trifolii* and the epistasis and/or dominance of the *R. meliloti* host-specific nodulation genes over the *R. trifolii* genes. A specific rhizobial signal (either a surface component or a diffusible molecule) is synthesized or modified by the host range gene products. This molecule(s) is able to specifically interact with clover and elicit the infectious process. When the *R. meliloti* host range genes are introduced into *R. trifolii*, they modify the clover-specific signal to an alfalfa-specific signal which is not recognized by clover. Alternatively, the clover-specific signal could still be synthesized by *R. trifolii* but the presence of an alfalfa-specific signal inhibits the recognition of the clover-specific signal by clover.

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LITERATURE CITED

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman, and K. Struhl.** 1987. Current protocols in molecular biology 1987–1988, p. 2.4.1–2.4.5. Greene Publishing Associates and John Wiley & Sons, Inc., New York.
2. **Banfalvi, Z., V. Sakanyan, C. Koncz, A. Kiss, I. Dusha, and A. Kondorosi.** 1981. Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *Rhizobium meliloti*. Mol. Gen. Genet. **184**:318–325.
3. **Beringer, J. E., J. Beynon, A. V. Buchanan-Wollaston, and A. W. B. Johnston.** 1978. Transfer of the drug resistance transposon Tn*5* to *Rhizobium*. Nature (London) **276**:633–634.
4. **Beynon, J. L., J. E. Beringer, and A. W. B. Johnston.** 1980. Plasmids and host range in *Rhizobium leguminosarum* and *Rhizobium phaseoli*. J. Gen. Microbiol. **120**:421–429.
5. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138**:179–207.
6. **Datta, N., R. W. Hedges, E. J. Shaw, R. B. Sykes, and M. H. Richmond.** 1971. Properties of an R factor from *Pseudomonas aeruginosa*. J. Bacteriol. **108**:1244–1249.
7. **Debellé, F., C. Rosenberg, J. Vasse, F. Maillet, E. Martinez, J. Dénarié, and G. Truchet.** 1986. Assignment of symbiotic devel-

opmental phenotypes to common and specific nodulation (*nod*) genetic loci of *Rhizobium meliloti*. J. Bacteriol. **168**:1075–1086.
8. **Debellé, F., and S. B. Sharma.** 1986. Nucleotide sequence of *Rhizobium meliloti* RCR2011 genes involved in host specificity of nodulation. Nucleic Acids Res. **14**:7453–7472.
9. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for Gram negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**:7347–7351.
10. **Djordjevic, M. A., D. W. Gabriel, and B. G. Rolfe.** 1987. *Rhizobium*, the refined parasite of legumes. Annu. Rev. Phytopathol. **25**:145–168.
11. **Djordjevic, M. A., W. Zurkowski, J. Shine, and B. G. Rolfe.** 1983. Sym plasmid transfer to various symbiotic mutants of *Rhizobium trifolii*, *Rhizobium leguminosarum*, and *Rhizobium meliloti*. J. Bacteriol. **156**:1035–1045.
12. **Dudley, M. E., T. W. Jacobs, and S. R. Long.** 1987. Microscopic studies of cell divisions induced in alfalfa roots by *Rhizobium meliloti*. Planta **171**:289–301.
13. **Figurski, D., R. Meyer, and D. R. Helinski.** 1979. Suppression of ColE1 replication properties by the Inc-P1 plasmid RK2 in hybrid plasmids constructed in vitro. J. Mol. Biol. **133**:295–318.
14. **Fisher, R. F., J. A. Swanson, J. T. Mulligan, and S. R. Long.** 1987. Extended regions of nodulation genes in *Rhizobium meliloti* 1021. II. Nucleotide sequence, transcription start sites and protein products. Genetics **117**:191–201.
15. **Gabriel, D. W., A. Burges, and G. R. Lazo.** 1986. Gene-for-gene interactions of five cloned avirulence genes from *Xanthomonas campestris* pv. malvacearum with specific resistance genes in cotton. Proc. Natl. Acad. Sci. USA **83**:6415–6419.
16. **Gottfert, M., B. Horvath, E. Kondorosi, P. Putnoky, F. Rodriguez-Quinones, and A. Kondorosi.** 1986. At least two *nodD* genes are necessary for efficient nodulation of alfalfa by *Rhizobium meliloti*. J. Bacteriol. **167**:881–887.
17. **Honma, M. A., and F. M. Ausubel.** 1987. *Rhizobium meliloti* has three functional copies of the *nodD* symbiotic regulatory gene. Proc. Natl. Acad. Sci. USA **84**:8558–8562.
18. **Hooykaas, P. J. J., A. A. N. van Brussel, H. den Dulk-Ras, G. M. S. van Slogteren, and R. A. Schilperoort.** 1981. Sym plasmid of *Rhizobium trifolii* expressed in different rhizobial species and *Agrobacterium tumefaciens*. Nature (London) **291**: 351–353.
19. **Horvath, B., C. W. B. Bachem, J. Schell, and A. Kondorosi.** 1987. Host specific regulation of nodulation genes in *Rhizobium* is mediated by a plant signal, interacting with the *nodD* gene product. EMBO J. **6**:841–848.
20. **Horvath, B., E. Kondorosi, M. John, J. Schmidt, I. Torok, Z. Gyorgypal, I. Barabas, U. Wieneke, J. Schell, and A. Kondorosi.** 1986. Organisation, structure and symbiotic function of *Rhizobium meliloti* nodulation genes determining host specificity for alfalfa. Cell **46**:335–343.
21. **Innes, R., M. Djordjevic, B. Rolfe, J. Denarie, C. Rosenberg, and P. Kuempel.** 1987. Interactions between *Rhizobium meliloti* and *Rhizobium trifolii* nodulation genes: what is the basis for dominance by *Rhizobium meliloti*, p. 229–231. *In* D. P. S. Verma and N. Brisson (ed.), Molecular genetics of plant-microbe interactions. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
22. **Johnston, A. W. B., J. L. Beynon, A. V. Buchanan-Wollaston, S. M. Setchell, P. R. Hirsch, and J. E. Beringer.** 1978. High frequency transfer of nodulating ability between strains and species of *Rhizobium*. Nature (London) **276**:634–636.
23. **Kondorosi, E., Z. Banfalvi, and A. Kondorosi.** 1984. Physical and genetic analysis of a symbiotic region of *Rhizobium meliloti*: identification of nodulation genes. Mol. Gen. Genet. **193**:445–452.
24. **Leigh, J. A., E. R. Signer, and G. C. Walker.** 1985. Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. Proc. Natl. Acad. Sci. USA **82**:6231–6235.
25. **Long, S. R.** 1984. Genetics of *Rhizobium* nodulation, p. 265–306. *In* T. Kosuge and E. W. Nester (ed.), Plant-microbe interactions, vol. 1. Macmillan Publishing Co., New York.
26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Martin, M. O., and S. R. Long.** 1984. Generalized transduction in *Rhizobium meliloti*. J. Bacteriol. **159**:125–129.
28. **Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149**:114–122.
29. **Mulligan, J. T., and S. R. Long.** 1985. Induction of *Rhizobium meliloti nodC* expression by plant exudate requires *nodD*. Proc. Natl. Acad. Sci. USA **82**:6609–6613.
30. **Putnoky, P., and A. Kondorosi.** 1986. Two gene clusters of *Rhizobium meliloti* code for early essential nodulation functions and a third influences nodulation efficiency. J. Bacteriol. **167**: 881–887.
31. **Rolfe, B. G., P. M. Gresshoff, and J. Shine.** 1980. Rapid screening for symbiotic mutants of *Rhizobium* and white clover. Plant Sci. Lett. **19**:277–284.
32. **Rosenberg, C., P. Boistard, J. Denarie, and F. Casse-Delbart.** 1981. Genes controlling early and late functions in symbiosis are located on a megaplasmid in *Rhizobium meliloti*. Mol. Gen. Genet. **184**:326–333.
33. **Ruvkun, G. B., and F. M. Ausubel.** 1981. A general method for site-directed mutagenesis in procaryotes. Nature (London) **289**: 85–88.
34. **Schmidhauser, T. J., and D. R. Helinski.** 1985. Regions of broad-host-range plasmid RK2 involved in replication and stable maintenance in nine species of gram-negative bacteria. J. Bacteriol. **164**:446–455.
35. **Schofield, P. R., R. W. Ridge, B. G. Rolfe, J. Shine, and J. M. Watson.** 1984. Host-specific nodulation is encoded on a 14kb DNA fragment in *Rhizobium trifolii*. Plant Mol. Biol. **3**:3–11.
36. **Spaink, H. P., C. A. Wijffelman, E. Pees, R. J. H. Okker, and B. J. J. Lugtenberg.** 1987. *Rhizobium* nodulation gene *nodD* as a determinant of host specificity. Nature (London) **328**:337–340.
37. **Staskawicz, B. J., D. Dahlbeck, and N. T. Keen.** 1984. Cloned avirulence gene of *Pseudomonas syringae* pv. glycinea determines race-specific incompatibility on *Glycine max* (L.) Merr. Proc. Natl. Acad. Sci. USA **81**:6024–6028.
38. **Swanson, J. A., J. K. Tu, J. Ogawa, R. F. Fisher, and S. R. Long.** 1987. Extended region of nodulation genes in *Rhizobium meliloti* 1021. I. Phenotypes of Tn*5* insertion mutants. Genetics **117**:181–189.
39. **Thomas, C. M., and C. A. Smith.** 1987. Incompatibility-group P plasmids: genetics, evolution, and use in genetic manipulation. Annu. Rev. Microbiol. **41**:77–101.
40. **Truchet, G., F. Debelle, J. Vasse, B. Terzaghi, A. M. Garnerone, C. Rosenberg, J. Batut, F. Maillet, and J. Dénarié.** 1985. Identification of a *Rhizobium meliloti* pSym 2011 region controlling the host specificity of root hair curling and nodulation. J. Bacteriol. **164**:1200–1210.
41. **Truchet, G., C. Rosenberg, J. Vasse, J. S. Julliot, S. Camut, and J. Dénarié.** 1984. Transfer of *Rhizobium meliloti* Sym genes into *Agrobacterium tumefaciens*: host specific nodulation by atypical infection. J. Bacteriol. **157**:134–142.
42. **Vasse, J. M., and G. L. Truchet.** 1984. The *Rhizobium*-legume symbiosis: observation of root infection by bright-field microscopy after staining with methylene blue. Planta **161**:487–489.