

Identification of a *Rhizobium meliloti* pSym2011 Region Controlling the Host Specificity of Root Hair Curling and Nodulation

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In *Rhizobium meliloti* 2011 nodulation genes (*nod*) required to nodulate specifically alfalfa are located on a pSym megaplasmid. Nod⁻ derivatives carrying large pSym deletions were isolated. By complementation of these strains with in vivo- and in vitro-constructed episomes containing pSym sequences and introduction of these episomes into *Agrobacterium tumefaciens*, we show (i) that from a region of pSym of about 360 kilobases, genes required for specific alfalfa nodulation are clustered in a DNA fragment of less than 30 kilobases and (ii) that a *nod* region located between *nifHDK* and the common *nod* genes is absolutely required for alfalfa nodulation and controls the specificity of root hair curling and nodule organogenesis initiation.

Rhizobium strains are soil bacteria which induce the formation of nodules on the roots of leguminous hosts, in which they fix nitrogen. The induction of nodule organogenesis is specific: a *Rhizobium* strain can generally form root nodules with a limited range of plants, e.g., *Rhizobium meliloti* strains with species of *Medicago*, *Melilotus*, and *Trigonella*, or *Rhizobium trifolii* with *Trifolium* species (38). In *Medicago sativa*-*R. meliloti* symbiosis the developmental steps leading to the initiation of nodule formation can be divided schematically as follows: attachment to root hairs, root hair curling, formation of infection threads within root hairs, growth of the threads toward the inner cortex of the root, initiation of a nodule meristem in the inner root cortex, and nodule organogenesis (17, 19, 29, 39, 40). This multistep process is likely to be controlled by both host and bacterial genes (for reviews, see references 41 and 43). Whether host specificity is involved for each of the early steps or only for some is not known (26).

The formation of shepherd's crooks (i.e., marked root hair curling) on root hairs (43) is considered to be a very specific host reaction to infection by the compatible bacteria (45). In *R. meliloti* it has been shown that genes controlling alfalfa root hair curling are located on a pSym megaplasmid (2, 19, 27, 30) of about 1,500 kilobase pairs (kb) (7). In *R. meliloti* 2011 insertional mutations that simultaneously alter root hair curling and nodulation have been mapped at about 25 kb from the nitrogenase genes (21, 24). These *nod* mutations, which can be complemented not only by the corresponding wild-type sequences from *R. meliloti* (27) but also by nodulation genes from other *Rhizobium* species (16), can be defined as altering common *nod* genes (16, 18, 25).

Indeed, DNA sequencing studies recently have revealed a considerable degree of homology between the common *nod* genes from *R. meliloti* (37) and *Rhizobium leguminosarum* (32). Transfer of *R. meliloti* pSym genes into *Agrobacterium tumefaciens* enables this bacterium to induce nodule forma-

tion on alfalfa roots (20, 24, 39, 44) but not on clover (18, 39), demonstrating that genes coding for nodule initiation and host range specificity are also located on pSym.

In this report, we describe the isolation of Nod⁻ derivatives of strain 2011 which carry large deletions in pSym. Complementation studies of these strains with in vivo- and in vitro-constructed episomes containing pSym sequences and introduction of these episomes into *A. tumefaciens* showed that (i) genes required for alfalfa nodulation are clustered in a DNA fragment of less than 30 kb, (ii) a *nod* region located between *nifHDK* and the common *nod* genes described previously (27) is absolutely required for alfalfa root hair curling and nodulation, and (iii) sequences located in this new *nod* region control the specificity of root hair curling and nodule organogenesis initiation.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are shown in Table 1. *A. tumefaciens* GMI9050 is a spontaneous streptomycin-resistant (100 µg/ml) and rifampin-resistant (100 µg/ml) derivative of C58C1. The Nod⁻ mutants were characterized as *R. meliloti* 2011 derivatives by sensitivity tests with the specific bacteriophage π1. *Escherichia coli*, *Rhizobium*, and *Agrobacterium* strains were grown as described previously (30). Phage sensitivity tests were performed by the double-layer technique on yeast extract-mannitol C medium (6).

Mating conditions. Donor and recipient strains were grown in TY liquid medium (4) to mid-log phase, mixed in equal volumes, and incubated overnight on a TY agar plate, directly in the case of spot mating, or after collection on a filter membrane. *E. coli* and *R. meliloti* crosses resulting in the insertion of Tn7 into pSym2011 have been described elsewhere (23). For the introduction of plasmids into *R. meliloti* and *A. tumefaciens*, *E. coli* donor strains (GMI3180, GMI3540, 803, and HB101) were counterselected with 100 µg of streptomycin per ml, 100 µg of rifampin per ml, or 100 µg of spectinomycin per ml. Transfer of pRmSL26, pGMI515, pGMI149, and pIJ1089 was selected with 10 µg of

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

Bacterial strain, bacteriophage, or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i>		
ED8767	<i>supE supF hsdS met recA56</i>	23
GMI3180	Spontaneous Rif ^r derivative of ED8767	23
GMI3540	Spontaneous Nal ^r derivative of ED8767	23
HB101	<i>ara gal lac met pro xyl supE rpsL (Sm^r) recA hsdR hsdM</i>	28
GMI3506	<i>thy deoC (Mu cts62) (Tn7)</i>	23
GMI3232	<i>thr leu rpsL hsdR rpoB (Mu cts62)</i>	23
803	<i>met recA</i>	A. Downie
<i>A. tumefaciens</i>		
C58C1	Derivative of C58 cured of Ti plasmid	30
GMI9050	Spontaneous Sm ^r Rif ^r derivative of C58C1	This study
<i>R. meliloti</i>		
RCR2011 = SU47	Wild-type; Nod ⁺ Fix ⁺ ^a	30
RCR2011 derivatives carrying pSym deletions ^b		
GMI963	Nod ⁺ Fix ⁻ $\Delta(\text{fix-2.3})298$ (Tn7) ^c	This study
GMI255	Nod ⁻ $\Delta(\text{fix-1074 nod nifHDK})7125$ (Tn5) ^d , Nal ^r	B. Terzaghi
GMI766	Nod ⁻ $\Delta(\text{nod nifA})766$ Sp ^r	T. Huguet (this laboratory)
GMI956	Nod ⁻ $\Delta(\text{nod nifA})29$ (Tn7)	This study
GMI957	Nod ⁻ $\Delta(\text{nod nifA})80$ (Tn7)	This study
GMI958	Nod ⁻ $\Delta(\text{nod nifA})166$ (Tn7)	This study
GMI932	Nod ⁻ $\Delta(\text{nod nifA})105$ (Tn7)	This study
GMI357	Nod ⁻ $\Delta(\text{nodABC})\text{HGO.1}$ Nm ^r Rif ^r	T. Huguet
GMI361	Nod ⁻ $\Delta(\text{nodABC})\text{HG4.1}$ Nm ^r Rif ^r	T. Huguet
IncP1 plasmids		
RP4	Tc ^r Ap ^r Km ^r	10
pGM142	RP4-prime (in vivo); 290-kb insert of pSym2011 with Tn5 insertion (no. 20) in the <i>nif</i> region; Tc ^r Ap ^r Nm ^r ^e	23
pGM1104	Derivative of pGM142 with a Mu <i>c</i> ⁺ insertion in the RP4 <i>tet</i> gene; Ap ^r Nm ^r	23
pGM171	RP4-prime (in vivo); 70-kb insert of pSym2011 with Tn5 insertion (no. 20) in the <i>nif</i> region; Tc ^r Ap ^r Nm ^r ^e	This study
pGM1515	RP4-prime (in vitro); Tc ^r , Ap ^r ^f	This study
pRK290	<i>ori</i> (RK2); Tc ^r	11
pGM1149	pRK290-prime, Tc ^r ^f	This study
pRmSL26	pLAFR1-prime, Tc ^r	27
pIJ1089	pLAFR1-prime with <i>nod</i> genes from pRL1JI; Tc ^r	12
Other plasmids		
pGM1160	pACYC184 carrying a Tn7 insertion	23
pRK2013	Helper plasmid for mobilization of pRK290 derivatives; <i>tra</i> (RK2) <i>ori</i> (ColE1); Km ^r	11
Bacteriophage π 1		
		6

^a On *M. sativa* cv. Gemini.^b See Fig. 2 and 4.^c Tn7, Sp^r Sm^r Tp^r.^d Tn5, Nm^r Sm^r.^e See Fig. 2.^f See Fig. 4.

tetracycline per ml for *R. meliloti* and 4 μ g of tetracycline per ml for *A. tumefaciens*. Transfer of pGM142 and pGM171 was selected with 100 μ g of neomycin per ml for *R. meliloti* and 100 μ g of kanamycin per ml for *A. tumefaciens*. Transfer of pRmSL26, pIJ1089, and pGM1149 was achieved by triparental crosses with *E. coli* HB101 carrying the pRK2013 helper plasmid (11).

Plant assays. Seeds of *Medicago sativa* cv. Gemini were obtained from Tourneur Frères (F77120 Coulommiers, France); seeds of *Trifolium repens* cv. Ladino and cv. Louisiana Nolin were a gift from F. B. Dazzo (East Lansing, Mich.). For nodulation tests, seeds were surface sterilized and germinated, and the seedlings were aseptically grown in

test tubes on Jensen nitrogen-free agar slants (42). Twenty plants were used for each treatment.

For microscopy, seeds germinated for 36 h were transferred onto Fahraeus agar plates (14) in square Petri dishes (23 by 23 cm; eight plants per dish). Five days later seedlings were inoculated with a suspension of bacteria (approximately 10⁸/ml) grown for 48 h on TY agar medium. Root hairs were observed by 3, 5, 7, 10, and 14 days after inoculation on seedlings previously transferred between a slide and a cover slip and stained with 0.01% methylene blue in liquid Fahraeus medium as described previously (40). For each of the three parameters studied (the bacterial strain, the host, and the time after inoculation at which root hairs were

observed) the whole root system of a minimum of 12 plants was observed. Light and electron microscopy of root deformations were performed as described previously (39). Anatomical studies of root deformations were also performed on sections (50 μm) of fixed and frozen plant material (Cryomicrotome Frigipel; Decimu, Paris).

General DNA techniques. The in-gel lysis method of Eckhardt (13) as modified by Rosenberg et al (31) was used, and plasmid content was determined by electrophoresis on 0.7 or 0.5% agarose gels. For preparative isolation, plasmid DNA was separated from chromosomal DNA by the cleared lysate technique (8) for pGMI160 and by alkaline denaturation (22) for pGMI42, pGMI71, and pGMI149. Plasmid DNA was purified in a cesium chloride-ethidium bromide density gradient (65,000 rpm in a VT180 Beckman rotor for 4 h).

Total DNA from *R. meliloti* was prepared as follows (T. Hugué, personal communication). Cells from a 10-ml stationary-phase culture in TY medium were incubated for 20 min at 20°C in 3.8 ml of the following mixture: 25 mM Tris hydrochloride (pH 8.0), 10 mM EDTA, 50 mM sucrose, 2 mg of lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml (5). Bacteria were lysed by adding 0.2 ml of 20% (wt/vol) Sarkosyl (CIBA-GEIGY Corp., Summit, N.J.). The lysate was added to 4.32 g of cesium chloride and 0.1 ml of ethidium bromide (15 mg/ml). After centrifugation (65,000 rpm in a VT180 Beckman rotor for 12 h) the DNA band was recovered and extracted with salt-saturated isopropanol before ethanol precipitation. Digestions were performed in UREB restriction buffer (33).

Transfer of DNA to a Biodyne membrane was as recommended by the manufacturer (Pall). DNA was labeled with [α - ^{32}P]dCTP (3,200 Ci/mmol) using a nick-translation kit (New England Nuclear Corp., Boston, Mass.). Hybridizations were carried out at 41°C for 24 h in 2 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% (vol/vol) formamide. Filters were washed three times in 2 \times SSC–0.2% (wt/vol) sodium dodecyl sulfate at 65°C and three times in 0.1 \times SSC at room temperature.

Construction of episomes. The pGMI71 episome was constructed by in vivo cloning of a 70-kb pSym insert into RP4 by the method used previously to construct pGMI42 (23). In spite of its size, this episome was shown to be stable in *E. coli* as well as in *R. meliloti*. pGMI515 is one of the plasmids constructed by subcloning of pGMI42 DNA, after partial *Hind*III digestion, into the *Hind*III site of pGMI500; pGMI500 is a derivative of the self-transmissible RP4 plasmid which no longer carries an *Eco*RI site (3). Physical characterization of pGMI515 was done by comparing the *Hind*III digestion patterns of pGMI515 and pGMI42. The cloned *Hind*III fragments were found to be adjacent on the pSym map (3). pGMI149 was generated by subcloning pGMI71 DNA, after partial *Eco*RI digestion, into the *Eco*RI site of pRK290 (F. Debelle, F. Maillat, J. Dénarié, E. Martinez, C. Rosenberg, J. Vasse, and G. Truchet, manuscript in preparation).

RESULTS

Isolation of Nod⁻ mutants. pGMI42 is an RP4-prime plasmid which contains a 290-kb insert of pSym2011 (23). It carries sequences required for specific nodulation of alfalfa (39). In a previous study we have reported the use of this episome to mutagenize the corresponding pSym2011 region (the 42 region) with transposon Tn7 (23). Bacteriophage Mu was inserted into the RP4 region of pGMI42 to generate a

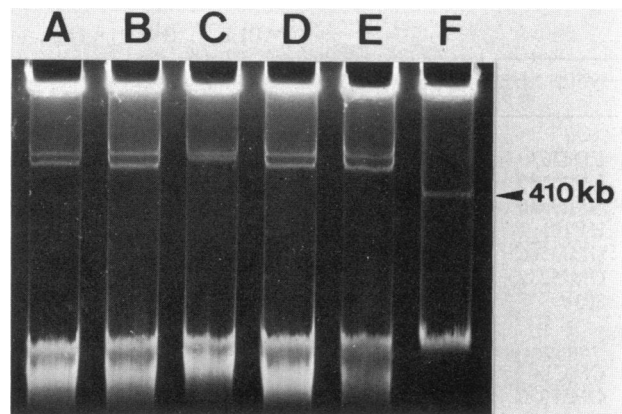


FIG. 1. Electrophoretic characterization of megaplasmids of symbiotically defective *R. meliloti* mutants in 0.5% agarose gels. Lane A, strain GMI956; lane B, GMI255; lane C, the wild-type *R. meliloti* 2011 strain; lane D, GMI963; lane E, GMI766; lane F, the *A. tumefaciens* GMI9050 strain with a plasmid of 410 kb. In lanes A through E, the upper bands correspond to the cryptic megaplasmid (35); the lower bands correspond to the pSym megaplasmid.

suicide vector (23). The resulting pGMI42::Mu hybrid (pGMI104) was mutagenized with Tn7 in *E. coli* by mating strain GMI3506(pGMI104) with strain GMI3232. The resulting population of *E. coli* GMI3232 (pGMI104::Tn7) transconjugants was then mated with *R. meliloti* 2011 (experiment 1). Among 793 Tn7-containing *R. meliloti* 2011 transconjugants 3 Nod⁻ and 10 Fix⁻ mutants were isolated. The *nod* mutations were located in the 42 region of pSym since the introduction of pGMI42 restored the Nod⁺ phenotype.

In a second similar experiment (experiment 2) the proportion of symbiotically defective mutants obtained was much higher: 20 Nod⁻ and 8 Fix⁻ mutants among the 115 Tn7-containing transconjugants tested. It has been observed that in the *E. coli* Rec⁺ donor strains GMI3506 and GMI3232 deletions into the pSym insert of pGMI104 occur frequently (T. Hugué, personal communication). This suggests that the increased proportion of Nod⁻ and Fix⁻ mutants could be due to the cointroduction into pSym2011 of such deletions together with Tn7. Introduction of pGMI42 restored the nodulating ability of all the Nod⁻ mutants tested.

Physical characterization of Nod⁻ mutants. The megaplasmid profile of various Nod⁻ mutants was studied by gel electrophoresis using a low concentration of agarose (0.5%). In the wild-type strain two very close bands of low motility could be seen, thus confirming the presence of a cryptic megaplasmid in addition to pSym (35). In all the Nod⁻ mutants tested (from experiments 1 and 2), the upper band remained at the same position, while the lower band showed an increased migration (Fig. 1). These results indicate that all these Nod⁻ mutants carry a rather large pSym deletion.

Figure 2 shows the physical map (*Hind*III sites) of the 42 region calibrated in kilobases starting from the *nifHDK* promoter region with positive values in the direction of the *nod* genes and negative values toward the *nifA* gene(3). To localize the various deletions in the 42 region, *Hind*III-digested total DNA from the mutants was hybridized with a pGMI42 probe. In addition to the bands which strongly hybridized and corresponded to homologous bands of pSym, some bands hybridized at various degrees, suggesting the presence of repeated sequences in the 42 region (Fig. 3). The precise end of deletions within the *Hind*III border fragments was not determined.

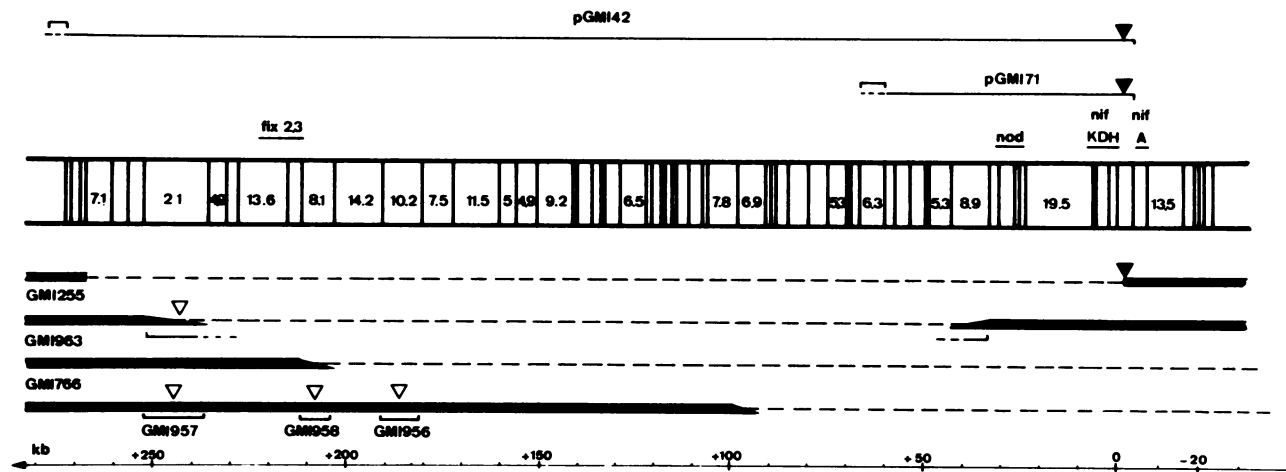


FIG. 2. Map of the 42 region of the pSym megaplasmid and physical characterization of symbiotically defective mutants of *R. meliloti* 2011. In the middle of the figure is shown the *Hind*III physical map of the 42 region of the pSym of approximately 300 kb (3). The *Hind*III fragments are numbered according to their estimated size (in kilobases). Fragments smaller than 0.7 kb were not recorded. The symbiotic regions are located according to the following previous results: *fix*2.3 (3), *nod* (16), *nif*HDK (34), *nif*A (36). Above the map are shown the episomes pGMI42 and pGMI71. Below the map the deletions are represented by interrupted lines. The slanting borders of a deletion mean that the deletion ends in the fragments but that the precise end within the fragments has not been studied. Similarly, the insertions of Tn7 represented by a bracket means that the point of insertion within the *Hind*III fragment has not been determined. On the scale the coordinates (in kilobases) are given from the promoter of the *nif*HDK operon as localized by Sundaresan et al. (36). Symbols: ▽, Tn7 insertions; ▼, Tn5 insertions.

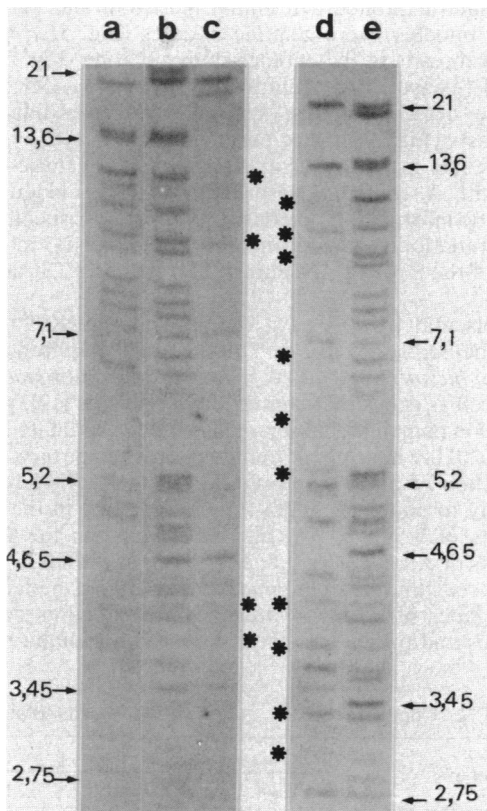


FIG. 3. Mapping of deletions in the 42 region of pSym plasmid. GMI957 (lane a), GMI963 (lane c), GMI766 (lane d), Wild-type control (lanes b and e). A pGMI42 probe was hybridized against *Hind*III-digested total DNA from the various strains. Asterisks indicate weakly hybridizing bands, suggesting that sequences present in the 42 region are repeated in the *R. meliloti* genome. Sizes are given in kb.

Interpretation of these hybridization data is given in Fig. 2, in which a map of the various deletions is proposed. As a control, strain GMI255, generated by a site-directed deletion procedure described elsewhere (M. H. Renalier, B. Terzaghi, A. M. Garnezone, G. Truchet, J. Vasse, J. Dénarié, and P. Boistard, manuscript in preparation), was shown to contain a deletion of approximately 280 kb covering almost the whole 42 region. The deleted pSym plasmid carried by strain GMI255 is much larger than the pAtC58 plasmid of 410 kb (32), confirming that the size of 2011 megaplasmids is much larger than 700 kb.

GMI766, a spontaneous Nod⁻ mutant, carries a deletion larger than that of GMI255 (which is more than 280 kb), according to the results of gel electrophoresis; this deletion, which has its left end at approximately 210 kb, thus was suspected to extend more than 70 kb to the right of *nif*HDK. This was confirmed by hybridization of GMI766 *Hind*III-digested total DNA with a radioactive probe of pGMI53, an RP4-prime plasmid carrying a pSym insert of about 70 kb from a region located on the right of *nif*HDK (Renalier et al., in preparation). No hybridizing bands could be detected. In all the Tn7 Nod⁻ mutants isolated from experiments 1 and 2, for example strains GMI956, GMI957, GMI958, and GMI932, the deletions had the same left end (approximately +100 kb) and extended further than the right extremity of the 64 region (approximately -70 kb).

The Tn7 insertions were mapped by hybridization of *Hind*III-digested total DNA with a radioactive pGMI160 used as a Tn7 probe. In mutants GMI956 and GMI957 (experiment 1) the Tn7 insertion was located in *Hind*III fragments of 10.2 and 21.0 kb, respectively, whereas in mutants GMI958 (experiment 1) and GMI932 (experiment 2) it was in a fragment of 8.1 kb (Fig. 2). In the Nod⁺ Fix⁻ mutant GMI963 the deletion was shown to extend over about 210 kb in the 42 region (from +45 to +257 kb); the Tn7 insertion was located at the junction of the two borders of the deletion (Fig. 2).

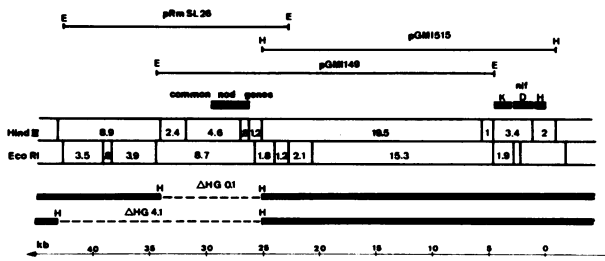


FIG. 4. Physical characterization of the *nod* region of pSym2011. The *Hind*III (H) and *Eco*RI (E) physical maps; the pRmSL26, pGMI149, and pGMI1515 episomes; and the Δ HG0.1 and Δ HG4.1 deletions are shown (see legend to Fig. 2).

Cloning of a pSym2011 region controlling alfalfa nodulation. The set of physically characterized deletions covers the 42 and 64 pSym regions, which is more than 360 kb. None of these *Nod*⁻ deletion mutants could be restored to *Nod*⁺ by the pRmSL26 plasmid containing the common *nod* genes (Fig. 4), showing that other *nod* gene(s) required for alfalfa nodulation are present in the pSym-deleted region. The fact that strain GMI963 was *Nod*⁺ *Fix*⁻ showed that no *nod* genes essential for alfalfa nodulation lies between coordinates +46 and +257 kb. To locate pSym regions required for alfalfa nodulation, complementation studies were performed by introducing into *Nod*⁻ mutants a set of episomes carrying pSym DNA inserts of various sizes, ranging from 20 to 290 kb (Fig. 2 and 4). The RP4-prime pGMI42 restored the *Nod*⁺ phenotype in GMI766, suggesting that no essential *nod* genes are located in the 64 region. pGMI71 complemented the *Nod*⁻ mutants GMI255 and GMI766. It complemented also the Tn7-deleted strains, showing that the *Nod*⁻ phenotype of these mutants is not due to Tn7 insertions in the *Hind*III fragments of 8.1, 10.2, and 21.0 kb but to genetic rearrangements within the 71 region. pGMI71 was thus used as a source of *nod* DNA for subcloning into the broad-host-range vector pRK290 after partial *Eco*RI digestion (Debellé et al., in preparation). The pRK290-prime population was mobilized from *E. coli* into *R. meliloti* GMI255 and GMI766 recipients, and *Nod*⁺ transconjugants were selected on alfalfa seedlings by the procedure described by Long et al. (27). All the pRK290-prime plasmids present in the *R. meliloti* clones isolated from the nodules carried in common, like the plasmid pGMI149 shown in Fig. 4, the *Eco*RI fragments of 8.7, 1.8, 1.2, 2.1, and 15.3 kb. These fragments are adjacent on the pSym map (3, 27).

Introduction of pGMI149 into GMI766 resulted in a *Nod*⁺ *Fix*⁻ phenotype (Table 2). That the root deformations were genuine nodules was shown by cytological studies which revealed the presence of peripheral vascular bundles and typical nodule zonation of the central tissue (Fig. 5A), the presence of infection threads (Fig. 5B), and the release of bacteria into the host cells (Fig. 5C and D). However, abnormalities in the infection process at the root hair level and in the differentiation into bacteroids were observed. The frequency of infection threads within root hairs was estimated to be lowered by at least an order of magnitude. A degeneration of released bacteria could be observed in the submeristematic zone of infection (ZII) associated with an increase of lysosome-like organelles (Fig. 5H). In the central tissue (ZIII) the host cells were surcharged with amyloplast (Fig. 5C), and the bacterial population comprised a majority of completely disorganized bacteria in addition to well-

differentiated bacteroids (Fig. 5D). Introduction of pGMI149 in GMI255 resulted also in a *Nod*⁺ *Fix*⁻ phenotype (Table 2).

A pSym region controlling the specificity of nodulation. The *R. meliloti* GMI357 strain carries a site-directed deletion of 9 kb (Δ HGO.1) which removed the 8.7-kb *Eco*RI fragment which has been shown previously (16, 27) to carry common *nod* genes (T. Huguet, M. Ghérardi, and J. Batut, submitted for publication). GMI361 carries a site-directed 17.9-kb deletion (Δ HG4.1) covering approximately the SL26 region (Fig. 4). These two *Nod*⁻ mutants were restored to *Nod*⁺ by introduction of pRmSL26. In contrast, pRmSL26 could not complement all the other *Nod*⁻ deletion mutants (GMI255, GMI766, GMI956, GMI980, etc.) which could be restored to *Nod*⁺ with pGMI149 (Table 2). Thus, in addition to the common *nod* genes already described in strain 2011 (27), sequences absolutely required for alfalfa nodulation are located in the *Eco*RI fragments of 2.1 kb, 15.3 kb, or both (Fig. 4).

When pRmSL26 was introduced into *A. tumefaciens* GMI9050, a C58 derivative cured of its Ti plasmid, no nodules could be observed on alfalfa seedlings; however, nodule-like deformations could be seen on a white clover root system (Fig. 6A and B). Cytological studies revealed that these deformations were swollen secondary roots and not nodules; their origin was not cortical and they contained central vascular bundles (Fig. 6C). In contrast, GMI9050 (pGMI149) induced root deformation on alfalfa (28 plants among the 100 tested) and not on clover. These deformations, which exhibited a terminal meristem and peripheral vascular bundles, were genuine nodules (Fig. 5E). Neither infection threads in the submeristematic zone (Fig. 5F) nor bacterial release in the central host cells (Fig. 5G and H) could be detected. These central host cells filled with amyloplasts (Fig. 5G) were enriched with dictyosomes and lysosome-like organelles (Fig. 5H). Results of these experiments with *A. tumefaciens* provide positive evidence that the genetic information responsible for the host specificity of nodule initiation is located in pGMI149, that is, in one or several of the *Eco*RI fragments of 8.7, 1.8, 1.2, 2.1, and 15.3 kb.

The plasmid pIJ1089 is a derivative of pRK290 which carries the *nod* genes of *R. leguminosarum* required for pea and *Vicia hirsuta* nodulation. It contains common *nod* genes (32) as well as genes that control the host range (12). pIJ1089 was used in complementation studies to discriminate regions of pSym2011 which carry common or specific *nod* genes. Introduction of pIJ1089 into GMI357 and GMI361 restored the ability to nodulate alfalfa (Table 2). Thus, in the *Eco*RI fragments of 3.5, 0.8, 3.9, and 8.7 kb and in the *Hind*III fragments of 8.9, 2.4, 4.6, 0.8, and 1.2 kb of the SL26 region, it can be seen that (i) all the *nod* genes can be complemented by *nod* genes of *R. leguminosarum* and are thus common *nod* genes and (ii) no sequences required for alfalfa-specific

TABLE 2. Complementation studies of *Nod*⁻-deleted strains^a

Bacterial strains	Episomes ^b		
	pRmSL26	pIJ1089	pGMI149
GMI357	+	+	+
GMI361	+	+	+
GMI766	-	-	+
GMI255	-	-	+
GMI956	-	-	+

^a Nodulation tests were performed on *M. sativa* cv. Gemini.

^b -, No nodule observed on the roots of 20 plants; +, abundant nodulation.

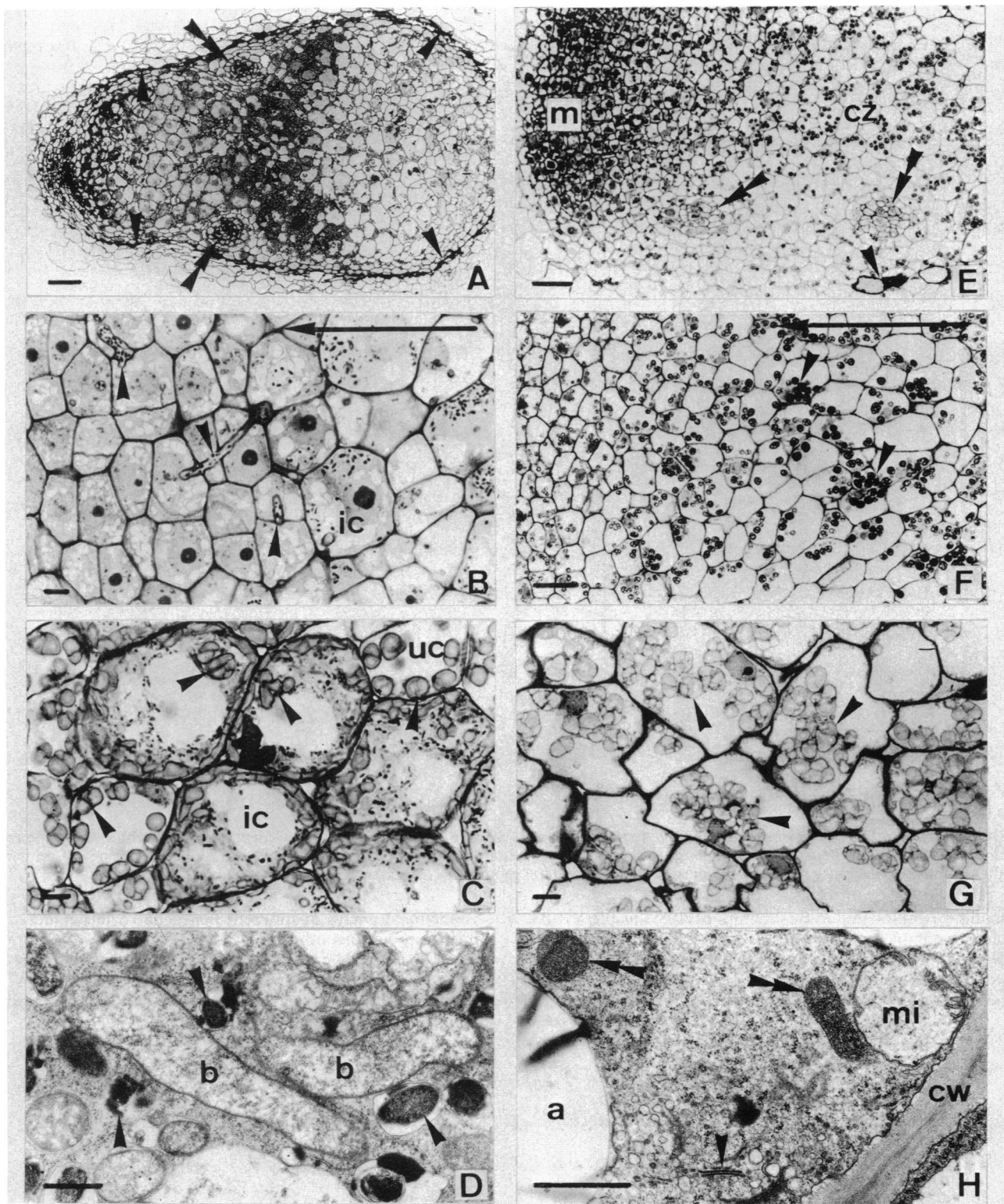


FIG. 5. Light and electron microscopy of nodules induced on *M. sativa* by *R. meliloti* GMI766(pGMI149) (A, B, C, and D) and *A. tumefaciens* GMI9050(pGMI149) (E, F, G, and H). (A) Semithin section of a nodule showing typical zonation. Endodermis (arrows) and nodular vascular bundles (double arrows) are seen at the periphery of the nodule. Bar, 100 μm. (B) Semithin section of the submeristematic zone. Infection threads (arrows) and infected cells (ic) are visible. The large arrow (upper right) is pointed to the apical meristem. Bar, 100 μm. (C) Semithin section of the bacteroid zone. Infected cells (ic) as well as uninfected cells (uc) are densely packed with amyloplasts (arrows). Bar, 10 μm. (D) Electron micrograph of an infected cell of the bacteroid zone. Some bacteria differentiated into bacteroids (b) or they prematurely senesced (arrows). Bar, 1 μm. (E) Semithin section of a nodule showing the apical meristem (m) and the unique central zone (cz). Endodermis (arrow) and nodular vascular bundles (double arrows) are peripheral. Bar, 100 μm. (F) Semithin section of the submeristematic zone. No infection threads are visible. The large arrow (upper right) is pointed to the apical meristem. Arrows, amyloplasts; bar, 100 μm. (G) Semithin section of the central zone. Plant cells, devoid of bacteria, are filled with large amyloplasts (arrows). Bar, 10 μm. (H) Electron micrograph of a plant cell of the central zone. Dictyosomes (arrows) and lysosome-like organelles (double arrows) lie in the plant cell cytoplasm. a, amyloplast; m, mitochondria; cw, plant cell wall; bar, 1 μm.

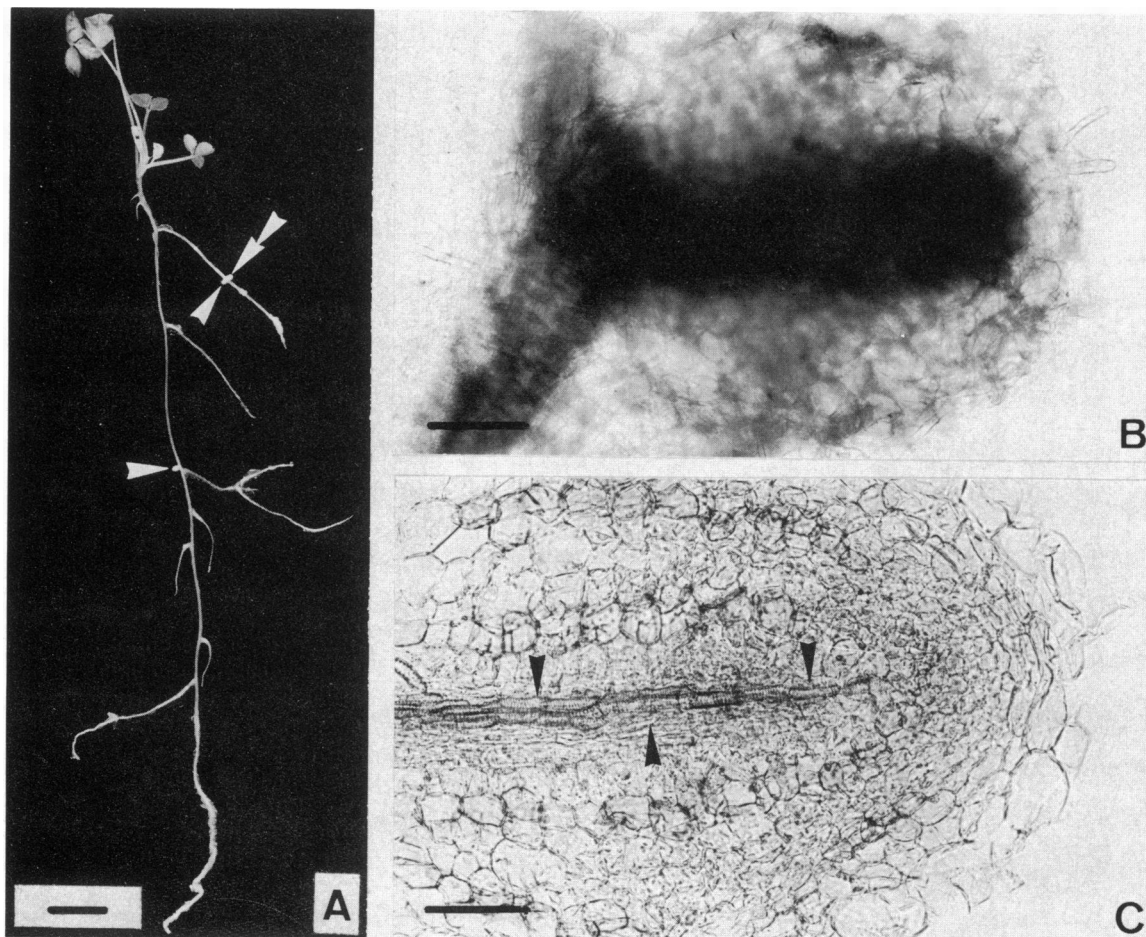


FIG. 6. *T. repens* cv. L. Nolin inoculated by *A. tumefaciens* GMI9050(pRmSL26). (A) General view of a plant with nodule-like root deformations (arrows). The double arrow points to the root deformation further shown after fixation (B) and freeze sectioning (C). In panel C, the central vascular trace (arrows) is typical of a swollen lateral root. Bars: 1 cm (A); 200 μ m (B); 100 μ m (C).

recognition are located in this region. pIJ1089, as pRmSL26, could not complement all of the other *Nod*⁻ deletions which could be complemented by pGMI149 (Table 2). Thus, in the *Hind*III fragments of 19.5 kb, or 1 kb, or both are located noncommon (specific) *nod* sequences.

Functional assays of different *nod* gene region clones. As opposed to various other root hair deformations, the formation of shepherd's crooks (marked curling) on root hairs is considered to be a very specific host reaction to infection by the compatible bacteria (45). In this study, shepherd's

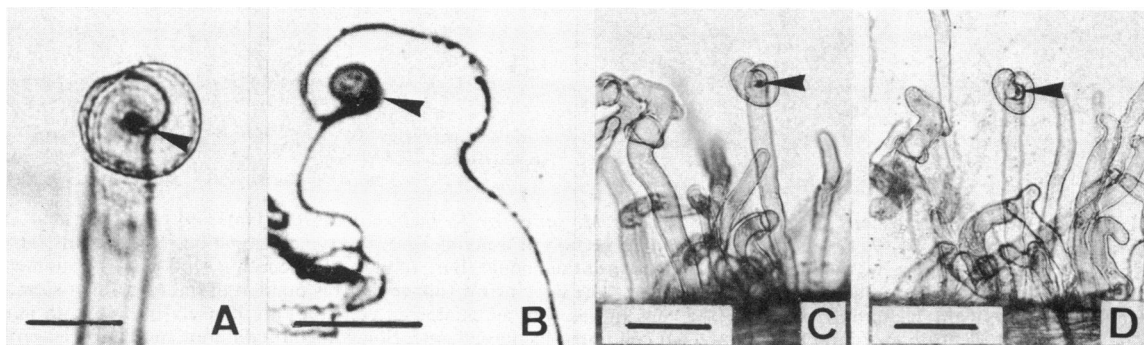


FIG. 7. Micrographs illustrating the criteria used in this study to define a shepherd's crook. (A) *M. sativa*-*A. tumefaciens* GMI9050(pGMI149). Typical shepherd's crook showing the 360° curvature of the root hair tip and the hyaline spot (arrow) in the middle of the curvature. Bar, 10 μ m. (B) Semithin section of a curled clover root hair induced by *A. tumefaciens* GMI9050(pRmSL26). Arrow, hyaline spot; bar, 10 μ m. (C and D) *M. sativa*-*R. meliloti* 2011. Shepherd's crook deformation of an individual root hair before (C) and after (D) pressure on the cover slip. Bars, 25 μ m.

crooks were distinguished from other types of root hair deformations by the following criteria: (i) a curvature of the tip of the root hair of at least 360° (Fig. 7A, C, and D); (ii) in the middle of the curvature, a spot intensively stained by methylene blue, corresponding to the hyaline spot described previously (9, 14) (Fig. 7, arrows in panels A, B, C, and D); (iii) a shepherd's crook deformation which withstands a firm pressure exerted on the cover slip (Fig. 7, compare panels C and D). In addition, to prevent confusion with root hair tip deformations due to a contact with adjacent root hairs, shepherd's crooks were always searched for on isolated root hairs.

To initiate a study of the respective roles of the left (the 8.7-kb *EcoRI* fragment) and right (the four *EcoRI* right fragments) *nod* regions of pSym2011 which control the specificity of marked root hair curling, *R. meliloti* GMI766 and *A. tumefaciens* GMI9050, carrying pRmSL26, pGMI515, or pGMI149 (Fig. 4), were inoculated on the *R. meliloti* homologous host (*M. sativa*) or on an heterologous host (two cultivars of *T. repens*).

With the two control strains *A. tumefaciens* GMI9050 and *R. meliloti* GMI766 root hairs remained straight on all the host plants for up to three weeks. Similarly, the inoculation of strains GMI9050 and GMI766 carrying the pGMI515 plasmid to alfalfa or clover plants did not produce any root hair deformation (Fig. 8A). Inoculation of white clover varieties with GMI766(pRmSL26) or GMI9050(pRmSL26) resulted in the formation of numerous shepherd's crooks (*Hac*⁺ phenotype) (Fig. 8C and D). The proportion of clover root hairs exhibiting shepherd's crooks was of the same order of magnitude as that on the seedlings inoculated by the *R. trifolii* 0403 control strain. The fact that an *A. tumefaciens* strain carrying pRmSL26 was *Hac*⁺ suggests strongly that the gene(s) encoding for the product(s) required for marked root hair curling is located on this episome. In contrast, GMI766(pGMI149) and GMI9050(pGMI149) did not induce shepherd's crook formation on the two clover varieties (Fig. 8G and H).

Opposite results were obtained by inoculation of the homologous host. Strains carrying pRmSL26 were *Hac*⁻ (Fig. 8B), whereas those carrying pGMI149 were *Hac*⁺ (Fig. 8E and F). These results show that the *EcoRI* fragments of 15.3 kb, 2.1 kb, or both are required for alfalfa root hair curling. In addition, it seems that this region interacts negatively with the SL26 region for hair curling on heterologous hosts. The plant reactions for the marked root hair curling character provoked by pRmSL26 (*Hac*⁻), pGMI515 (*Hac*⁻), and pGMI149 (*Hac*⁺) were similar in the *R. meliloti* or *A. tumefaciens* genetic background (Table 3), suggesting that the DNA sequences required for hair curling and the control of its specificity are cloned in pGMI149.

We propose to call the various root hair deformations *Had* (such as branching, waviness, bulging, swelling, moderate curling) to distinguish them from marked hair curling (*Hac*, as proposed by Vincent [43]). *A. tumefaciens* and *R. meliloti* strains which were *Hac*⁺ were also *Had*⁺. In contrast, *Hac*⁻ strains could be *Had*⁺ or *Had*⁻. Thus, GMI766(pGMI149) and GMI9050(pGMI149) were *Had*⁺ *Hac*⁺ on alfalfa and *Had*⁺ *Hac*⁻ on clover (Table 3).

In general, for a given episome the proportion of deformed root hairs (*Had* and *Hac*) was higher in *A. tumefaciens* GMI9050 than in *R. meliloti* GMI766. This was particularly the case for GMI9050(pRmSL26) and GMI766(pRmSL26) on clover.

No infection thread could be detected on clover root system whatever the *R. meliloti* or *A. tumefaciens* strain

inoculated, or on both plants when inoculated with an *A. tumefaciens* strain. Thus *A. tumefaciens* GMI9050 (pGMI149) was found to be *Inf*⁻ *Nod*⁺ on alfalfa. No infection threads could be detected in the root hairs, even when they were markedly curled; and no threads could be detected in the submeristematic zone of the nodules. Infection threads in root hairs could be seen only in the association alfalfa *R. meliloti* GMI766(pGMI149).

DISCUSSION

pGMI42, an RP4-prime plasmid carrying a 290-kb pSym insert, contains the host-specific nodulation genes of *R. meliloti* 2011 (39). In the course of Tn7 mutagenesis of the corresponding 42 region of pSym, several *Nod*⁻ mutants were isolated which were shown to carry large deletions of pSym. Electrophoresis of lysates of deleted strains in soft agarose gels revealed the presence of a second band of low motility, confirming the presence of a cryptic megaplasmid, in strain *R. meliloti* 2011 (35). A cryptic megaplasmid, in addition to pSym, has also been reported in *R. meliloti* 41 (1, 2) and in *R. meliloti* L5-30 (C. Rosenberg, unpublished data). Thus, the presence of two megaplasmids of about 1,500 kb each (7) seems to be the rule in this bacterial species.

In some deleted strains, for instance GMI963, it was shown that the Tn7 transposon was located between the two borders of the deletion, suggesting that the transposon could be involved in the generation of the deletion. Since during the Tn7 mutagenesis of pGMI42::Mu in *E. coli* spontaneous deletions occurred frequently in the pSym insert, deletions could also be introduced in the *R. meliloti* plasmid by cotransfer with Tn7. The mechanism by which the deletions extending outside the 42 region were generated on the right is not clear. The left end of many deletions, for instance those of strains GMI956, GMI957, and GMI958, is located in the *HindIII* fragment of 6.9 kb which contains a repeated sequence (A. M. Garnerone, unpublished data).

All of these *Nod*⁻ deletions could be restored by pGMI42 but not by pRmSL26 which carries the common *nod* genes (16, 21, 27), showing that some gene(s) essential for alfalfa nodulation is located in the 42 region outside the fragment cloned in pRmSL26. One such deleted strain was used to allow the selection on alfalfa seedlings, by the procedure of Long et al. (27), of subclones which contain sequences required to nodulate alfalfa. The isolated clones contained a piece of 29.1 kb in common: the *EcoRI* fragments of 8.7, 1.8, 1.2, 2.1, and 15.3 kb. Due to the fact that the two extreme bands were large, 8.7 and 15.3 kb, the minimal size of DNA required for alfalfa nodulation could be much smaller.

When introduced into *A. tumefaciens* GMI9050, a strain cured of its Ti plasmid, the clone pGMI149 carrying the 29.1-kb insert conferred to the transconjugants the ability to induce marked root hair curling and genuine nodule formation on the homologous host alfalfa and not on clover. These experiments have shown that this clone carries the genetic information required for host-specific root hair curling and nodule initiation on alfalfa.

An *R. meliloti* strain carrying a site-directed deletion removing the 8.7-kb *EcoRI* fragment and containing pJ1089 which carries common and host range nodulation genes of *R. leguminosarum* (12, 32) was able to nodulate alfalfa. This result shows that no gene essential for alfalfa recognition is present in the 8.7-kb fragment and indicates that such genes are likely to be located in the right part of pGMI149 that is in one or several of the *EcoRI* fragments of 1.8, 1.2, 2.1, or 15.3 kb. This is in agreement with the finding of Kondorosi et al. (25) that in *R. meliloti* 41, a strain of a different geographical

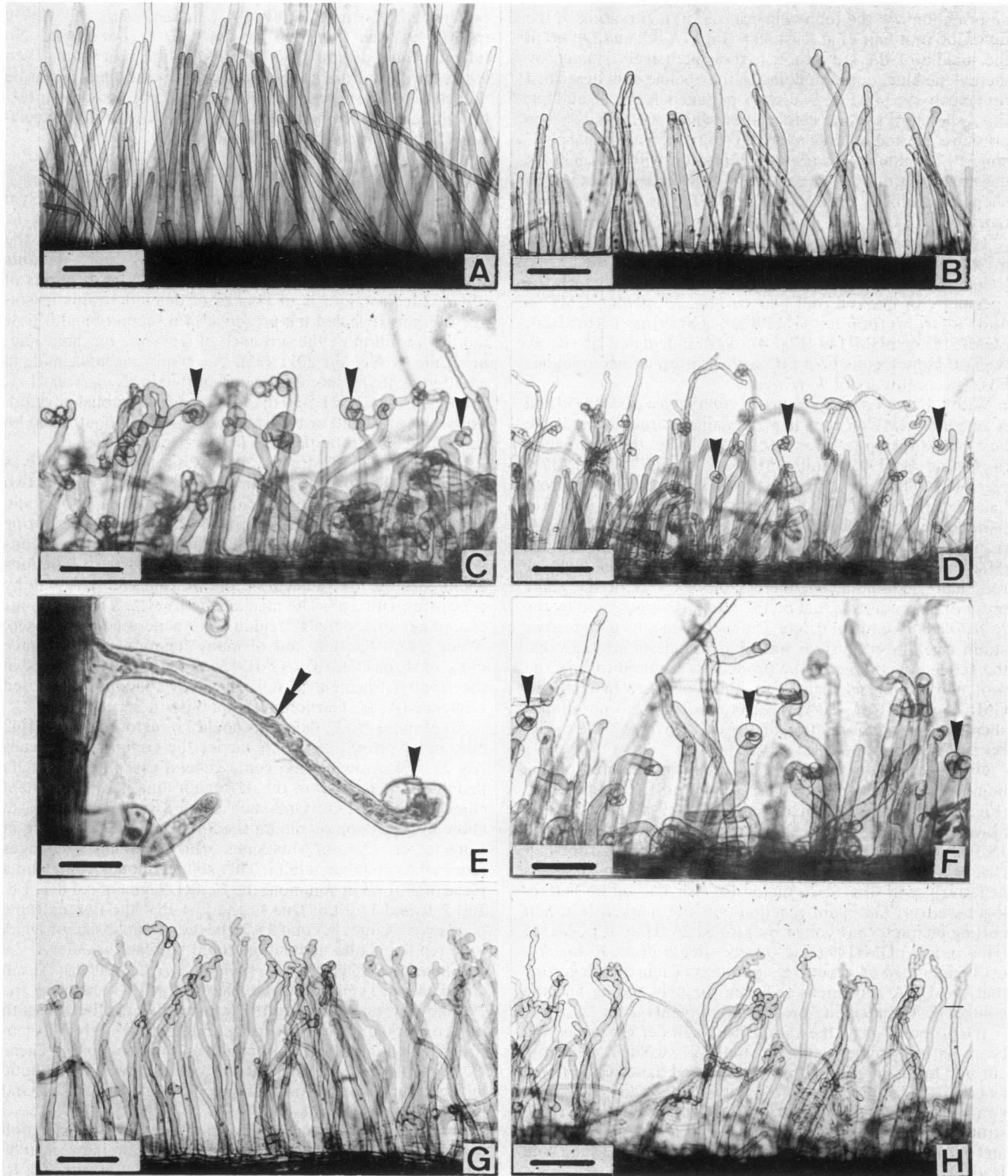


FIG. 8. Root hair curling. (A and B) *M. sativa* inoculated by *R. meliloti* GMI766(pGMI515) (A) or *R. meliloti* GMI766(pRmSL26) (B). Straight root hairs are shown (Had⁻ phenotype). Bar, 30 μ m. (C and D) *T. repens* inoculated with *A. tumefaciens* GMI9050(pRmSL26) (C) or *R. meliloti* GMI766(pSL26) (D). Arrows point to shepherd's crooks (Hac⁺ phenotype). Bars: 20 μ m (C); 25 μ m (D). (E and F) *M. sativa* with *R. meliloti* GMI766(pGMI149) (E) or *A. tumefaciens* GMI9050(pGMI149) (F). Both strains induced shepherd's crook deformations (arrows), but infection threads (double arrows) were initiated only by *R. meliloti* GMI766(pGMI149) (E). Bars: 10 μ m (E); 15 μ m (F). (G and H) *T. repens* inoculated with *R. meliloti* GMI766(pGMI149) (G) and *A. tumefaciens* GMI9050(pGMI149) (H). Deformations other than shepherd's crook are shown (Had⁺ Hac⁻ phenotype). Bars, 25 μ m.

TABLE 3. Symbiotic phenotypes of GMI766 and GMI9050 containing episomes with various fragments of the *nod* region^a

Strains and plasmids	<i>M. sativa</i>				<i>T. repens</i> ^b			
	Had	Hac	Inf	Nod	Had	Hac	Inf	Nod
<i>R. meliloti</i> 2011	+	+	+	+	±	-	-	-
<i>R. trifolii</i> 0403	-	-	-	-	+	+	+	+
<i>R. meliloti</i> GMI766	-	-	-	-	-	-	-	-
pRmSL26	-	-	-	-	+	+	-	- ^c
pGMI515	-	-	-	-	-	-	-	-
pGMI149	+	+	+	+	+	-	-	-
<i>A. tumefaciens</i> GMI9050	-	-	-	-	-	-	-	-
pRmSL26	-	-	-	-	+	+	-	-
pGMI515	-	-	-	-	-	-	-	-
pGMI149	+	+	-	+	+	-	-	-

^a Had⁺, root hair deformations (branching, waviness, swelling, etc.); Hac⁺, shepherd's crooks; Inf⁺, infection threads in root hairs; Nod⁺, nodule formation (43).

^b The same phenotypes were observed for the two white clover cultivars tested.

^c Root deformations (Fig. 6).

origin, a cluster of nodulation genes which cannot be complemented by *R. leguminosarum* nodulation genes lies between the common *nod* genes and the *nifHDK* genes.

By insertional mutagenesis, Long and co-workers have shown that genes located in the 8.7-kb fragment control alfalfa root hair curling (19, 21, 27). The fact that an *A. tumefaciens* strain containing pRmSL26 elicits very effectively the formation of shepherd's crooks on clover roots provides positive evidence that the left *nod* region contains all the genetic information to induce this plant reaction. Surprisingly, the introduction of neither the 8.7-kb fragment nor pRmSL26 into *A. tumefaciens* (18) or into *R. meliloti* GMI766, a strain that carries a pSym deletion which removes the whole *nod* region, results in an Hac⁺ phenotype on alfalfa. This indicates that another gene(s) is required for shepherd's crook formation on the homologous host.

Plasmid pGMI149, containing the whole *nod* region, elicited the opposite plant reactions: shepherd's crook formation on the homologous host and not on the heterologous plant. Strains carrying the pGMI515 episome (containing the right part of the *nod* region) did not induce any visible plant reaction. These results suggest that in the right portion of pGMI149 (*Eco*RI fragments of 2.1 and 15.3 kb) is located a gene(s) which is absolutely required, in addition to genes in the common *nod* region, for root hair curling of the homologous host. On the other hand the right *nod* region interacts negatively with the left region to suppress the shepherd's crook formation on heterologous hosts. Tn5 mutagenesis of the right *nod* region is in progress to locate the DNA sequences which interact with the common *nod* genes to control positively and negatively the host specificity of root hair curling. This mutagenesis is a prerequisite to investigate whether these controls of host specificity are mediated at the gene expression level or at the gene product level.

Typical hyaline spots could be observed in the middle of shepherd's crooks of white clover inoculated with *R. meliloti* GMI766(pRmSL26) and *A. tumefaciens* GMI9050 (pRmSL26) and of alfalfa inoculated with *A. tumefaciens* GMI9050(pGMI149), in spite of the fact that in these plant-bacteria combinations no infection threads could be detected in the root hairs. In previous studies it has been suggested that these bright refractile spots are the point at which rhizobia enter the root and initiate infection thread formation (9, 43). Our results indicate that the gene(s) required for spot

formation is located in pRmSL26 and that it is not sufficient for infection thread formation.

pGMI149 provoked alfalfa root hair curling and nodulation when introduced into *R. meliloti* GMI766 and *A. tumefaciens* GMI9050, but in the latter case no infection threads could be detected either in the root hairs or in the nodules. This confirms the results of previous studies in which it has been reported that alfalfa nodule initiation does not require the formation of infection threads (15, 18, 20, 39) and can be triggered at a distance by rhizobial cells. The lack of thread formation in *A. tumefaciens*, even when the whole pSym megaplasmid is introduced (39), could mean that *R. meliloti* *inf* genes either (i) are located on the chromosome or on the second megaplasmid or (ii) cannot be expressed in *A. tumefaciens*. In contrast to *R. meliloti* GMI766(pRmSL26), *R. meliloti* GMI766(pGMI149) is Inf⁺ on alfalfa. This could be interpreted by two hypotheses concerning the *inf* genes: (i) they are located in pGMI149 or (ii) they are located elsewhere in the *R. meliloti* GMI766 genome but pGMI149 makes the infection thread formation possible by allowing the previous steps of the infection process, root hair curling and hyaline spot formation, to occur.

R. meliloti GMI766(pGMI149) induced a much reduced proportion of shepherd's crook and infection thread formation on alfalfa than did the wild-type strain; this suggests that ancillary nodulation genes involved in the efficiency of the infection process are located on the pSym plasmid in the deleted area (from about 200 kb to more than -60 kb on the pSym map). This is in agreement with the results of a previous report (39) in which it has been shown that ancillary nodulation genes are located on the pSym2011 fragment outside the 42 region.

The pSym2011 fragment which contains genes coding for host-specific root hair curling and nodulation has been cloned. This was a prerequisite to study the molecular genetics of host range specificity in *R. meliloti* 2011.

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