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Rhizobium meliloti Nodulation Genes Allow *Agrobacterium tumefaciens* and *Escherichia coli* to Form Pseudonodules on Alfalfa

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Regions of the *Rhizobium meliloti* symbiotic plasmid (20 to 40 kilobase pairs long) containing nodulation (*nod*) genes were transferred to *Agrobacterium tumefaciens* or *Escherichia coli* by conjugation. The *A. tumefaciens* and *E. coli* transconjugants elicited root hair curling and the formation of ineffective pseudonodules on inoculated alfalfa plants. *A. tumefaciens* elicited pseudonodules formed at a variable frequency, ranging from 15 to 45%, irrespective of the presence of the Ti plasmid. These pseudonodules developed characteristic nodule meristems, and in some nodules, infection threads were found within the interior of nodules. Infrequently, infection threads penetrated deformed root hairs, but these threads were found only in a minority of nodules. There was no evidence of bacterial release from the infection threads. In addition to being found within threads, agrobacteria were also found in intercellular spaces and within nodule cells that had senesced. In the latter case, the bacteria appeared to invade the nodule cells independently of infection threads and degenerated at the same time as the senescing host cells. No peribacteroid membranes enclosed any agrobacteria, and no bacteroid differentiation was observed. In contrast to the *A. tumefaciens*-induced pseudonodules, the *E. coli*-induced pseudonodules were completely devoid of bacteria; infection threads were not found to penetrate root hairs or within nodules. Our results suggest that relatively few *Rhizobium* genes are involved in the earliest stages of nodulation, and that curling of root hairs and penetration of bacteria via root hair infection threads are not prerequisites for nodule meristem formation in alfalfa.

Bacteria in the genus *Rhizobium* establish nitrogen-fixing symbioses with host plants from the legume family. In the symbiosis, nitrogen fixation occurs in nodules, highly differentiated organs that usually develop on the roots. The nodulation response is a multistep process, as evidenced by the many different stages at which it may arrest (35, 47). Such arrest is generally observed when plants are inoculated with mutant bacteria (47), although there are also recessive plant mutations that prohibit effective nodule formation (2, 6, 46).

During the past several years, a major approach to the study of the symbiosis has involved the combined use of transposon mutagenesis and recombinant DNA techniques to identify and isolate *Rhizobium* symbiotic (*sym*) genes (for examples, see references 4, 8, 12, 31, 36, 39, 40; M. Hahn and H. Hennecke, personal communication). This approach seeks to identify *sym* genes by the specific loss of symbiotic function. A complementary approach is to look for the acquisition of symbiotic functions by bacterial species which previously lacked them. For example, in several cases in which *Rhizobium* plasmids carrying symbiotic genes (Sym plasmids) were transferred to *Agrobacterium tumefaciens* recipients, the recipients gained the capacity to induce small, white, non-nitrogen-fixing (ineffective) nodules on an appropriate legume host (19, 20, 23, 44, 50).

One advantage to the strategy of identifying *sym* genes by transfer to foreign hosts is that it simplifies the task of distinguishing between the role of bacterial and plant genes at various stages of the differentiation process; that is, by transferring *Rhizobium* genes into a species that does not exhibit symbiotic interactions (for example, *Escherichia coli*), any symbiosis-like host response obtained must be due either to the transferred *Rhizobium* genes or to plant host genes.

The work reported here is based on the prior construction of a recombinant plasmid, pRmSL26, carrying *Rhizobium meliloti* nodulation (*nod*) genes (24). In this paper, we report that when pRmSL26 and related plasmids were transferred to *A. tumefaciens* and *E. coli*, the transconjugants elicited the formation of ineffective pseudonodules on inoculated alfalfa plants. Irrespective of the presence of the Ti plasmid, *A. tumefaciens* (pRmSL26) induced pseudonodules that contained characteristic nodule meristems. Some of these pseudonodules developed infection threads; however, the *A. tumefaciens* cells were not released from the infection threads, and the bacteria found in the nodules outside of infection threads were found in intercellular spaces or in host cells that had senesced. Agrobacteria present in host cells were not individualized by a peribacteroid membrane and were degenerate. *E. coli* carrying *Rhizobium* DNA elicited the formation of pseudonodules as well, although in contrast to the nodules induced by the *A. tumefaciens* strains, the *E. coli*-elicited nodules were completely devoid of bacteria and did not appear to contain infection threads.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* MM294 and MM294(pRK2013) (7, 38), *A. tumefaciens* A348 (*onc*$^+$) (14) and A136 (*onc*$^-$) (49), and *R. meliloti* 1021 (26) have been described previously. Recombinant plasmids, carrying *R. meliloti* DNA fragments, are shown in Fig. 1. Plasmids pRmSL26 (24) and pRmWB564, pRmBE11, and pRmBE2 (3)

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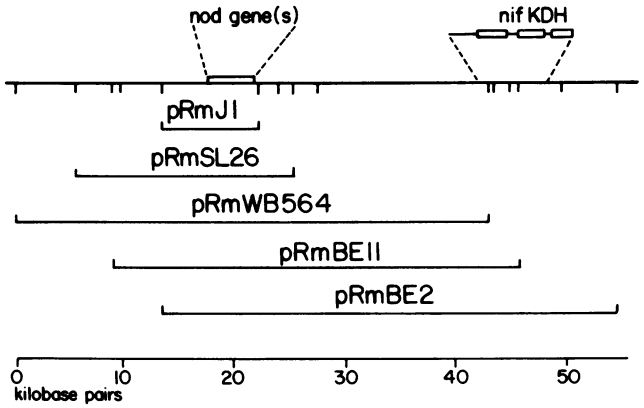

FIG. 1. Map of the recombinant plasmids used in this study. Map of pRmJ1 and *nod* genes is from T. Jacobs and S. Long (Stanford University).

have been described previously. Plasmid pRmJ1 was a gift from T. Jacobs and S. Long (Stanford University, Stanford, Calif.).

Media. LB (26) and M9 (27) media have been described previously.

Strain construction. To introduce *R. meliloti* sequences cloned in pRmWB564 and pRmBE2 (Fig. 1) into *A. tumefaciens*, cointegrates between pRmSL26 and pRmWB564 and between pRmSL26 and pRmBE2 were constructed as follows. First, the two desired plasmids were transformed sequentially into *E. coli* MM294, using the calcium shock method of Mandel and Higa (25). Second, to select for cointegrate formation, the doubly transformed strains were mated with *A. tumefaciens* A348 or A136, in a triparental mating using *E. coli* MM294 containing the mobilizing plasmid pRK2013 as described previously (7, 38). After overnight growth on LB media at 32°C, cells were plated on M9–sucrose medium containing 200 μg of carbenicillin and 10 μg of tetracycline per ml. To verify the presence of cointegrate plasmids in selected *A. tumefaciens* transconjugants, individual clones were examined by the plasmid detection method of Eckhardt (9). A large plasmid of approximately 50 kilobase pairs (kb) was observed in the strains, indicating that cointegrate formation had occurred (data not shown). Strains containing the presumptive cointegrates were maintained on selective media containing both carbenicillin and tetracycline. When these strains were not continually subjected to carbenicillin selection, carbenicillin-sensitive derivatives appeared at a high frequency, indicating that the cointegrate plasmids had undergone intramolecular recombination, generating the two parental plasmids. Because pRmWB564 and pRmBE2 cannot replicate in *A. tumefaciens*, these plasmids presumably were lost. Plasmid pRmSL26 was conjugated into *A. tumefaciens* A348 and A136, using the triparental mating technique as described above.

DNA biochemistry. Extraction and purification of DNA (26), agarose gel electrophoresis of DNA fragments (32), transfer of gel-fractionated DNA fragments to nitrocellulose sheets (32), labeling of DNA fragments by nick translation (32), hybridization conditions (32), colony hybridizations (37), and autoradiography of hybridized nitrocellulose sheets (32) followed procedures described previously. DNA fragments used for hybridization probes were extracted from agarose gels as described previously (48). Hybridization probes were derived from pRmSL26, pRmBE11, and from pRmJ1 (S. Long and T. Jacobs, personal communication); the latter contained an 8.7-kb *Eco*RI fragment from pRmSL26 cloned into pBR322. Restriction endonucleases were purchased from Bethesda Research Laboratories, Rockville, Md., and used according to the manufacturer's instructions.

Nodule formation. Seedlings of alfalfa (*Medicago sativa* cv. Iroquois) were germinated as described previously (26). Root hair curling was examined by using Fahraeus slide assemblies containing liquid medium (10, 17).

Extraction of bacteria from nodules. Approximately five nodules from each *Agrobacterium-Rhizobium* or *Escherichia-Rhizobium* hybrid were squashed on LB agar after surface sterilization for 15 s in 95% ethanol and for 2 min in 20% sodium hypochlorite (5% wt/vol), followed by three rinses in sterile water. After incubation at 30°C for 1 to 2 days, individual colonies were tested for tetracycline and carbenicillin resistance phenotypes. Selected clones were tested further by extraction of total DNA and restriction endonuclease digestion, by Southern blotting, or by colony hybridization techniques as described above and below.

Light and electron microscopy. For light and electron microscopy, nodules were prepared and examined as described previously (16).

Histochemical determination of polysaccharide material was performed at both the light and electron microscope levels. For light microscope examination, 1.0-μm plastic sections were collected on slides and treated overnight in saturated dimedone (11) to block intrinsic and fixative-derived aldehyde groups. After two rinses in running water to remove the excess dimedone, the sections were treated with freshly prepared 1% periodic acid for 15 min, followed by another rinse in running water and a 30-min treatment in Schiff reagent (11). After three successive rinses (2 min each) in 0.5% sodium metabisulfate, the slides were rinsed again in running water, allowed to dry, and mounted.

For electron microscopic examination, ultrathin sections were collected on gold grids and stained with the Thiery (42) reaction, following a procedure outlined by Roland (34). Sections were treated for 30 min with freshly prepared 1% periodic acid. After rinsing, thiocarbohydrazide staining was performed for 12 h. Controls for the selectivity of the periodate oxidation consisted of substituting 10% H_2O_2 for the 1% periodic acid pretreatment and also of preparing untreated sections with periodic acid before the thiocarbohydrazide staining. All controls were negative for polysaccharides.

RESULTS

Experimental strategy. A cluster of several *nod* genes in *R. meliloti* 1021 is located on a large Sym plasmid (megaplasmid) (1, 12, 38; S. Long, personal communication) approximately 25 kb distal to the nitrogenase genes (*nifHDK* operon) (3, 24; S. Long, personal communication; Fig. 1). Transposon Tn*5* insertional mutations in this *nod* gene region completely block any readily observable interaction between the mutant bacteria and roots, suggesting that the mutated region contains a gene(s) that acts near or at the beginning of the nodulation process (17; T. Jacobs and S. Long, personal communication). In the experiments described here, we conjugated cloned DNA fragments containing this *nod* region into *A. tumefaciens* and *E. coli* strains to determine whether the cloned *R. meliloti nod* genes would confer on the recipients the ability to interact with alfalfa roots.

TABLE 1. Properties of *A. tumefaciens* (A348 and A136) strains carrying *R. meliloti nod* genes

Strain	Property			
	Root hair curling (Hac$^+$)[a]	Infection thread formation (Inf$^+$)[b]	Nodulation of alfalfa seedlings (Nod$^+$)[c]	Nitrogen fixation (Nif$^+$)
R. meliloti 1021	+++	+	++	+
A. tumefaciens A348 or A136(pRmSL26)	+/−	+/−	+	−
A. tumefaciens A348(pRmSL26 + pRmWB564)	+++	+	+	−
A. tumefaciens A348(pRmSL26 + pRmBE2)	+++	+	++	−

[a] +++, Extensive root hair curling or shepherd's crooks or both; +/−, hypertrophy; no curling.
[b] +, Infection threads present in root hairs and nodules; +/−, infection threads present in nodules.
[c] ++, Five or more nodules per plant; +, fewer than five nodules per plant.

The cloned DNA fragments used in these experiments were carried on plasmids pRmSL26, pRmWB564, and pRmBE2 (Fig. 1). Plasmid pRmSL26 contains the mobilizable cosmid vector pLAFR1 (13), which can replicate stably in *R. meliloti* and *A. tumefaciens*. In contrast, pRmWB564 and pRmBE2 contain the cosmid vector pHC79 (18), which cannot replicate in *A. tumefaciens*. To introduce *R. meliloti* DNA sequences on pRmWB564 and pRmBE2 into *A. tumefaciens*, we constructed recombinational cointegrates in vivo between pRmSL26 and pRmWB564 (pRmSL26 + pRmWB564) and between pRmSL26 and pRmBE2 (pRmSL26 + pRmBE2), which we conjugated into *A. tumefaciens*.

***Agrobacterium-Rhizobium* hybrids form pseudonodules on alfalfa.** *A. tumefaciens* strains A348 (Ti$^+$ Onc$^+$) containing pRmSL26, (pRmSL26 + pRmWB564), or (pRmSL26 + pRmBE2); and A136 (Ti$^-$ Onc$^-$) carrying pRmSL26 were tested for the ability to induce root hair curling (Hac phenotype) and nodule development (Nod phenotype) on alfalfa seedlings (see above); these responses were compared with those elicited by wild-type *R. meliloti* 1021. The results showed that all of the *A. tumefaciens* strains containing *R. meliloti nod* genes which we tested elicited the formation of pseudonodules on alfalfa (Table 1). Moreover, bacteria recovered from excised, surface-sterilized pseudonodules (see above) elicited the formation of nodules at a slightly lower frequency than found originally, except for *A. tumefaciens* (pRmSL26 + pRmBE2), in which there was a significant decrease in the frequency of nodule development upon reinoculation (Table 2). On approximately 100 alfalfa plants, the control inoculations of *A. tumefaciens* A136 and A348 without the *R. meliloti nod* genes did not form pseudonodules.

To verify that the pseudonodules were elicited by the inoculating *A. tumefaciens-R. meliloti* hybrids, and not, for example, by contaminating *R. meliloti* strains, bacteria recovered from nodules were tested for their antibiotic resistance phenotype and for the presence of *R. meliloti nod* gene sequences. Of 600 bacterial clones isolated from pseudonodules, all exhibited the expected antibiotic-resistant phenotypes: tetracycline resistance for pRmSL26 and carbenicillin resistance for pRmWB564 and pRmBE2. Selected clones were tested for the presence of *R. meliloti* DNA sequences carried in pRmSL26, pRmWB564, and pRmBE2, using the Grunstein-Hogness colony hybridization procedure (15). Gel-purified, ^{32}P-labeled *Eco*RI fragments of pRmSL26, pRmBE11, or pRmJ1 were used as hybridization probes. A total of 150 clones were tested by this procedure, and among batches of 25 clones isolated from a particular nodule, 15 to 60% contained the expected hybridizing sequences. This latter result was somewhat surprising since all clones tested carried the drug resistance markers of the starting plasmid(s). Ten clones were examined further by isolating total DNA, digesting with *Eco*RI, and examining the digests for the presence of pRmSL26 by using the Southern gel transfer and hybridization procedure (41). Only one digest contained the complete complement of pRmSL26 *Eco*RI fragments, verifying the colony hybridization results which indicated that the *R. meliloti* DNA inserts in pRmSL26 and in the cointegrate plasmids were unstable during passage through the plant (data not shown). Although the plasmids carrying the *R. meliloti nod* genes appeared to be unstable in *A. tumefaciens*, the overall pattern of *Eco*RI fragments observed for clones isolated from pseudonodules was indistinguishable from the pattern observed for the *A. tumefaciens-R. meliloti* hybrid used to inoculate the plant and was clearly different from the pattern observed for *R. meliloti* 1021.

In the experiments described above, and in those that will be described below, identical results were obtained with *A. tumefaciens* strains A348(Ti$^+$)(pRmSL26) and A136(Ti$^-$)(pRmSL26), indicating that the Ti plasmid did not carry genes required for the formation of pseudonodules. We have not yet tested whether the Ti plasmid plays a role in the pseudonodule phenotypes associated with the cointegrate plasmids (pRmSL26 + pRmBE2) and (pRmSL26 + pRmWB564). In the sections that follow, all experiments were carried out with A348(pRmSL26), A136(pRmSL26), A348(pRmSL26 + pRmBE2), and A348(pRmSL26 + pRmWB564). However, for the sake of simplicity, in some cases we will refer to these strains collectively as *A. tumefaciens-R. meliloti* hybrids.

Root hair curling elicited by *Agrobacterium*. Root hair deformation was evident 24 h after inoculation with *R. meliloti* 1021 (wild type) and with all *A. tumefaciens-R. meliloti* hybrids. No root hair deformation was observed with the parental *A. tumefaciens* strains. Root hairs inoculated with A348(pRmSL26) and A136(pRmSL26) generally exhibited hypertrophy and branching (Fig. 2A), and those inoculated with A348(pRmSL26 + pRmWB564) often developed tightly coiled corkscrews or loosely curled shepherd's crooks (Fig. 2B). The shepherd's crook morphology that typifies a wild-type *R. meliloti* interaction was observed only

TABLE 2. Frequency of nodule development on alfalfa roots induced by *A. tumefaciens-R. meliloti* hybrids

Time of testing	Inoculation frequency (%)[a]			
	A348-(pRmSL26)	A136-(pRmSL26)	A348-(pRmSL26 + pRmWB564)	A348-(pRmSL26 + pRmBE2)
Initial inoculation (>100 tubes)	15.4	12.1	16.1	45.8
Reinoculation after isolation from nodules (50 tubes)	10.0	5.0	NT[b]	7.1

[a] Calculated by the formula [(number of tubes with nodules)/(total number of tubes inoculated)] × 100.
[b] NT, Not tested.

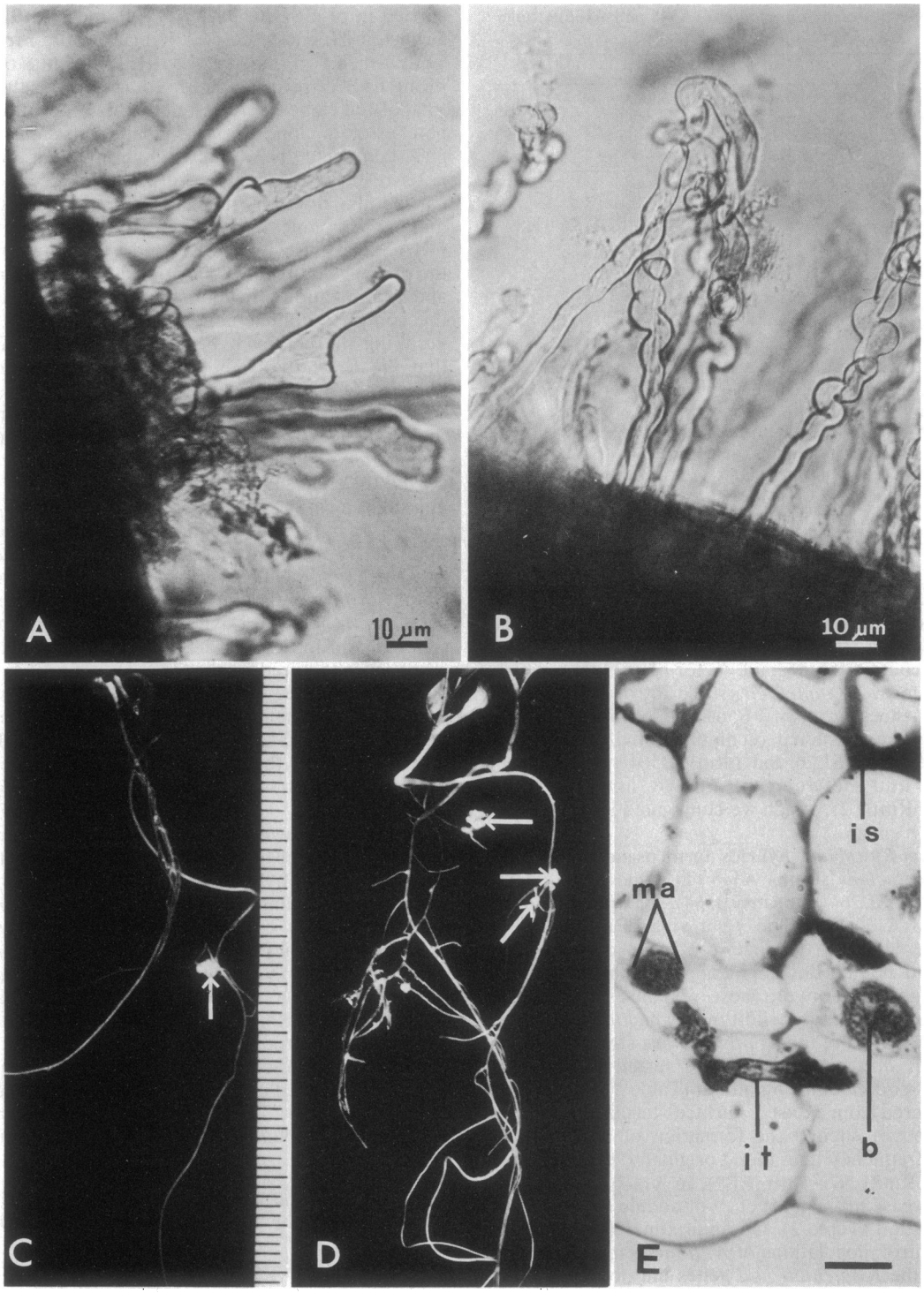

FIG. 2. (A) Hypertrophy and branching of root hairs inoculated with *A. tumefaciens*(pRmSL26). ×600. Bar, 10 μm. (B) Loosely curled "shepherd's crook" and corkscrew-curled root hairs inoculated with *A. tumefaciens*(pRmSL26 + pRmWB564). ×600. Bar, 10 μm. (C) Tumorous-appearing pseudonodule on alfalfa root (arrow). Inoculation was with *A. tumefaciens*(pRmSL26). (D) Pseudonodules on alfalfa roots. Inoculation was with *A. tumefaciens*(pRmSL26 + pRmBE2). (E) Light micrograph of a section through a pseudonodule induced by *A. tumefaciens*(pRmSL26 + pRmWB564). Bacteria (b) are found within infection threads (it), intercellular spaces (is), and within a matrix (ma). ×90.

when roots were infected by A348(pRmSL26 + pRmWB564) or A348(pRmSL26 + pRmBE2) (data not shown).

In all *A. tumefaciens-R. meliloti* hybrids, when root hairs were examined after 14 days of growth in Fahraeus slide assemblies, no infection threads were found in root hair cells. However, after 21 days, infection threads were found in root hairs inoculated with A348(pRmSL26 + pRmWB564) and A348(pRmSL26 + pRmBE2) (data not shown). We did not observe threads in hairs infected by *A. tumefaciens*(pRmSL26); however, a significant root hair curling

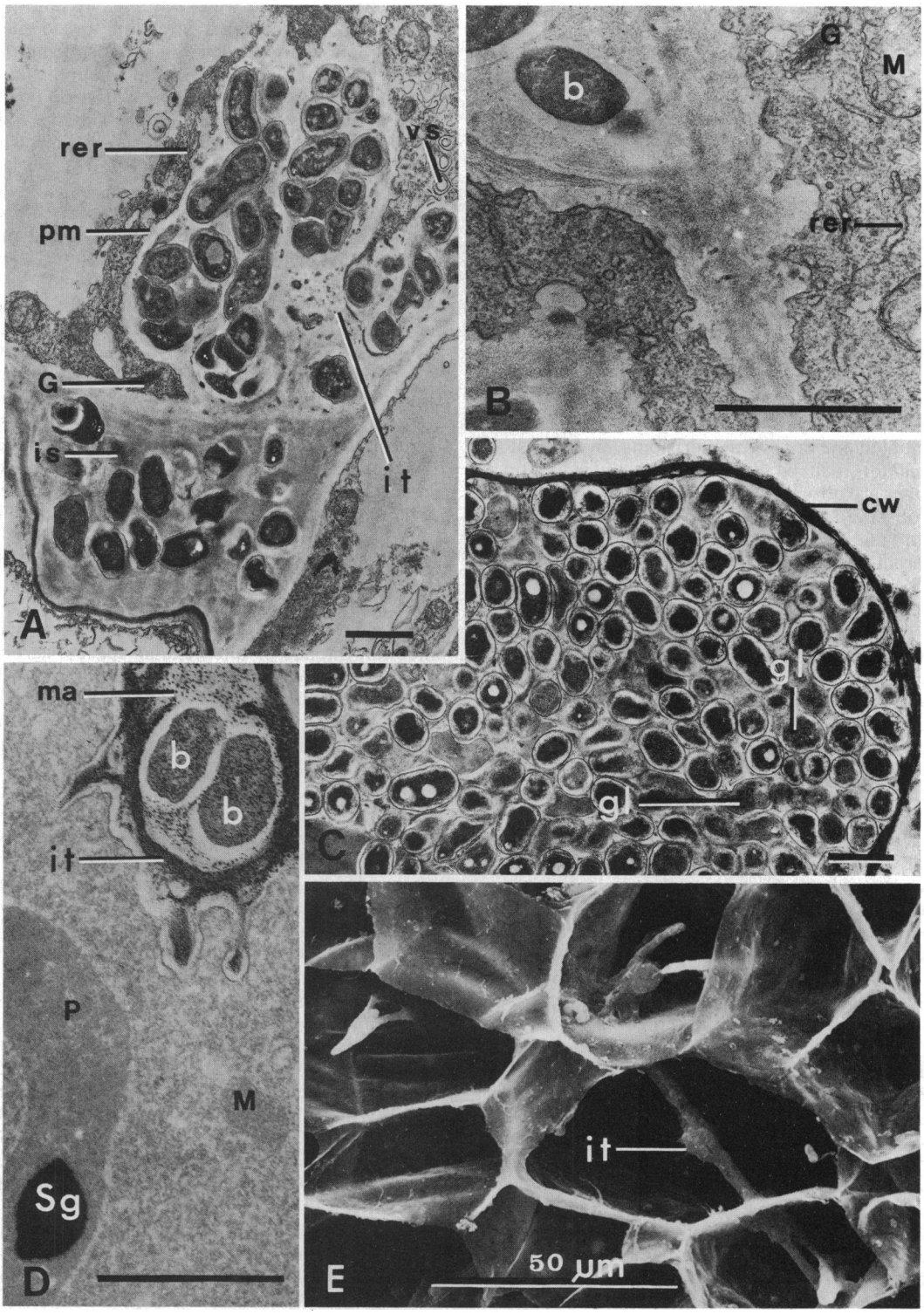

FIG. 3. (A) Transmission electron micrograph (TEM) through an infected cell of a pseudonodule induced by *A. tumefaciens*(pRmSL26). Numerous vesicles (vs), rough endoplasmic reticulum (rer), and Golgi bodies (G) are adjacent to the plasma membrane (pm) surrounding the infection thread (it). Bacteria (b) are also present within intercellular spaces (is). ×9,800. Bar, 1 μm. (B) TEM showing numerous organelle profiles around infection threads. The pseudonodule was induced by *A. tumefaciens*(pRmSL26 + pRmWB564). M, Mitochondrion. ×19,000. Bar, 1 μm. (C) TEM section stained with Thiery reagent. The cell wall (cw) surrounding the intercellular spaces stains positively; the bacteria contain glycogen (gl) granules. The pseudonodule was induced by *A. tumefaciens*(pRmSL26 + pRmWB564). ×9,800. Bar, 1 μm. (D) TEM section stained with Thiery reagent. Plastids (P), mitochondria (M), and other cellular components do not stain, whereas starch grains (Sg), the infection thread (it), and the matrix (ma) surrounding the bacteria (b) stain positively for polysaccharides. ×19,000. Bar, 1 μm. (E) Scanning electron micrograph of infection threads (it) in pseudonodules induced by *A. tumefaciens*(pRmSL26 + pRmBE2). Threads are distorted and attenuated. ×760. Bar, 50 μm.

response did not result after infection with this strain (Fig. 2A; Table 1).

Pseudonodule formation elicited by *Agrobacterium*. All of the *A. tumefaciens-R. meliloti* hybrids induced white, ineffective nodules on alfalfa roots which generally appeared 3 to 4 weeks after inoculation. In contrast, nodules elicited by *R. meliloti* 1021 were relatively large, pink, and effective, and appeared within 7 to 14 days. Alfalfa seedings inoculated with A348(pRmSL26), A136(pRmSL26), or A348(pRmSL26 + pRmWB564) developed one to two nodules per root system per plant (Fig. 2C). The tumorous appearance of these nodules resulted from the initiation of several discrete meristems. Some of these nodules were very large (2 to 3 mm diameter). In contrast, A348(pRmSL26 + pRmBE2) induced the development of numerous (5 to 10) small, ineffective nodules (Fig. 2D). These were usually less than 1 mm long, but at times large nodules were found also (Fig. 2D).

All nodules induced by the *A. tumefaciens-R. meliloti* hybrids had initiated one or several meristems. In contrast to wild-type *R. meliloti*-induced nodules, in which four distinct histological zones are observed (17, 21, 22, 29, 30, 45, 46), no zonation was observed other than the region comprising the meristem. Thus, there was no evidence for the early symbiotic zone characteristic of wild-type-induced nodules, in which bacteria are released into the host cell cytoplasm and are surrounded by peribacteroid membranes. Likewise, there was no development of a late symbiotic or elongate bacteroid-containing zone. The majority of nodules induced by *A. tumefaciens-R. meliloti* hybrids consisted of host cells empty of bacteria but packed with starch grains. Some nodules did contain bacteria, but these were restricted to infection threads, intercellular spaces, or senesced cells.

Nodules induced by A348(pRmSL26), A136(pRmSL26), and A348(pRmSL26 + pRmWB564) developed infection threads (of normal or larger than normal diameter) that surrounded bacteria (Fig. 3A, B, and D). In contrast, nodules induced by A348(pRmSL26 + pRmBE2) frequently appeared to contain aborted infection threads which were very attenuated, seemed to end blindly, and were empty (Fig. 3E). In those nodules in which we found infection threads, considerable vesiculation was associated with the membrane delimiting the infection thread from the host cell cytoplasm (Fig. 3A and B). In addition to single membrane bound vesicles, rough endoplasmic reticulum and Golgi profiles were found. The presence of these organelles suggests continued membrane synthesis which is most likely related to the progressive invasion of the host cell by the *Agrobacterium* cells. This type of increase in vesiculation has been reported for normal infection thread development (33). Some infection threads observed resembled those found in *Rhizobium*-induced nodules, whereas others were distorted grossly and may represent different stages of the invasion process (see below).

In some nodules induced by the agrobacterial strains, we observed bacterial cells enclosed within intercellular spaces and embedded in an amorphous matrix (Fig. 2E and 3C). Often the spaces were expanded greatly in size, which may have resulted from the accumulation of bacteria and surrounding matrix. It was difficult to determine whether the origin of the surrounding matrix was bacterial or plant derived; spaces lacking bacteria were devoid of the matrix. The bacteria may have secreted the polysaccharides as they entered the intercellular spaces, or the plant may have produced the matrix as a wound response.

The matrix frequently contained fibrillar material similar in density to the cell wall as well as an amorphous component; thus, it resembled infection thread material. Light microscopic histochemistry of sections from the same nodule (Fig. 2E) indicated that the material surrounding the agrobacteria was periodic acid-Schiff stain positive and thus consisted of polysaccharide (data not shown). Similar results were obtained by using the Thiery reaction, which localizes polysaccharides at the electron microscope level (Fig. 3C and D). Starch grains and host cell walls exhibited a positive staining reaction (Fig. 3D). Other cellular components, such as mitochondria, plastids, and rough endoplasmic reticulum, did not stain with silver proteinate after 1% periodic acid pretreatment. The infection thread itself, as well as the matrix surrounding the bacteria, generally stained positively for polysaccharides (Fig. 3D). Although Newcomb and McIntyre (28) found that the thread matrix was not stained by the Thiery reaction in their study of root nodules of mung bean, we found that the intensity of stain reaction, although variable, was positive. In addition, deposits within the bacteria themselves stained with the Thiery reaction. In Fig. 3D, this material is diffuse and evenly scattered throughout the bacterial cytoplasm. In other sections, bacteria exhibited several small, positively staining granules (Fig. 3C), and some exhibited one large, polarly located body. Because of their staining properties and appearance, which are similar to those previously reported for such granules, these bodies most likely consist of glycogen (5).

In addition to those bacteria enclosed within defined infection threads or intercellular spaces, agrobacteria were present within certain nodule cells, in which bacteria surrounded by matrix and membrane appeared to be within the host cell cytoplasm. This observation held true only for nodules induced by *A. tumefaciens* carrying pRmSL26 or A348(pRmSL26 + pRmWB564) (Fig. 2E). Most likely, this appearance was due to sections having been obliquely cut through irregularly shaped infection threads. For example, the bulge to the side of the infection thread shown in Fig. 3E, if sectioned obliquely, would yield the view of bacteria surrounded by matrix and membrane seen in Fig. 2E and 3A.

Certain nodule cells appeared to lack the densely packed matrix surrounding the agrobacteria. In these, the bacteria appeared to be suspended within a vacuole (Fig. 4A) or within the cell itself (Fig. 4B). In Fig. 4A, the bacteria and remnants of the fibrillar matrix were enclosed by such a membrane.

We did not observe the endocytosis of bacteria from the confines of this membrane; thus, they were not individualized by peribacteroid membranes. When bacteria were contained within the nodule cell cytoplasm (Fig. 4B), both the bacterial and host cells appeared degenerated. Similar observations have been reported by S. Lancell and J. Torrey for pre-nodule of *Parasponia rigida* (personal communication). It is difficult to determine from electron micrographs whether host cell senescence preceded or followed the breakdown of the matrix surrounding the agrobacterial cells. Some host cell degeneration is evident in Fig. 4A. An increase in electron density—which is diagnostic of host cell senescence—and considerable vesiculation of the cytoplasm have occurred.

Nodules are induced by *R. meliloti* sequences in *E. coli*. To determine the contribution of the *Agrobacterium* genetic background to nodule induction, plants were inoculated with *E. coli* transformants containing pRmSL26, (pRmSL26 + pRmWB564), and (pRmSL26 + pRmBE2). After several weeks, a small percentage of plants (10 of 75 tubes) developed nodules in response to infection with these bacteria

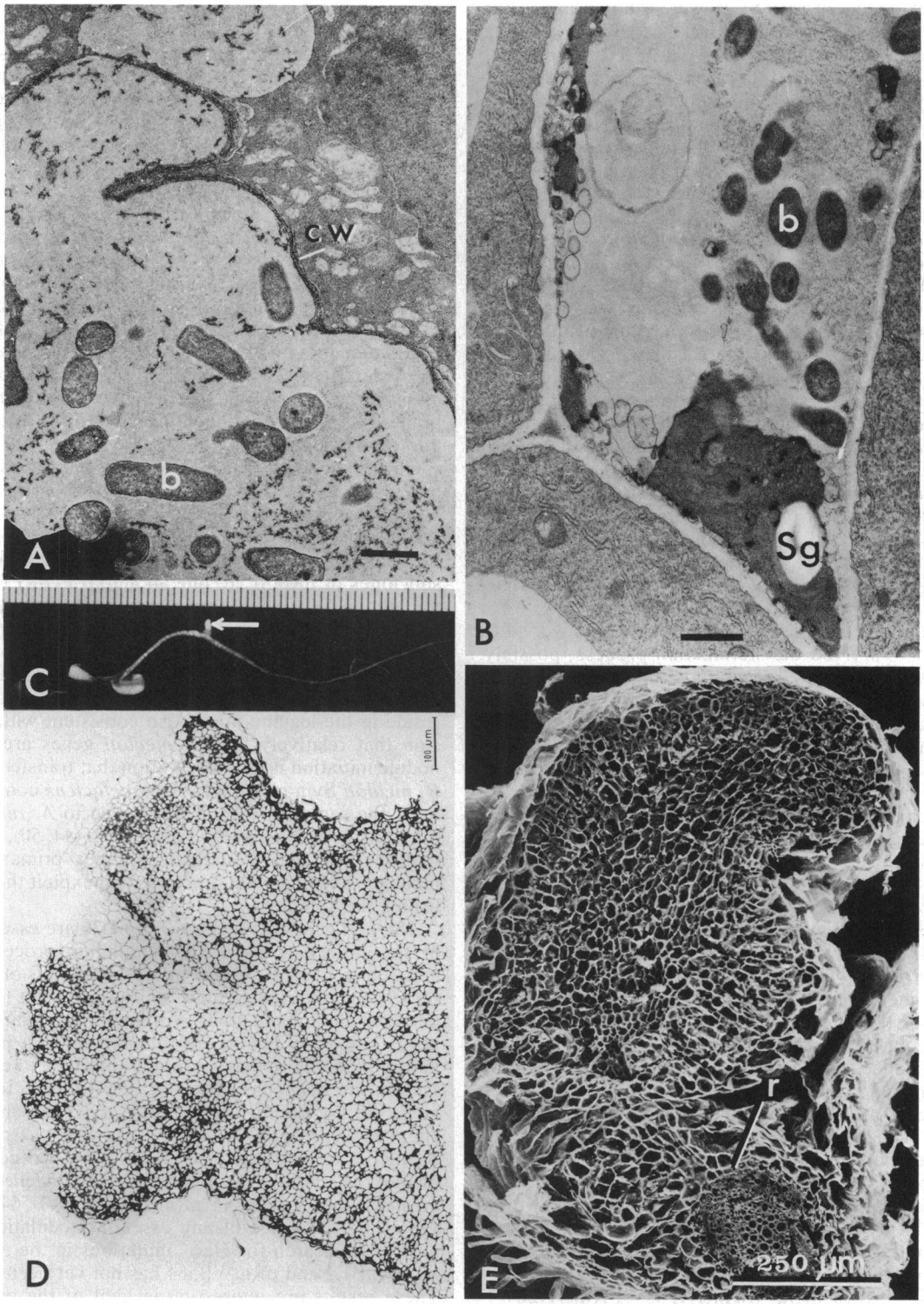

FIG. 4. (A) TEM section stained with Thiery reagent. Shown is an infected cell of a pseudonodule induced by *A. tumefaciens*(pRmSL26 + pRmWB564). Bacteria (b) appear suspended in a vacuole formed by the dissolution of matrix. Vacuole is surrounded by cell wall (cw), which reacts positively with the Thiery reagent. ×9,800. Bar, 1 µm. (B) TEM of host and bacteria (b) undergoing senescence. The adjacent, uninfected cells are intact. Sg, Starch grains. ×9,800. Bar, 1 µm. (C) Alfalfa plant with nodule induced by *E. coli* MM294(pRmSL26). (D) Light micrograph of one-half of a pseudonodule induced by MM294(pRmSL26 + pRmBE2). Neither bacteria nor infection threads are apparent. ×80. (E) Scanning electron micrograph of a longitudinal cut through a pseudonodule induced by MM294(pRmSL26 + pRmBE2). The point of attachment to the root is designated by r. ×90. Bar, 250 µm.

(Fig. 4C). The effects of pRmSL26 were studied in greater detail, although all three plasmids were capable of eliciting this response. In general, the results paralleled those found with *Agrobacterium*-induced nodules. *E. coli*(pRmSL26)-induced nodules were few in number per root system, whereas several nodules per plant were induced by (pRmSL26 + pRmBE2). Like the *A. tumefaciens*-induced nodules, the nodules that developed in response to *E. coli* inoculation initiated several discrete meristems (Fig. 4D and E). Unlike the *A. tumefaciens*-induced nodules, however, those induced by *E. coli* carrying *R. meliloti* DNA were consistently devoid of bacteria. No infection threads were observed within root hairs inoculated with the *E. coli* strains. The hair curling response for all three clones was comparable to that induced by *A. tumefaciens* carrying pRmSL26.

A small number of colonies were isolated from several squashed nodules induced by pRmSL26. These colonies exhibited the expected antibiotic-resistant phenotype, and DNA isolated from one such colony showed a restriction pattern after *Eco*RI digestion that was identical to that of the inoculating *E. coli* strain (data not shown). Inoculation of alfalfa seedlings with bacteria isolated from these nodules resulted in the induction of new pseudonodules that were identical in structure to those originally induced. We also have been able to isolate *E. coli* carrying clone (pRmSL26 + pRmBE2) from nodules. These colonies were carbenicillin resistant.

It is likely that the occasional colonies recovered were due to bacteria that survived the sterilization process, possibly because of their deep-seated location within an intercellular space. *E. coli*-induced pseudonodules were obtained in several independent experiments carried out at Harvard, at Wellesley, and at Stanford in S. Long's laboratory. The replication of the experimental results in three different laboratories, and the failure to recover *Rhizobium* or *Agrobacterium* from these pseudonodules, indicate that the pseudonodules were not caused by *Rhizobium* or *Agrobacterium* contaminants.

DISCUSSION

Our experiments showed that nodulation genes from *R. meliloti* could be expressed in *A. tumefaciens* and *E. coli*, conferring on *A. tumefaciens* and *E. coli* the ability to form small, white, ineffective nodules on alfalfa. In addition, our results indicate that, at most, 20 kb of the *R. meliloti* Sym plasmid is required for nodule induction. Most of the *A. tumefaciens*-induced nodules and all of the *E. coli*-induced nodules examined appeared to be completely devoid of infection threads and bacteria. Some nodules elicited by *A. tumefaciens*, which we have described in detail above, contained infection threads either containing or devoid of bacteria, or bacteria located in intercellular spaces, or both. Very infrequently, we found bacterial cells suspended within a membrane in dying host cells or found dead bacteria in completely senescent nodule cells. Infection threads were found in the internal regions of nodules induced by *Agrobacterium* cells containing pRmSL26, (pRmSL26 + pRmWB564), and (pRmSL26 + pRmBE2). However, we found direct evidence for infection thread penetration through root hairs only for roots inoculated with *A. tumefaciens* A348(pRmSL26 + pRmWB564) and A348(pRmSL26 + pRmBE2). As mentioned above, the majority of nodules examined were devoid of bacteria and infection threads. These results suggest that the initiation of nodule development was independent of infection thread formation.

In those nodules containing infection threads, we never observed normal release of *A. tumefaciens* from infection threads into the host cell cytoplasm and the enveloping of these bacteria within peribacteroid membranes. However, atypical distortions of infection threads did occur, and these bulged into the host cell. We propose that bacteria which appeared to be intracellular and were surrounded by a polysaccharide matrix resulted from these protrusions into the nodule cell.

The infrequent appearance of agrobacteria within senescent nodule cells either within or without a surrounding membrane most likely represents further protrusion and subsequent dissolution of the infection thread. From this appearance, we infer that the plant is responding to an ineffective (or pathological) infection and those cells invaded by infection threads undergo necrosis. Although we did not observe endocytosis from the infection thread of individual *Agrobacterium* cells, we did observe extensive association of single membrane-bound organelles with the thread membrane, suggesting continued invasion of the infection thread into the nodule.

One of the most interesting aspects of the work reported here is that a relatively small region of the *R. meliloti* megaplasmid appears to be sufficient to elicit a significant nodulation response. Moreover, A. Hirsch, T. Jacobs, and S. Long (unpublished data) have shown recently that two subclones of pRmSL26, one an 8.7-kb *Eco*RI fragment and the other a 5.5-kb *Pst*I fragment, elicited the same response in *A. tumefaciens* on roots of alfalfa as did pRmSL26. This result argues strongly that a limited number of *R. meliloti* genes are involved in the initial stages of nodule formation and that most of the genes required for nodule formation reside in the legume host. Also consistent with the conclusion that relatively few *R. meliloti* genes are involved in nodule initiation is the observation that transfer of the entire *R. meliloti* Sym plasmid to *A. tumefaciens* conferred essentially the same nodulation phenotype to *A. tumefaciens* as did transfer of (pRmSL26 + pRmBE2) (44, 50). It is possible that the *Rhizobium* nodulation genes primarily serve to influence existing plant functions and exploit them for establishing the symbiosis.

The *nod* genes carried on pRmSL26 are essential for one of the earliest steps in the nodulation process, since no apparent root hair or nodulation response is obtained when these genes are inactivated by Tn*5* insertion (17; S. Long, personal communication). Although inactivation of these genes results in a Hac^- (root hair curling) phenotype, it appears that these genes are not sufficient to elicit the root hair curling response. Transfer of pRmSL26 to *A. tumefaciens* did not confer on *A. tumefaciens* the ability to form shepherd's crooks, whereas transfer of (pRmSL26 + pRmBE2) or (pRmSL26 + pRmWB564) did confer a Hac^+ phenotype. On the other hand, Tn*5* mutagenesis of the *R. meliloti* DNA cloned in pRmBE2 or pWB564 has not revealed the presence of any essential nodulation genes. (A systematic search for Hac^- mutations in the region cloned on pRmBE2 and pRmWB564 has not yet been carried out.) These results are interesting in light of the generally held view that root hair curling, and especially shepherd's crook formation, only occurs in compatible *Rhizobium*-legume interactions and is directly on the pathway to infection thread formation and nodule initiation. Our results, however, are consistent with the view that the *R. meliloti* genes required for shepherd's crook formation may not be essential nodulation genes and that the nodulation genes carried on pRmSL26 act in the nodulation pathway before the formation of shepherd's crooks.

It could be argued that *A. tumefaciens*, as a plant pathogen with a broad host range, already possesses genes that complement the action of the nodulation genes carried on pRmSL26. If *A. tumefaciens* does contain "plant-interaction" genes that help it initiate nodule formation, then these interaction genes must be located on the *A. tumefaciens* chromosome (or large cryptic plasmid) because the presence or absence of the Ti plasmid appears to have no effect on the nodulation phenotype. The likelihood that *A. tumefaciens* contains genes that complement the transferred *R. meliloti* nodulation genes appears lower in light of the fact that *E. coli*(pRmSL26) also elicited nodule initiation on alfalfa. Although the frequency of nodule development was low (13% of the plants inoculated), the nodules formed exhibited a consistent phenotype. They were large, tumorous, and bacteria-free. We have not determined with complete certainty whether or not infection threads were present within these nodules or within root hairs. More studies are in progress.

A result somewhat different from ours was obtained by Hooykaas et al. (20) and F. Dazzo (personal communication) in a study of clover roots infected with *A. tumefaciens* carrying the entire pSym of *Rhizobium trifolii*. Hooykaas et al. found that within pseudonodules formed on clover, *Agrobacterium* cells are released from infection threads into the host cell cytoplasm and become surrounded by peribacteroid membranes. Dazzo found moderate curling, shepherd's crook deformations, and infection threads in clover root hairs infected with *A. tumefaciens*(pSym) after 14 days of incubation. In contrast, longer time periods (greater than 21 days) are necessary for infection thread formation in alfalfa root hairs.

According to the nomenclature introduced by Vincent (47), the phenotype of the pseudonodules examined in this paper is Bar$^-$ (arrest before release from infection threads). *R. meliloti* mutants with a Bar$^-$ phenotype have also been reported by others. Leu$^-$ auxotrophs of *R. meliloti* induced alfalfa root nodules in which the host cells were devoid of bacteria (43). Infection threads were observed, but there was no release of bacteria unless leucine was added to the medium. Bar$^-$ *R. meliloti* cell surface mutants have been isolated by E. Johansen and E. Signer (personal communication). These mutants also induced bacteria-free nodules (S. Deegan and A. Hirsch, unpublished data).

Mutant plant lines, when infected with wild-type *Rhizobium*, can display a phenotype similar to that induced by Bar$^-$ bacteria. For example, Vance et al. (46) found that the ineffective nodules formed on particular alfalfa mutants were bacteria-free; these nodules appear to be similar to the ones described in this paper. Infection threads were formed early in development in these nodules, and some bacteria were released into host cells. They failed to develop into bacteroids, rapidly senesced, and then the host cells degenerated. In a second example, ineffective nodules that formed on the roots of red clover *ie/ie* homozygotes exhibited abnormal tumorization adjacent to regions of infection (2). Infection threads also formed in these nodules, and bacteria were released into the nodule cells, but once again the bacteroids degenerated. Finally, a non-nodulating cultivar of *Pisum sativum* cv. Afghanistan developed infection threads within root hairs, but nodules were not formed (6). The infection process aborted, and root swellings developed at the points where nodules would have occurred.

In previous studies, a common feature of nodules formed by Bar$^-$ bacterial mutants and of nodules formed on plant nodulation mutants was the presence of infection threads. These observations are consistent with the generally held view that there is a causal relationship between the formation of infection threads and the induction of mitoses in root cortical cells. In contrast, we find and report in this paper that the majority of nodules induced by *A. tumefaciens-R. meliloti* and *A. tumefaciens-E. coli* hybrids are devoid not only of bacteria but of infection threads as well. This suggests that penetration of bacteria via root hair infection threads may not be a prerequisite for nodule meristem formation in alfalfa. Recently, H. Calvert and W. Bauer (personal communication) came to a similar conclusion for soybeans by analyzing root cortical cell division patterns in inoculated roots. They found that many cell division centers in the root did not have curled hairs or hairs with infection threads associated with them.

In summary, our results strongly suggest that a limited region of the *R. meliloti* Sym plasmid is capable of initiating a cascade of developmental events in the plant and may serve as some sort of biochemical cue or trigger leading to nodule formation. Numerous questions now come to mind with regard to the exact biochemical nature of this signal, how it is regulated by the bacterium and the plant to establish a symbiosis, and what is the mechanism whereby the plant responds to this cue. The results presented here show that our overall strategy to dissect the nodulation process by transfer of symbiotic genes into foreign bacterial hosts has been successful and indicates that shepherd's crook formation, possibly infection thread penetration into root hairs, and certainly thread persistence, are not prerequisites for nodule formation. This strategy, therefore, should prove a useful tool in evaluating the contributions of plant and bacterial genes to nodule development.

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