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Nodule Initiation Elicited by Noninfective Mutants of *Rhizobium phaseoli*

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***Rhizobium phaseoli* CE106, CE110, and CE115, originally derived by transposon mutagenesis (Noel et al., J. Bacteriol. 158:149–155, 1984), induced the formation of uninfected root nodule-like swellings on bean (*Phaseolus vulgaris*). Bacteria densely colonized the root surface, and root hair curling and initiation of root cortical-cell divisions occurred normally in mutant-inoculated seedlings, although no infection threads formed. The nodules were ineffective, lacked leghemoglobin, and were anatomically distinct from normal nodules. Ultrastructural specialization for ureide synthesis, characteristic of legumes that form determinate nodules, was absent. Colony morphology of the mutant strains on agar plates was less mucoid than that of the wild type, and under some cultural conditions, the mutants did not react with Cellufluor, a fluorescent stain for β-linked polysaccharide. These observations suggest that the genetic lesions in these mutants may be related to extracellular polysaccharide synthesis.**

The legume root nodule, site of biological nitrogen fixation by the microsymbiont *Rhizobium* spp., is a structure unique to this symbiosis and distinct from other plant organs. Intricate interactions between the symbionts lead to the establishment of an effective nodule (reviewed in references 2, 28, and 42). Before infection via a root hair, rhizobia bind to the host root and elicit root hair curling. The host cell wall is degraded in a pocket formed by the curled root hair, and the bacteria invade the cell through the degraded portion of the wall (9). Plant cell wall material deposited around the bacteria forms the infection thread. Meanwhile, meristematic activity induced in the root cortex gives rise to the cells that will form the nodule. The infection thread grows into many of these new cells, and the rhizobia are then released into the cytoplasm, surrounded by a peribacteroidal membrane. Bacteria assume the bacteroid state, differentiation of the plant cells occurs, and nitrogenase activity appears.

Nodule initiation and development depend on the expression of host and microsymbiont genes; variation in both host and bacterial genomes may affect the sequence of nodule development. In both symbionts, a portion of the genome is expressed only in the symbiotic state. The plant produces a number of nodule-specific proteins, or nodulins, some of which have now been identified (7, 41). In the bacterium, many genetic loci have been identified by symbiotic function, and some loci have been identified by gene product (16, 30).

In fast-growing rhizobia, many genes for symbiotic functions, including nodulation and nitrogenase activity, occur on large plasmids, referred to as Sym plasmids (see references 5 and 42 for reviews). Early symbiotic functions encoded by the Sym plasmid include binding of the bacteria to the root hairs (46), hair curling (23), and host specificity (3, 17). Sym plasmid genes expressed during nodule development include genes for nitrogenase polypeptides (33) and a regulatory gene of nitrogenase activity (38).

Interest in the Sym plasmid has overshadowed the possible symbiotic roles of chromosomal genes in fast-growing *Rhizobium* species. However, some symbiotic mutations have been localized on the chromosome. Forrai et al. (11) mapped chromosomal mutations causing nodulation or fixation deficiencies in *R. meliloti*, but did not further characterize the mutant phenotypes. Noel et al. (31) found that half of the symbiosis-specific mutations of *R. phaseoli* resulting from random Tn*5* mutagenesis were not located on plasmids.

We have chosen to characterize more completely the defects in nodule development caused by three *R. phaseoli* chromosomal mutations. We have found that the mutants are unable to infect *Phaseolus vulgaris* (common bean), but that root hair curling and nodule initiation occur normally. Our results indicate that the expression of certain chromosomal genes of *R. phaseoli* is necessary for the infection of bean roots and that nodule initiation and infection are separable events in beans.

MATERIALS AND METHODS

Cultivation of bacterial strains. *R. phaseoli* CE3 is a symbiotically proficient strain derived from a field isolate, strain CFN42 (35). Strains CE106, CE110, and CE115 were obtained by transposon mutagenesis of strain CE3. The mutants were selected by screening for prototrophic strains that induced ineffective nodules on sterile been seedlings (31). Each contains a single insertion of Tn*5* (J. Cava, personal communication).

To prepare *R. phaseoli* as inoculum, the bacteria were grown overnight in liquid TY medium (4) containing 0.5% tryptone, 0.3% yeast extract, and 10 mM $CaCl_2$. Minimal medium for *R. phaseoli* contained (per liter) 0.10 g of $MgSO_4 \cdot 7H_2O$, 0.22 of K_2HPO_4, 0.15 g of $CaCl_2 \cdot 2H_2O$, 0.04 g of $FeCl_3 \cdot 6H_2O$, 1 mg of biotin, 1 mg of thiamine, 1 mg of calcium pantothenate, 1.0 g of sodium glutamate, and 1.0 g of sodium succinate, pH 7.0. Yeast-mannitol medium (AMA) was made as described in Wacek and Brill (44).

Plant cultivars and growth of inoculated seedlings. *P. vulgaris* cv. Negro Jamapa was used for most experiments. *P. vulgaris* cvs. Sanilac and Puebla were used to check the phenotypic stability of the mutant *R. phaseoli* strains on beans of different genotype. All *P. vulgaris* cultivars were

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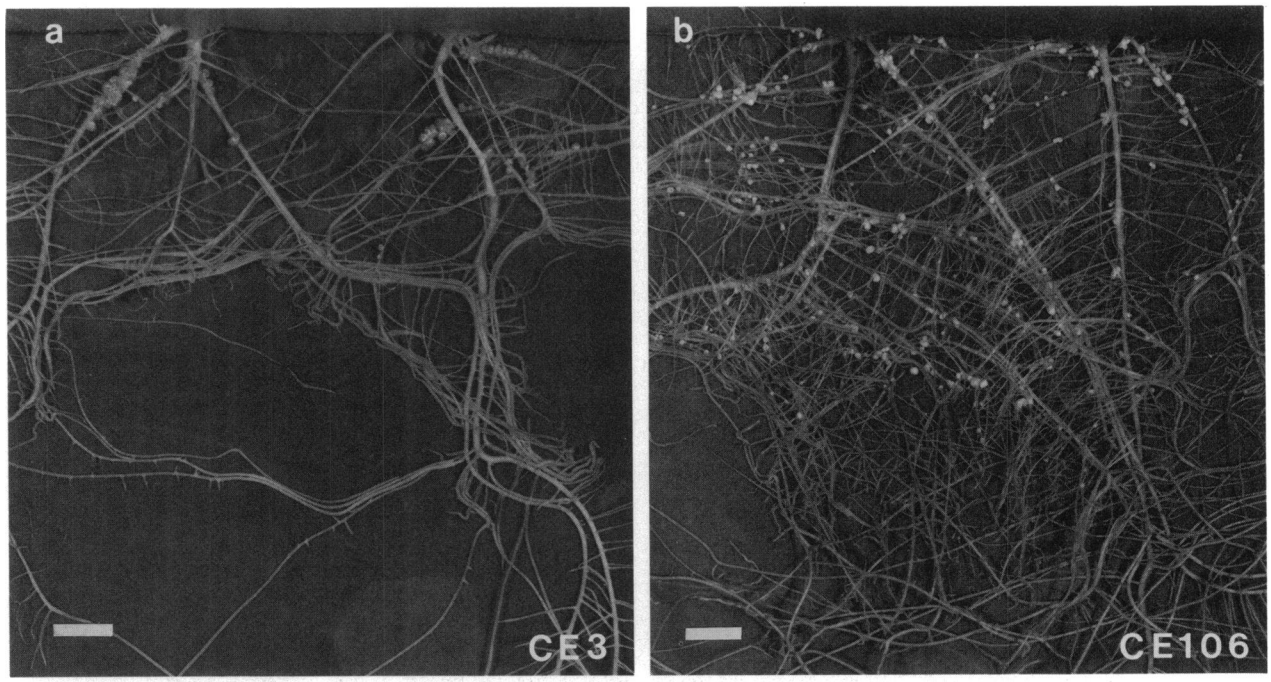


FIG. 1. Inoculated root systems of beans grown in growth pouches. 0.75×; bar, 1 cm. (a) At 17 days after inoculation with *R. phaseoli* CE3 (WT). (b) At 21 days after inoculation with *R. phaseoli* CE106.

obtained from the Department of Horticulture, University of Wisconsin, Madison.

Two-day-old surface-sterilized seedlings were transferred to growth pouches, inoculated with a suspension of bacteria in TY medium (32), and fertilized with nitrogen-free RBN medium (44). The inoculated seedlings were grown in a light chamber at 25 to 27°C with 16 h of light daily.

Light and electron microscopy. For examination of root hair curling, root segments from 1-week-old seedlings were examined by phase optics. For anatomical and ultrastructural observations, root segments and nodules were excised from seedlings 1 to 4 weeks after inoculation and fixed for 1.5 h at room temperature in freshly prepared 3% glutaraldehyde in 0.05 M potassium phosphate buffer, pH 6.8. Specimens were then washed three times for 15 min each in buffer and postfixed for 2 h in buffered 2% osmium tetroxide. Root and nodule segments were dehydrated in an acetone series and embedded in Spurr resin. For light microscopy, thick sections (about 0.5 μm) were cut on a Sorvall MT-2 ultramicrotome, mounted on gelatin-coated slides (6), and stained over an alcohol lamp with 0.05% toluidine blue O in 1% sodium borate. For electron microscopy, ultrathin sections (60 to 90 nm) were cut with a diamond knife, mounted on uncoated copper grids, and stained for 20 min in aqueous 2% uranyl acetate and for 10 min in lead citrate. The sections were observed at an accelerating voltage of 75 kV with either a Hitachi HU-11A or a Hitachi H-600 electron microscope.

Protein analysis. Nodules or root segments were crushed in cold buffer, pH 7.5, containing 10 mM Tris-hydrochloride, 5 mM $MgCl_2$, and 10 mM β-mercaptoethanol. The soluble portion obtained after centrifugation at 13,000 × *g* (31) was subjected to one-dimensional gel electrophoresis (18). Acrylamide and bisacrylamide concentrations were 12.5 and 0.33%, respectively. Protein was visualized with Coomassie blue staining.

Immunological detection of the plant protein leghemoglobin was by an Ouchterlony double diffusion assay. Antiserum raised in rabbits against soybean leghemoglobin A (Lba) was directed against crude extracts of bean nodules induced by *R. phaseoli* CE3, CE106, CE110, and CE115. Extracts were prepared by grinding nodules, which had been excised 3 weeks after inoculation, in cold potassium phosphate buffer, pH 7.0, containing 1% insoluble polyvinylpyrrolidone and 1% Triton X-100. Cell debris and polyvinylpyrrolidone were removed by brief centrifugation. Protein was determined by a biuret assay, preceded by precipitation of proteins with deoxycholate-trichloroacetic acid in the presence of sodium dodecyl sulfate (34). Antiserum to Lba was a gift from Paul Ludden.

Staining of bacterial slime in culture. *R. phaseoli* strains were streaked onto TY, AMA, or minimal agar medium containing 0.02% Cellufluor (purified Calcofluor from Polysciences, Inc.), a fluorescent stain known to bind to cellulose, chitin, and callose, and other β-linked polysaccharides (15, 24). Colonies were allowed to grow for 3 days, were exposed to long-wave UV radiation, and were scored for fluorescence. This procedure was adapted from that of J. A. Leigh, E. R. Singer, and G. C. Walker (unpublished manuscript) and Matthysse (26).

RESULTS

Infection and nodule initiation. Nodules were visible on seedlings inoculated with strain CE3 (wild type) within 7 days; nodulation on mutant-inoculated seedlings occurred within 8 to 9 days. The mutant strains (CE106, CE110, and CE115) formed numbers of nodules similar to the numbers of the wild-type strain. The nodules formed by the mutants were dispersed over the root system, whereas wild-type inoculum produced nodule clusters (Fig. 1).

The responses of *P. vulgaris* root hairs to inoculation with the various *R. phaseoli* strains are illustrated in Fig. 2. Uninoculated bean seedlings, to which TY medium alone had been applied, showed no root hair curling. Seedlings inoculated with the wild-type strain (CE3) showed a variety

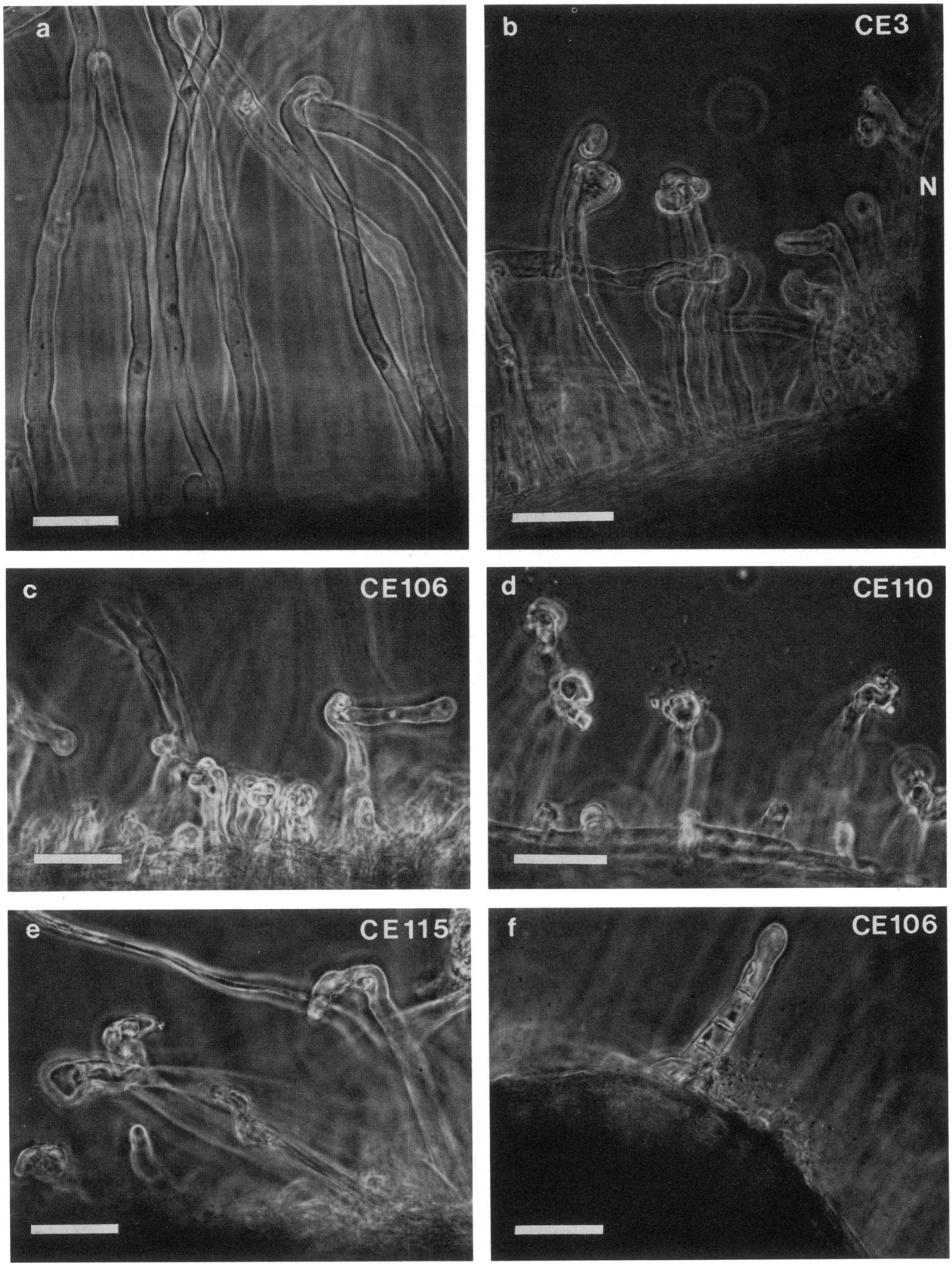


FIG. 2. Views of bean root hairs from living seedlings, photographed with phase optics. Bars, 50 μm. (a) Uncurled root hairs from a seedling inoculated with TY medium (control). 290×. (b) Seedling inoculated with strain CE3 (WT). Root hairs of different lengths show curling and deformation. A nodule (N) is visible at the right edge of the micrograph. 350×. (c, d, and e) Curled root hairs on mutant-inoculated seedlings resemble normal curled hairs. (c) strain CE106, 290×; (d) strain CE110, 325×; (e) strain CE115, 300×. (f) Young seedling inoculated with strain CE106. A multicellular root hair is clearly visible on the surface. 315×.

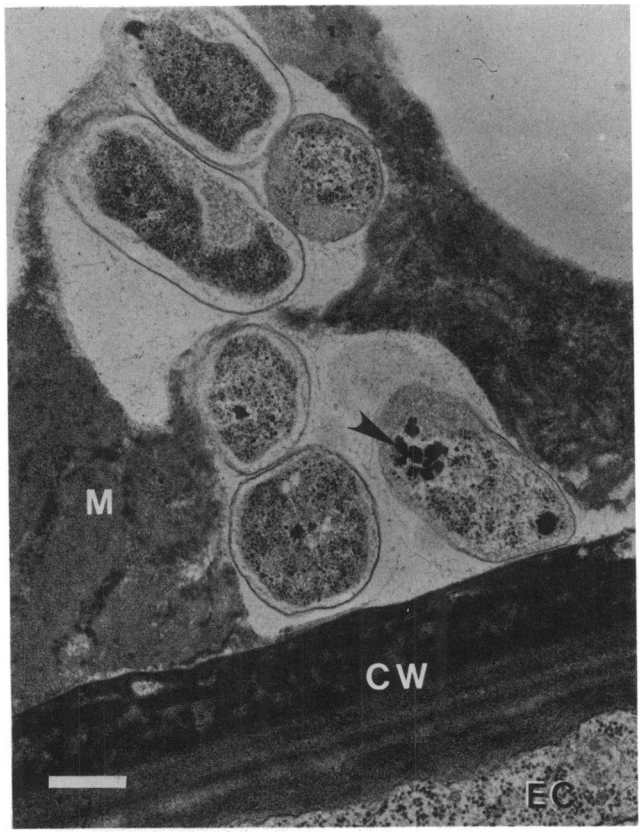


FIG. 3. Cells of *R. phaseoli* CE106 on the bean root surface. Bacteria are embedded in a fine, fibrillar matrix and surrounded by a thicker, more granular matrix (M). Some bacteria contain glycogen granules (arrowhead). CW, Wall of root epidermal cell; EC, wall of root epidermal cell; 21,500×; bar, 0.5 μm.

of root hair responses, from tightly curled short hairs to elongated hairs with markedly curled (>360°) or deformed tips. Curled root hairs had a patchy distribution on the root, but they were always found in regions of young developing nodules. The three mutant strains of *R. phaseoli* elicited the same marked root hair curling as did the wild type. Curling on roots inoculated with each of the three mutants also occurred in patches. Unlike the results of inoculation with the wild type, however, regions of curled root hairs sometimes lacked young nodular swellings, and sometimes nodules were found in regions of straight hairs. In addition, the epidermal cells, including hair cells, were sometimes stimulated to divide. Such multicellular root hairs were not observed in plants inoculated with wild-type bacteria.

Bacteria were visible in the rhizosphere in association with root hairs and sloughed root cap cells and on the surface of living epidermal cells. Typically, a layer of amorphous material surrounded the bacteria (Fig. 3). This fibrous material appeared to be loosely associated with the bacteria. Bacteria on the root surface were sometimes further encased in a granular matrix, perhaps a plant-derived mucilage. Inclusions, particularly storage granules of glycogen and poly-β-hydroxybutyrate, frequently were present in extracellular populations of the bacteria. In these aspects of rhizosphere bacteria, the mutant and wild-type strains were indistinguishable.

Anatomical examination of young nodules with wild-type bacteria revealed infection threads within curled root hairs (Fig. 4a and b). Localized cell division commenced in the outer root cortex adjacent to the infected root hairs, giving rise to a region of meristematic activity similar to that described for soybean (29, 40). Infection threads containing rhizobia invaded newly divided cells. In young, emergent bean nodules, extensive mitotic activity in the central tissue was accompanied by the release of the bacteria from the infection threads, as in soybean nodules (29).

Nodules induced by inoculation with strains CE106, CE110, and CE115 resembled one another. The development of these nodules is illustrated by specimens of CE106-induced nodules. In serial thick sections, infection threads were not visible at the light microscopy level in nodules induced by any of these three strains. Rhizobia also did not enter intercellular spaces via a break in the epidermis. Electron microscopy confirmed that these nodules at all ages lacked infected cells and intercellular bacteria.

Nodules induced by the mutant strains closely resembled normal nodules in early development except that they were uninfected. Meristematic activity began in the outermost layer of root cortical cells (Fig. 4a), forming derivative layers of cytoplasmically dense cells within the parental cell layer (Fig. 4d). As development proceeded, cell division occurred deeper in the cortex (Fig. 4e and f), giving rise to an emergent, nodulelike structure.

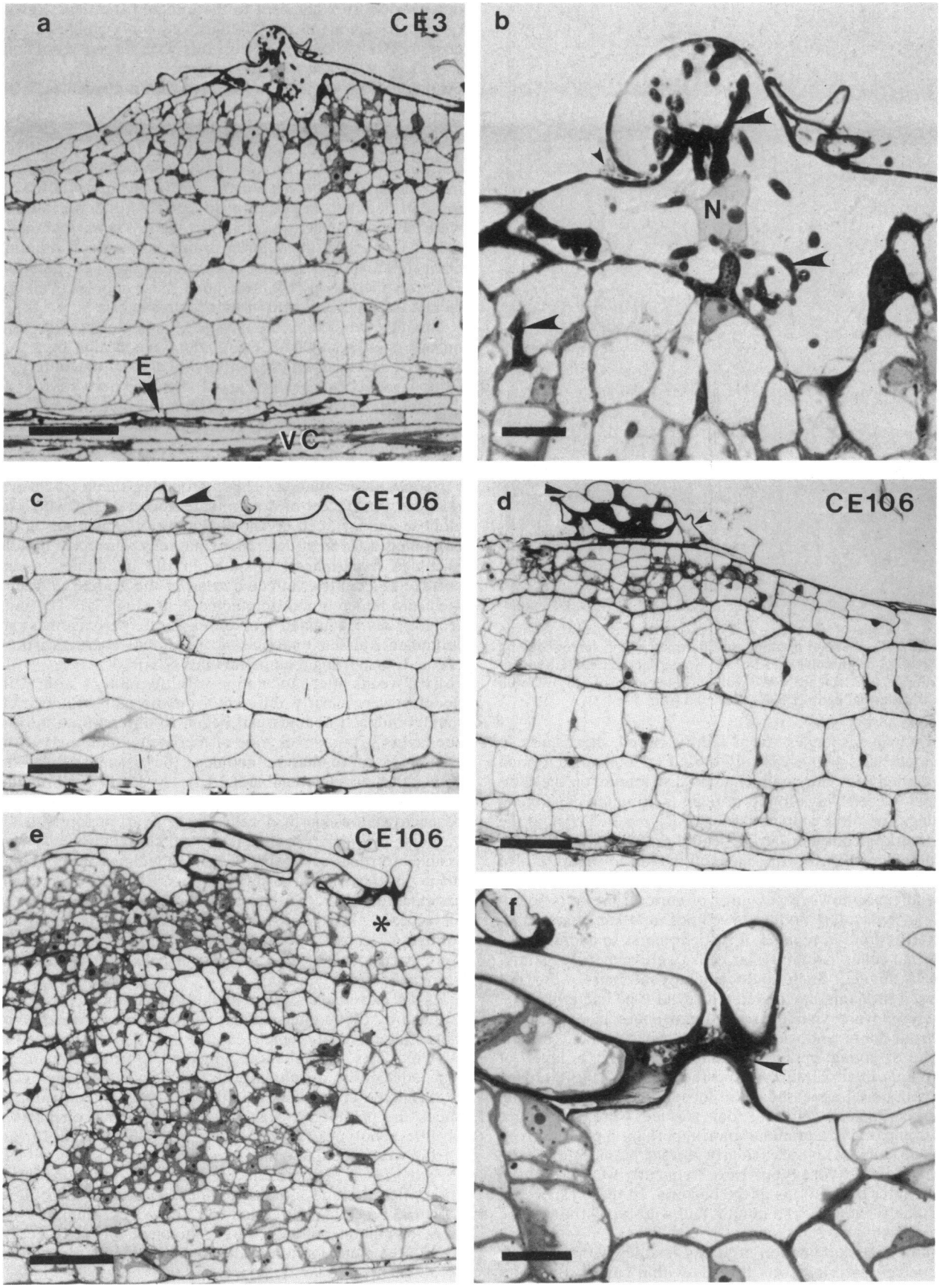
Nodule differentiation. The anatomy of mature wild-type nodules was typical of determinate nodules (37), which are globose and lack cell division at maturity. The outer cortex contained a layer of sclerenchyma cells. Vascular bundles occupied a peripheral position in the uninfected nodule cortex. The central, infected zone of the nodule differentiated into infected and uninfected cells (Fig. 5a). The uninfected cells contained numerous large peroxisomes and prominent tubular endoplasmic reticulum characteristic of ureide-transporting legume nodules (26a).

By 2 weeks after inoculation with the mutant strains, the nodules were clearly different from normal nodules. The mutant-induced nodules had begun to differentiate into tissue layers with a small zone of meristematic activity at the center. In more mature nodules, the tissues exhibited a concentric arrangement (Fig. 6a). A single or branched vascular cylinder occupied a central position, surrounded by parenchymatous cortical cells. In a layer surrounding the vascular cylinder, prominent crystal-containing cells were visible. Cortical cells were highly vacuolate and contained many plastids with large starch grains. Electron microscopic examination of the various cell types (Fig. 6b) showed that all types lacked ultrastructural specialization for ureide formation. Small microbodies, presumably peroxisomes, were occasionally found in cortical cells, but no enlarged peroxisomes were present.

As an extreme endpoint in the development of nodules induced by the mutant strains, some nodules gave rise to a single lateral root, which emerged at 3 to 4 weeks (not shown). These lateral roots appeared to arise from meristems adjacent to developing vasculature of the nodules.

Phenotype of mutants on other *P. vulgaris* cultivars. To check the phenotypic stability of the mutants on hosts of diverse genotypes, *R. phaseoli* CE3, CE106, CE110, and CE115 were inoculated into two additional cultivars of bean. *P. vulgaris* cvs. Puebla and Sanilac nodulated more slowly with wild-type and mutant *R. phaseoli* strains than did *P. vulgaris* cv. Jamapa. Ultimately, however, numbers and sizes of mutant nodules were equivalent on all three cultivars. The mutant strains induced white, ineffective nodules on all of the cultivars tested.

Analysis of nodule proteins. Plant proteins from nodules


a
CE3
E
VC
b
N
c
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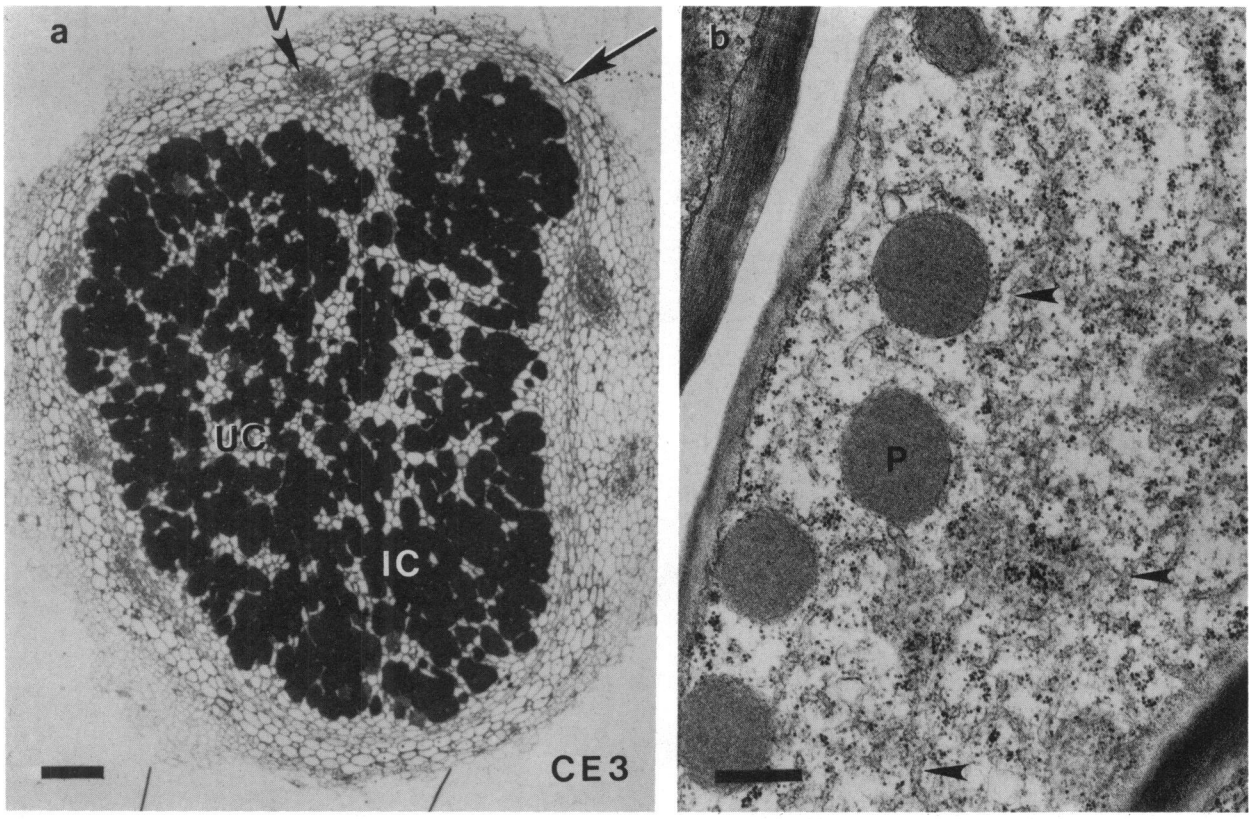

FIG. 5. Anatomy and ultrastructure of normal bean nodules. (a) Longitudinal section through a mature nodule. Darkly stained infected cells (IC) and vacuolate uninfected cells (UC) make up the central region, which is surrounded by a sclerenchymatous layer (arrow) and peripheral vascular bundles (V). 40×; bar, 200 μm. (b) Electron micrograph of an uninfected cell shows prominent peroxisomes (P) and tubular endoplasmic reticulum (arrowheads). 23,000×; bar, 0.5 μm.

induced by *R. phaseoli* CE3 and CE106 are compared in Fig. 7. Nodules induced by strains CE106, CE110, and CE115 yielded identical protein profiles (data not shown). The mutant-induced tissues were clearly different from nodules induced by the wild type, although they did share a signature protein band (N in Fig. 7). Neither did the mutant-induced tissues resemble normal young nodules or growing root tips (lanes 1 and 5 in Fig. 7). Mutant-induced tissues more closely resembled mature root tissues, but again were obviously distinct. In mature nodules carrying strain CE3, leghemoglobin migrated as an intense band at a molecular weight of about 14,000. This band was barely evident from young normal nodules. Whether it was absolutely lacking from the mutant-induced nodules was not clear from one-dimensional gels, owing to a faint band at this position; to answer this question, a more specific assay was available.

Ouchterlony double diffusion analysis with anti-soybean leghemoglobin was performed on extracts of bean nodules with purified soybean leghemoglobin as a standard. Strong precipitin bands were observed in the reaction against the standard or extracts from the nodules with wild-type bacteria, indicating that antiserum against soybean Lba is cross-reactive with the bean protein. The assay was able to detect leghemoglobin in wild-type nodule extracts down to a concentration of 2.4 μg of total nodule protein per ml. The double diffusion assay failed to detect leghemoglobin in extracts of strain CE106-induced nodules, which were 1,000 times more concentrated in protein (Fig. 8). This result is most clearly visible in the comparison between wells 10 and x in Fig. 8.

Colony morphology on agar plates. Mutant colonies were translucent and much less mucoid than colonies of strain CE3 when grown on minimal agar medium. On the two rich agar media, however, mutant and wild-type colonies were indistinguishable, being very mucoid on AMA medium and only moderately mucoid on TY medium. These differences

FIG. 4. Anatomy of early nodule development. (a) Normal infection in a seedling inoculated with strain CE3. At the site of an infected, curled root hair, cell divisions are mostly confined to the outer layers of cortical cells. The vascular cylinder (VC), surrounded by the endodermis (E), is visible at the bottom of the micrograph. 310×; bar, 50 μm. (b) Higher magnification of infected root hair pictured in (a). Infection threads (large arrowheads) branch throughout the hair cell. An infection thread has penetrated into the cortex. Bacteria (small arrowhead) are present on the root hair surface near the probable infection site. N, Nucleus; 1,150×; bar, 10 μm. (c through f) Early nodule development in strain CE106-inoculated seedlings. (c) Very early stage of nodule development, showing primary anticlinally divided cells in outer cortex. The bases of two root hairs are visible. One base (arrowhead) has become multicellular. 245×; bar, 50 μm. (d) In a slightly later stage of development, cell divisions in the outer cortex have given rise to a dense packet of cells. Bacteria are present between sloughed cells (large arrowhead) and root hair (small arrowhead). 245×; bar, 50 μm. (e) In a region of cortical cell division showing further development, divisions have occurred in all layers of the root cortex. The cells are densely cytoplasmic. No infection threads are present. A root hair (*) is illustrated at a higher magnification in (f). 290×; bar, 50 μm. (f) Bacteria (arrowhead) are visible on the surface of this curled root hair, but no infection threads have formed. 1,150×; bar, 10 μm.

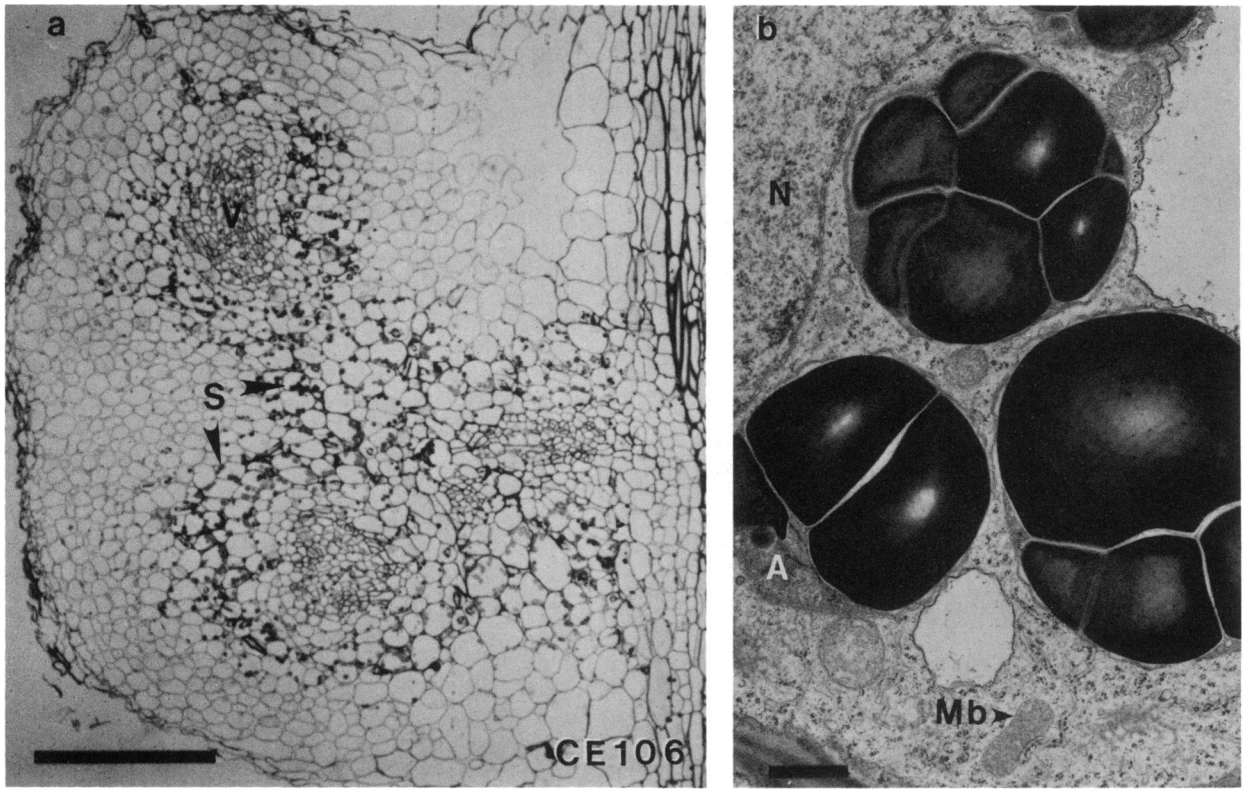

FIG. 6. Anatomy and ultrastructure of nodules induced by strain CE106. (a) Longitudinal section through a 2-week-old nodule induced by strain CE106. Vasculature (V) is differentiating in a central location. Cells in the central cortex contain large starch grains (S). 120×; bar, 200 μm. (b) Ultrastructure of a central cortical cell. Large amyloplasts (A) are the most prominent organelle. A small microbody (Mb) is present, but no large peroxisomes are visible. N, Nucleus; 21,000×; bar, 0.5 μm.

were highlighted by incorporating into the agar the fluorescent stain Cellufluor (Fig. 9), known to stain exopolysaccharides of *Agrobacterium* sp. (26). On minimal agar containing Cellufluor, colonies of strain CE3 exhibited intense blue-white fluorescence with UV irradiation. Mutant colonies on this medium fluoresced very weakly. On TY agar, however, mutants and wild type showed the same weak fluorescence, and all strains appeared bright on AMA medium with Cellufluor. It should be noted that the nodulation response to the mutant strains was not affected by the medium on which the cells were grown.

DISCUSSION

Genetic studies of early symbiotic interactions have concentrated on nodulation functions controlled by *Rhizobium* spp. Certain strains defective in nodulation ability (Nod^-) have been found to lack the ability to curl host root hairs (Hac^-) (23) or to fail in the recognition and nodulation of homologous hosts (3, 17). Hair curling and, presumably, recognition functions are among the earliest symbiotic interactions, occurring before infection thread formation. Defects in these early essential steps would, of necessity, preclude infection. This paper describes *R. phaseoli* mutants that are deficient specifically in infection, although earlier events occur normally.

R. phaseoli CE106, CE110, and CE115 induce the formation of small, white swellings on the roots of *P. vulgaris*. The structures are not infected and have attributes of both nodules and roots. Early development resembles normal nodulation of bean. Root hair curling appears normal. Meristematic activity begins in the outer cortex, as is characteristic of the determinate type of nodule development (27), and not in the pericycle, which is the case for lateral roots.

Later development of the mutant nodulelike structures lacks anatomical (37) and ultrastructural (26a) specializations for symbiosis that typify nodules of the determinate type. Instead of having peripheral vascular bundles and a central symbiotic zone, vasculature differentiates in a central position. Growth of some of the nodules is indeterminate, giving rise to a lateral root. No central cells in the nodules exhibit the proliferation of smooth endoplasmic reticulum or differentiation of large peroxisomes that is associated with the metabolism of recently fixed nitrogen in ureide-transporting legumes (26a). In addition, the mutant-induced nodules lack the symbiosis-specific protein leghemoglobin and differ from normal nodules in other plant proteins as well. Therefore, the later development of the structures induced by these strains more closely resembles that of roots than of normal nodules.

Several possibilities exist to explain the failure of the mutant *R. phaseoli* strains to infect the host roots. One explanation would be incomplete recognition between host and bacterium, perhaps due to altered cell surface features of the mutant rhizobia. Exopolysaccharide-deficient mutants of *R. meliloti* (J. Leigh et al., unpublished manuscript) do not elicit normal root hair curling and are unable to form infection threads in alfalfa, yet they induce nodule formation (T. M. Finan, A. M. Hirsch, E. Johansen, G. A. Kuldau, S. Deegan, and E. R. Signer, unpublished manuscript). In cul-

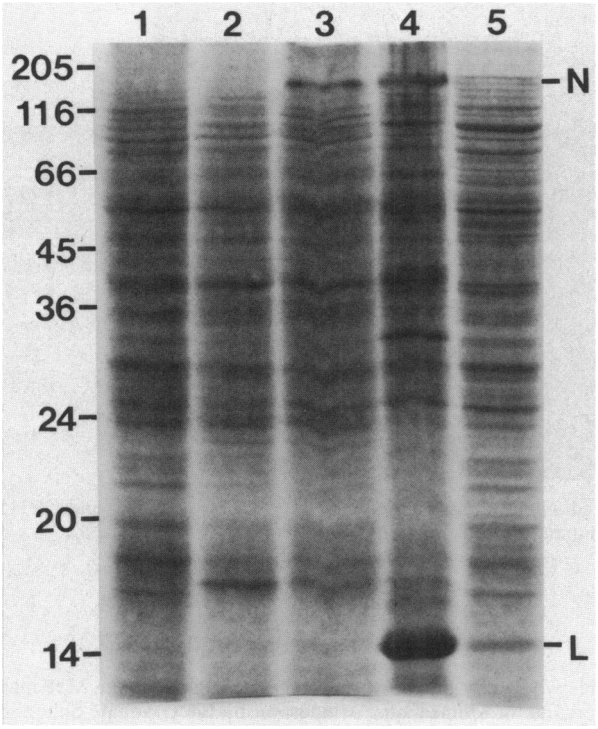

FIG. 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of soluble plant proteins extracted from 0.5-cm sections at root tips (lane 1), mature root segments (lane 2), nodules 21 days after inoculation by strains CE106 (lane 3) and CE3 (lane 4), and newly emergent nodules 8 days after inoculation by strain CE3 (lane 5). The samples were the total extracts from (in order, lanes 1 through 5) 12, 120, 65, 31, and 5 mg (fresh weight) of tissue. On the left are indicated the molecular weights (in kilodaltons) and positions of standard proteins. In order of decreasing size, these proteins are myosin (205), β-galactosidase (116), bovine serum album (66), egg albumin (45), glyceraldehyde-3-phosphate dehydrogenase (36), trypsinogen (24), soybean trypsin inhibitor (20), and α-lactalbumin (14). On the right are indicated the positions of leghemoglobin (L) and a prominent signature protein band of mature nodules (N).

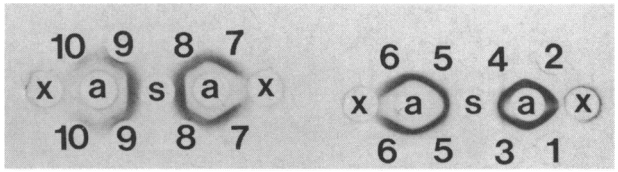

FIG. 8. Ouchterlony double diffusion assay for leghemoglobin. Wells labeled "a" contain antibody against soybean Lba. s, Soybean Lba standard, 20 μg/ml; x, extract from strain CE106-induced nodules, 2.4 mg of protein per ml. Numbered wells contain a dilution series of extract from strain CE3-induced nodules. Well 1 contains 1.2 mg of protein per ml. Wells 2 through 10 contain dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512, respectively.

ture, the mutant *R. meliloti* strains do not stain with Cellufluor, a fluorescent stain for β-linked polysaccharides.

The noninfective *R. phaseoli* mutants of this study also may be exopolysaccharide deficient, as indicated by differences in slime and Cellufluor staining in agar culture. However, the differences between mutants and wild type and the character of the wild type itself varied greatly depending on the medium composition. These facts suggest that regulation of exopolysaccharide synthesis may be affected by the mutations. Alternatively, the wild-type strain may synthesize different Cellufluor-staining molecules in response to different medium conditions, and the mutants may be defective in the synthesis of only one of these molecules. Defects in infection and slime production may also be independent reflections of a pleiotropic defect. These results do not necessarily predict the behavior of the bacteria in the rhizosphere. Electron micrographs of bacteria on the root surface of both wild-type and mutant-inoculated plants show the bacteria surrounded by an extracellular matrix which morphologically resembles the capsular polysaccharide of *R. japonicum* (1). Further studies are necessary to elucidate the nature of this matrix.

Alternatively, the bacteria may lack hydrolytic enzymes necessary for the penetration of root hairs. Ultrastructural studies of root hair infections (9) show evidence of host wall dissolution at the infection site. Because rhizobia produce low levels of hydrolytic enzymes in culture (14, 25), these enzymes may have a role in wall degradation at the infection site. Ljundggren and Fahreaus (21, 22) hypothesized that the host plant is the source of hydrolytic enzymes and that enzyme activity is enhanced by exopolysaccharide from homologous rhizobia. If this hypothesis is true, alterations in surface polysaccharides of the bacteria could prevent infection by not inducing host wall dissolution.

Nodulation in response to noninfective mutants suggests that infection thread formation is not required for the initiation of nodule development. This observation contrasts with earlier summaries of nodule morphogenesis that suggest that nodulation follows infection (20, 43). In a study of nodulation patterns in soybean, Calvert et al. (9a) also found that induction of meristematic activity may be separable from infection. Centers of cortical meristematic activity often occurred independently of infected or curled root hairs in inoculated soybean plants. These "pseudoinfections" aborted after limited cell division in the outer root cortex and so did not resemble the extensive proliferations induced in our study.

Other studies have reported the formation of nodules which lack infected tissue. *Agrobacterium* transconjugants, bearing nodulation genes from the Sym plasmid of *R. meliloti* (12) or carrying the entire plasmid (39, 45), are able to induce nodulation in homologous hosts, but the central tissue of these nodules remains uninfected. In the nodules induced by these transconjugants, abortive infection threads are sometimes found in root hair cells or show limited proliferation in the nodule interior. The bacteria may also invade several layers of nodule tissue via intercellular infection. In this regard, the *Agrobacterium* strains bearing *R. meliloti* nodulation genes differ from the mutant *R. phaseoli* strains, which appear unable to infect their host at all. All of these studies suggest, however, that nodule initiation can occur independently of successful infection.

The structures that result from inoculation with the *R. phaseoli* mutants become vascularized in the center. In other anomalous growths of plants, such as in wound callus or in the more extensive growth of *Agrobacterium* galls, nests of vascular tissue often differentiate in the center of the structure (8). In *Agrobacterium* galls, root meristems may form near centers of vascular development, similar to root formation in nodules induced by the *R. phaseoli* mutants in this study. This similarity suggests that in the current instance, the vascular development is not a specific response to the rhizobia, but a generalized response to the onset of meristematic activity.

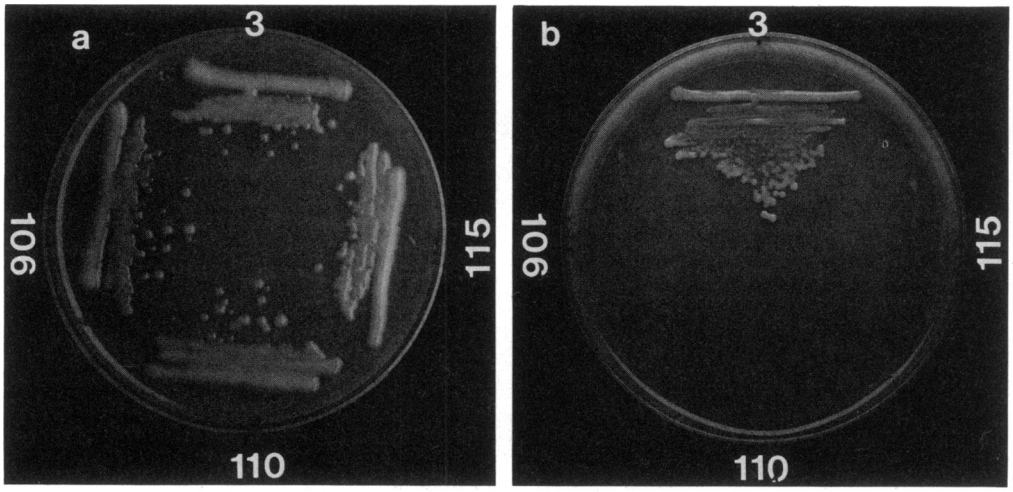

FIG. 9. Calcofluor-induced fluorescence of *R. phaseoli* cultures illuminated with UV light. (a) Strains CE3, CE106, CE110, and CE115, grown on AMA medium, exhibit fluorescence. (b) Same strains grown on minimal medium, on which only strain CE3 fluoresces.

Genes governing early symbiotic events are found in more than one replicon in *R. phaseoli*. As in other fast-growing rhizobia, genes governing early nodulation functions, including host specificity, are borne on large plasmids (3, 13, 19). However, Noel et al. (31) found that the mutations in *R. phaseoli* CE106, CE110, and CE115 could not be localized on detectable plasmids, certainly not on the same plasmid as *nifK*, *nifD*, and *nifH* (35), and probably occurred on the chromosome. Our findings indicate that these chromosomal mutations specifically prohibit infection. Each of the mutations examined in this study is found in a separate *Eco*RI restriction fragment (J. Cava, personal communication); from the sizes of these fragments, it may be inferred that each mutation occurs in a separate gene. This possibility suggests that at least three chromosomal genes may be involved in this step of the infection process.

The isolation of symbiotically defective mutants of *Rhizobium* spp. has proved a useful tool for elucidating bacterial genes involved in symbiosis. Phenotypic analysis, including microscopy, of mutant-induced nodules is important to both the geneticist and the developmental biologist; it provides insight into bacterial gene function and furthers understanding of complex plant-bacterial interactions.

It has often been commented that Nod^- mutants (which induce no nodule tissue) occur much less frequently than Fix^- mutants (which induce nodules unable to fix nitrogen) (5, 10, 11, 36). Some geneticists have concluded from this generalization and from the observation that apparently all plasmid genes for nodulation occur within 10 to 14 kilobases of the genes for nitrogenase structural polypeptides that very few bacterial genes may be involved in nodule development (10, 11, 36). These conclusions should be reassessed in light of the work reported here and the studies on nodule initiation by *Agrobacterium tumefaciens* carrying Sym plasmid genes (12, 13). Some Nod^+ Fix^- strains can induce a nodule but do not infect at all. Subsequent deficiencies in nodule development in this case should depend on *Rhizobium* gene expression. Therefore, genes involved in nodule development could outnumber the genes necessary specifically for bacteroid physiology and nitrogen fixatin. A large class of Fix^- mutants induces white nodules (5, 42). The anatomy and ultrastructure of these nodules should be examined.

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LITERATURE CITED

1. **Bal, A. K., S. Shantharam, and S. Ratnam.** 1978. Ultrastructure of *Rhizobium japonicum* in relation to its attachment to root hairs. J. Bacteriol. **133**:1393–1400.
2. **Bauer, W. D.** 1981. Infection of legumes by rhizobia. Annu. Rev. Plant Physiol. **32**:407–449.
3. **Benyon, 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**:421–231.
4. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84**:188–198.
5. **Beringer, J. E., N. B. Brewin, and A. W. B. Johnston.** 1980. The genetic analysis of *Rhizobium* in relation to symbiotic nitrogen fixation. Heredity **45**:161–186.
6. **Berlyn, G. P., and J. P. Miksche.** 1976. Botanical microtechnique and cytochemistry, p. 66–67. Iowa State University Press, Ames.
7. **Bisseling, T., F. Govers, R. Wyndael, J.-P. Nap, and J.-W. Taanman.** 1984. Expression of nodulin genes during nodule development from effective and ineffective root nodules, p. 579–586. *In* C. Veeger and W. E. Newton (ed.), Advances in nitrogen fixation research. Nijhoff/Junk, The Hague.
8. **Braun, A. C., and T. Stonier.** 1958. Morphology and physiology of plant tumors. Protoplasmatologia **10**:1–93.
9. **Callaham, D. A., and J. G. Torrey.** 1981. The structural basis for infection of root hairs of *Trifolium repens* by *Rhizobium*. Can. J. Bot. **59**:1647–1664.

9a. **Calvert, H. E., M. K. Pence, M. Pierce, N. S. A. Malik, and W. D. Bauer.** 1984. Anatomical analysis of the development and distribution of *Rhizobium* infections in soybean roots. Can. J. Bot. **62**:2375–2384.

10. **Downie, J. A., G. Hombrecher, G.-S. Ma, C. D. Knight, B. Wells, and A. W. B. Johnson.** 1983. Cloned nodulation genes of *Rhizobium leguminosarum* determine host-range specificity.

Mol. Gen. Genet. **190**:359–365.
11. **Forrai, T., E. Vincze, Z. Bánfalvi, G. B. Kiss, G. S. Randhawa, and A. Kondorosi.** 1983. Localization of symbiotic mutations in *Rhizobium meliloti*. J. Bacteriol. **153**:635–643.
12. **Hirsch, A. M., K. J. Wilson, J. D. G. Jones, M. Bang, V. V. Walker, and F. M. Ausubel.** 1984. *Rhizobium meliloti* nodulation genes allow *Agrobacterium tumefaciens* and *Escherichia coli* to form pseudonodules on alfalfa. J. Bacteriol. **158**:1133–1143.
13. **Hombrecher, G., N. J. Brewin, and A. W. B. Johnston.** 1981. Linkage of genes for nitrogenase and nodulation ability on plasmids in *Rhizobium leguminosarum* and *R. phaseoli*. Mol. Gen. Genet. **182**:133–136.
14. **Hubbell, D. H., V. M. Morales, and M. Umali-Garcia.** 1978. Pectolytic enzymes in *Rhizobium*. Appl. Environ. Microbiol. **35**:210–213.
15. **Hughes, J., and M. E. McCully.** 1975. The use of an optical brightener in the study of plant structure. Stain Technol. **50**:319–329.
16. **Kondorosi, A., and A. W. B. Johnson.** 1981. The genetics of *Rhizobium*. Int. Rev. Cytol. **13**(Suppl.):191–224.
17. **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.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Lamb, J. W., G. Hombrecher, and A. W. B. Johnson.** 1982. Plasmid-determined nodulation and nitrogen-fixation abilities in *Rhizobium phaseoli*. Mol. Gen. Genet. **186**:449–452.
20. **Libbenga, K. R., and R. J. Bogers.** 1974. Root-nodule morphogenesis, p. 430–472. *In* A. Quispel (ed.), The biology of nitrogen fixation. North Holland Publishers, Amsterdam.
21. **Ljunggren, H., and G. Fahraeus.** 1959. Effect of *Rhizobium* polysaccharide on the formation of polygalacturonase in lucerne and clover. Nature (London) **183**:1578–1579.
22. **Ljunggren, H., and G. Fahraeus.** 1961. The role of polygalacturonase in root-hair invasion by nodule bacteria. J. Gen. Microbiol. **26**:521–528.
23. **Long, S. R., W. J. Buikema, and F. M. Ausubel.** 1982. Cloning of *R. meliloti* nodulation genes by direct complementation of Nod$^-$ mutants. Nature (London) **298**:485–488.
24. **Maeda, H., and N. Ishida.** 1967. Specificity of binding of hexopyranosyl polysaccharides with fluorescent brightener. J. Biochem. **62**:276–278.
25. **Martinez-Molina, E., V. M. Morales, and D. H. Hubbell.** 1979. Hydrolytic enzyme production by *Rhizobium*. Appl. Environ. Microbiol. **38**:1186–1188.
26. **Matthysse, A. G.** 1983. Role of bacterial cellulose fibrils in *Agrobacterium tumefaciens* infection. J. Bacteriol. **154**:906–915.
26a. **Newcomb, E. H., S. R. Tandon, and R. R. Kowal.** 1985. Ultrastructural specialization for ureide production in uninfected cells of soybean root nodules. Protoplasma **125**:1–12.
27. **Newcomb, W.** 1979. Control of morphogenesis and differentiation of pea root nodules, p. 87–102. *In* W. E. Newton and W. H. Orme-Johnson (ed.), Nitrogen fixation, vol. 2. Symbiotic associations and cyanobacteria. University Park Press, Baltimore.
28. **Newcomb, W.** 1981. Nodule morphogenesis and differentiation. Int. Rev. Cytol. **13**(Suppl.):247–298.
29. **Newcomb, W., D. Sippel, and R. L. Peterson.** 1979. The early morphogenesis of *Glycine max* and *Pisum sativum* root nodules. Can. J. Bot. **57**:2603–2616.
30. **Noel, K. D.** 1984. Molecular genetics of nitrogen fixation, p. 53–85. *In* G. B. Collins and J. F. Petolino (ed.), Applications of genetic engineering to crop improvement. Nijhoff/Junk, The Hague.
31. **Noel, K. D., A. Sanchez, L. Fernandez, J. Leemans, and M. A. Cevallos.** 1984. *Rhizobium phaseoli* symbiotic mutants with transposon Tn*5* insertions. J. Bacteriol. **158**:148–155.
32. **Noel, K. D., G. Stacey, S. R. Tandon, L. E. Silver, and W. J. Brill.** 1982. *Rhizobium japonicum* mutants defective in symbiotic nitrogen fixation. J. Bacteriol. **152**:485–494.
33. **Nuti, M. P., A. A. Lepidi, R. K. Prakash, R. A. Schilperoff, and F. C. Cannon.** 1979. Evidence for nitrogen fixation (*nif*) genes on indigenous *Rhizobium* plasmids. Nature (London) **282**:533–535.
34. **Peterson, G. L.** 1977. A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. Anal. Biochem. **83**:346–356.
35. **Quinto, C., H. de la Vega, M. Flores, L. Fernandez, T. Ballado, G. Soberon, and R. Palacios.** 1982. Nitrogen fixation genes are reiterated in *Rhizobium phaseoli*. Nature (London) **299**:724–726.
36. **Schofield, P. R., R. W. Ridge, B. G. Rolfe, J. Shine, and J. M. Watson.** 1984. Host-specific nodulation is encoded on a 14 kb DNA fragment in *Rhizobium trifolii*. Plant Mol. Biol. **3**:3–11.
37. **Sprent, J. I.** 1980. Root nodule anatomy, type of export product and evolutionary origin in some Leguminosae. Plant Cell Environ. **3**:35–43.
38. **Szeto, W. W., J. L. Zimmerman, C. Sundaresan, and F. M. Ausubel.** 1984. A *Rhizobium meliloti* symbiotic regulatory gene. Cell **36**:1035–1043.
39. **Truchet, G., C. Rosenberg, J. Vasse, J.-S. Julliot, S. Camut, and J. Denarie.** 1984. Transfer of *Rhizobium meliloti* pSym genes into *Agrobacterium tumefaciens*: host-specific nodulation by atypical infection. J. Bacteriol. **157**:134–142.
40. **Turgeon, B. G., and W. D. Bauer.** 1982. Early events in the infection of soybean by *Rhizobium japonicum*. Time course and cytology of the initial infection process. Can. J. Bot. **60**:152–161.
41. **Verma, D. P. S., J. Lee, F. Fuller, and H. Bergmann.** 1984. Leghemoglobin and nodulin genes: two major groups of host genes involved in symbiotic nitrogen fixation, p. 557–564. *In* C. Veeger and W. E. Newton (ed.), Advances in nitrogen fixation research. Nijhoff/Junk, The Hague.
42. **Verma, D. P. S., and S. Long.** 1983. The molecular biology of *Rhizobium*-legume symbiosis. Int. Rev. Cytol. **14** (Suppl): 211–245.
43. **Vincent, J. M.** 1980. Factors controlling the legume: *Rhizobium* symbiosis, p. 103–129. *In* W. E. Newton and W. H. Orme-Johnson (ed.), Nitrogen fixation, vol. 2. Symbiotic associations and cyanobacteria. University Park Press, Baltimore.
44. **Wacek, T., and W. J. Brill.** 1976. Simple, rapid assay for screening nitrogen-fixing ability in soybean. Crop Sci. **16**:519–522.
45. **Wong, C. H., C. E. Pankhurst, A. Kondorosi, and W. J. Broughton.** 1983. Morphology of root nodules and nodule-like structures formed by *Rhizobium* and *Agrobacterium* strains containing a *Rhizobium meliloti* megaplasmid. J. Cell Biol. **97**:787–794.
46. **Zurkowski, W.** 1980. Specific adsorption of bacteria to clover root hairs, related to the presence of the plasmid pW72 in cells of *Rhizobium trifolii*. Microbios **27**:27–32.