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Mutations in *Rhizobium phaseoli* That Lead to Arrested Development of Infection Threads

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Two *Rhizobium phaseoli* mutants, isolated previously by Tn*5* mutagenesis, elicited infection threads which ceased development prematurely, usually within root hairs. These infection threads were wide, globular, and otherwise altered in morphology, compared with normal infection threads. Anatomy and division of the root cortical cells during initial stages of nodule morphogenesis appeared normal. However, later nodule differentiation deviated considerably from normal development, and release of bacteria from infection threads was not observed. In tryptone-yeast extract medium the mutants sedimented during growth in shaken cultures and formed rough colonies on agar. Electrophoresis of washed cultures solubilized in dodecyl sulfate revealed that the major carbohydrate band was absent from the mutants. The behavior of this carbohydrate in phenol-water extraction and gel chromatography, its apparent ketodeoxyoctonate content, and its susceptibility to mild acid hydrolysis suggested that it was a lipopolysaccharide. From the results of genetic crosses or reversion analysis, the defect in synthesizing this carbohydrate material and the defect in infection could be attributed to a single mutation in each mutant.

Nitrogen-fixing root nodules appear on legumes only in the presence of bacteria of the genera *Rhizobium* and *Bradyrhizobium*. Several early events in nodule development are common to most legumes that have been studied (1, 27), including *Phaseolus vulgaris* (bean). In these legumes the bacteria invade curled, or otherwise deformed, root hairs (1, 27). According to a theory best substantiated with soybean (1, 5, 37), the root hairs that will be invaded generally have not emerged from the developing root at the time of first bacterial contact. The presence of the bacteria causes the root hairs to curl as they develop (1). A bacterial colony at the root hair surface is thought to become trapped in a pocket created by this deformation, and from this pocket the bacteria penetrate the root hair cell wall (4, 37). A wall of host origin is deposited between the invading bacteria and the involuted plasmalemma (4, 27, 37). The bacteria, this tubular wall, and associated material constitute an infection thread, which grows through the root hair cell into the root cortex. During the course of this infection sequence, active cell division commences in the root cortex (5, 27). As the nodule develops from this region of meristematic activity, bacteria eventually are released from the infection thread into some of the resulting host cells (26), but remain separated from the host cytoplasm by peribacteroid membranes (27). Further development and cellular differentiation result in a unique organ.

The bacteria have properties that are necessary for both infection and the induction of meristematic activity. When roots are inoculated with rhizobial mutants classified as Nod$^-$ (15), meristematic activity does not occur or is not sustained long enough to generate nodule tissue. In the fast-growing rhizobia (14), including *Rhizobium phaseoli*, *R. leguminosarum*, *R. trifolii*, and *R. meliloti*, the mutations of Nod$^-$ mutants have been mapped to Sym plasmids, which also carry genes for nitrogen fixation (28, 29a). Some of the targeted genes have been characterized at the molecular level (11, 12, 15, 31).

In contrast to Nod$^-$ mutants, certain mutants of *R. meliloti* and *R. phaseoli* stimulate normal meristematic activity, but fail to elicit infection threads. In the nodulelike structures that result, little or no evident bacterial penetration is observed (13a, 17, 38). The mutations map in a plasmid other than the Sym plasmid (17, 23) and in the chromosome (29). In agar culture one major class of these mutants does not stain normally with Calcofluor, a fluorescent stain for β-linked polysaccharides (17, 23, 38). This property in *R. meliloti* mutants has been correlated with a deficiency in acidic extracellular polysaccharide (23).

In the present report we describe two *R. phaseoli* mutants that appear to have altered lipopolysaccharide (LPS). In the nodule structures induced by these mutants, infection threads exhibit abnormal morphology and usually cease development within the root hair. Both defects, in polysaccharide and infection, appear to be due to a single mutation in each mutant. Previous analysis indicated that one of these mutations was on the chromosome, rather than an indigenous plasmid (29). Hence a second class of *R. phaseoli* surface polysaccharides and a second set of chromosomal loci are important for infection thread development.

MATERIALS AND METHODS

Growth of bacteria. Bacteria were cultured at 30°C in vitamin-supplemented minimal salts with succinate and glutamate or in rich medium (TY) containing tryptone, yeast extract, and $CaCl_2$, as described previously (38). Solid media contained 1.5% Bacto-Agar (Difco Laboratories).

Cultivation of plants. Seeds of *P. vulgaris* L. cv. Negro Jamapa (obtained from the University of Wisconsin, Madison) were surface sterilized in hypochlorite and germinated on moist sterile filter paper (29). Two-day-old seedlings were transferred to plastic growth pouches, inoculated with a

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TABLE 1. *R. phaseoli* strains used in this work

Strain	Description	Reference(s)
CFN42[a]	Original isolate from *P. vulgaris* nodule	30
CE3[b]	Str^r (wild-type symbiotic proficiency)	29
CE8	Ery^r (wild-type symbiotic proficiency)	
CE106, CE107, CE110, CE115	Str^r Km^r Ndv$^-$ (noninfective) (chromosomal Tn*5* insertion)	29, 38
CE109, CE113	Str^r Km^r Ndv$^-$ (abortive infection) (chromosomal Tn*5* insertion)	29

[a] Immediate parent of spontaneous mutants CE3 and CE8.
[b] Immediate parent of mutants CE106, CE107, CE109, CE110, CE113, and CE115.

bacterial suspension in TY medium, and grown in nitrogen-free salts (38).

Light and electron microscopy. Root segments and nodules were excised from seedlings 9 to 21 days after inoculation. Root segments and small nodules were fixed whole in 3% glutaraldehyde in 0.05 M potassium phosphate buffer, pH 6.8, while larger nodules were cut into 1-mm slices directly into the fixative. Specimens in fixative were placed in a vacuum for 5 min to facilitate infiltration. After 1.5 h in fixative, specimens were rinsed in buffer, postfixed for 2 h in buffered 2% osmium tetroxide, dehydrated in acetone, and embedded in Spurr resin. For light microscopic observations, thick sections were stained with toluidine blue 0. Thin sections were poststained with uranyl acetate and lead citrate and examined in a Hitachi H-600 electron microscope operated at 75 kV. Further details of techniques are described by VandenBosch et al. (38).

Calcofluor staining. Bacteria were streaked on the surface of minimal agar containing 0.02% Cellufluor (purified Calcofluor from Polysciences, Inc.). After 3 days at 30°C they were examined for fluorescence upon exposure to long-wave-length UV light (38).

Hot phenol-water extraction and chromatography. Bacteria grown in 1 liter of TY broth were centrifuged at 6,000 × *g* for 15 min and washed three times in 1% NaCl and twice in distilled water. The washed cell pellet was stored at −20°C. For extraction, it was suspended in 9 ml of distilled water and put at 65°C, and 10 ml of 90% phenol at 65°C (39) was added with vigorous mixing. After 15 min at 65°C the mixture was cooled to 10°C and centrifuged at 6,000 × *g* for 30 min. The aqueous phase was dialyzed exhaustively against water and lyophilized. A portion was dissolved in 0.1 M EDTA–0.3 M triethylamine, applied to Sepharose 4B, and eluted with the same buffer (8). Fractions were assayed for ketodeoxyoctonate (KDO) content by the thiobarbiturate procedure (21). Total carbohydrate content was measured by the phenol-sulfuric acid assay (13), and hexose content was estimated by the anthrone procedure (33).

Gel electrophoresis. Washed cells, phenol-water extracts, or samples from chromatography were suspended in sodium dodecyl sulfate (SDS) sample buffer (22) and put in boiling water for 3 min. After discontinuous SDS-polyacrylamide gel electrophoresis (22), the gels were stained by Coomassie blue, the periodic acid (PA)-Schiff procedure (16), or the PA-silver procedure (19). Protein molecular weight standards were obtained from Sigma Chemical Co.

KDO content was measured in gel slices after electrophoresis of material eluted from a Sepharose 4B column. Fractions with apparent KDO content were pooled, dialyzed against water, and lyophilized. A 2-mg portion of the powder was prepared for SDS electrophoresis, and the sample was applied to adjacent wells of a slab gel. After electrophoresis, one lane was stained by the PA-silver procedure, while a second lane was maintained in plastic wrap at 4°C. Regions that corresponded to the staining of the first lane were cut from the second lane. Each gel slice was chopped into small pieces, sulfuric acid was added to 0.2 N (assuming access to the entire gel volume), and the resulting sample was processed by the method used for other KDO determinations (21). In this way 60 to 70% of the KDO applied to the gel was recovered in the two staining regions. Two controls were performed to detect spurious reaction or interference by polyacrylamide and electrophoresis buffers. When a slice of identical volume was obtained from the region above that which had staining material and processed in the same way, the reaction was identical to a water blank. When commercial KDO (Sigma Chemical Co.) was incubated with a parallel slice, color yield was unaffected.

Mild acid hydrolysis. The pooled fractions with KDO content from Sepharose 4B chromatography were dialyzed against several changes of water and lyophilized. Samples were treated with 2% acetic acid at 100°C for 4 h. They were centrifuged to remove a chloroform-soluble precipitate, and after chloroform extraction the aqueous phase was lyophilized. The dried powder was dissolved in 250 μl of water, and 200 μl was applied to a Sephadex G-50 column (1.0 by 35 cm; 28 ml) eluted with water. Fractions were assayed for uronic acid (2) and hexose (33) content.

Crosses. The donor strain (CE109 or CE113) carried conjugative plasmid pJB3 (3). Donor and recipient (strain CE8) were grown in liquid TY overnight, and 0.5 ml of each culture was spread on a TY agar plate. After 24 h at 30°C, the mating mixture was suspended in 0.1 M $MgSO_4$, and serial dilutions were plated on TY agar containing 30 μg of kanamycin (Km) and 10 μg of erythromycin (Ery) per ml. Km^r Ery^r colonies were tested for susceptibility to 200 μg of streptomycin (Str) per ml as evidence that they were recombinants carrying Tn*5* in the recipient chromosome (Ery^r Str^s). Presence of saccharide I (see Fig. 6) was monitored by gel electrophoresis, and nodulation was tested by the 15-day vial assay with bean plants (29).

Recovery of bacteria from nodules. Nodules were immersed for 5 min in commercial bleach (5.25% sodium hypochlorite) diluted 10-fold with water. They were soaked for 15 min in sterile water in a petri dish. Individual nodules were crushed by trituration with a sterile glass rod in 1 ml of TY medium in a small tube. After the tube was agitated on a Vortex mixer and let stand briefly, the contents above the settled debris were diluted serially in TY and samples were spread on TY agar plates. Colonies appeared in 2 days at 30°C. When surface-sterilized nodules were agitated in 1 ml of TY by vortexing, but not crushed, zero to two colonies per 0.1 ml were observed.

RESULTS

Isolation and nodulating ability. Strains CE109 and CE113 (Table 1) were isolated after mutagenesis by introducing the Tn*5* suicide plasmid pJB4JI into strain CE3, an Str^r derivative of *R. phaseoli* soil isolate CFN42 (29). They were among a group of strains designated as Ndv$^-$ (affecting nodule development). In gross appearance, nodulation by these two strains very closely resembled that of noninfective Ndv$^-$ *R. phaseoli* mutants CE106, CE110, and CE115 (38). Inocula-

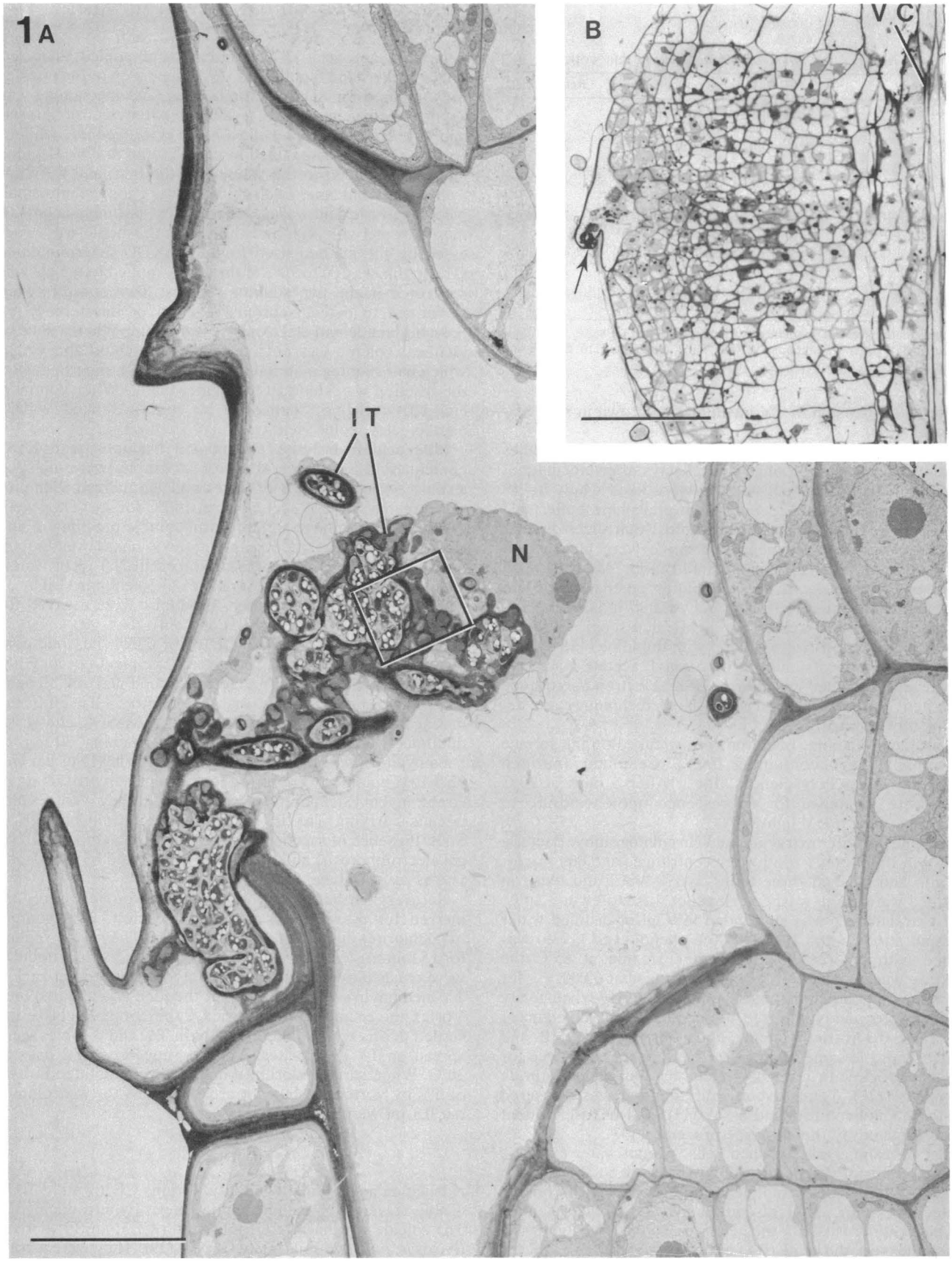


FIG. 1. Anatomy and ultrastructure of a bean root hair, infected by *R. phaseoli* CE109, 9 days after inoculation. (A) Electron micrograph of root hair. The infection thread (IT) is visible in the host cell cytoplasm. N, Host cell nucleus. The boxed area is shown enlarged (×2800) in Fig. 2. Bar, 10 μm. (B) Light microscopic view of a longitudinal section through the same infection site. Many cell divisions have taken place in the root cortex below the infected root hair (arrow). VC, Root vascular cylinder. Magnification, ×230. Bar, 100 μm.

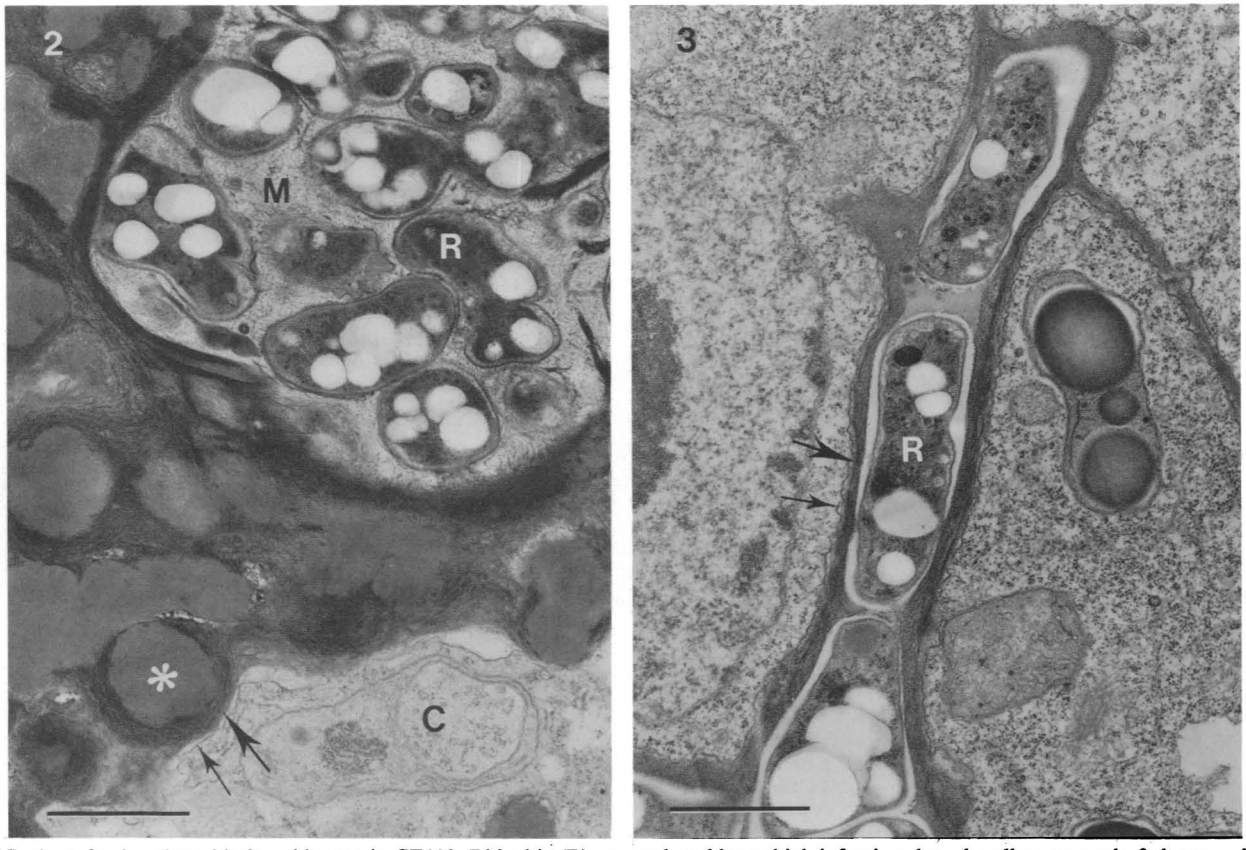


FIG. 2. Infection thread induced by strain CE109. Rhizobia (R) are enclosed by a thick infection thread wall composed of electron-dense fibrillar material (large arrow) and less dense, amorphous material (asterisk). Abundant matrix (M) surrounds the rhizobia. The host plasma membrane (small arrow) separates the infection thread from the host cytoplasm (C). Bar = 1 μm.

FIG. 3. Infection thread in nodule induced by strain CE3 (wild type). The thin infection thread is bounded by a layer of fibrillar wall material (large arrow) and the host plasma membrane (small arrow). Little thread matrix is present. Bar, 1 μm.

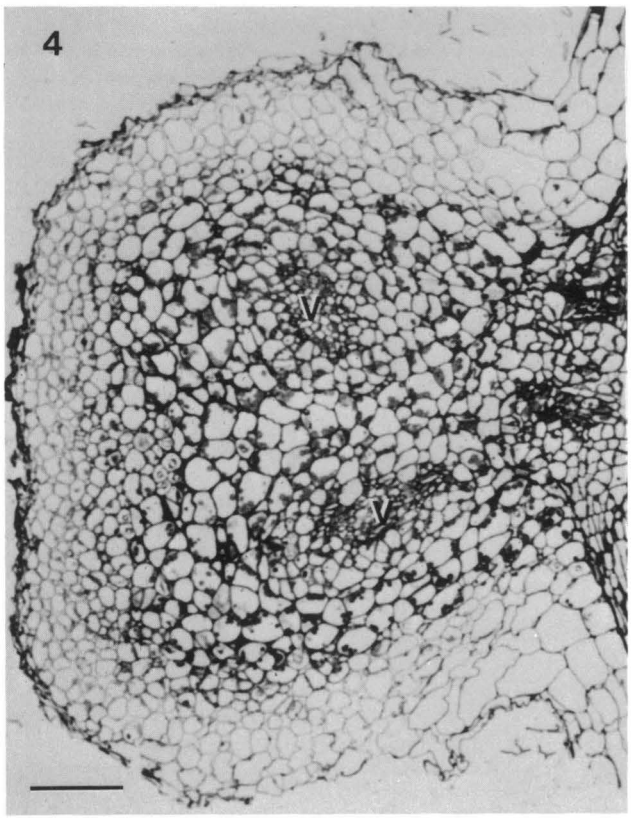
tion by strains CE109 and CE113 resulted in nodules distributed throughout the root system. Ultimately, they outnumbered nodules induced by the wild type, which were confined mainly to large clusters on upper lateral roots. Mutant-induced nodules appeared 2 to 3 days later than nodules from wild-type inoculation and at all times afterward were white, smaller than normal, and devoid of nitrogenase (acetylene-reducing) activity (29).

Early nodule development. Nodules embedded 9 to 14 days after inoculation were sampled for the study of early nodule development. Serial thick sections revealed that an infected root hair was present at most (19 of 21) sites of nodule initiation elicited by the mutant strains of *R. phaseoli*, CE109 and CE113. In all observed cases, the morphology of the infection thread was grossly altered in comparison with that of a normal infection induced by wild-type *R. phaseoli* (Fig. 1 to 3). The wide, globular infection threads induced by the mutant strains almost always remained confined to the infected root hair cell. In the wild-type infection at a comparable stage, infection threads had penetrated into underlying cortical cells (38). Release of rhizobia from the aberrant infection threads was not observed at any stage of development. Anatomy of the root cortex and division of the cortical cells were not noticeably different in the mutant-induced versus wild-type-induced nodules at this stage.

In ultrastructural observations of root hairs infected by either wild-type bacteria or strain CE109, the infection thread and enclosed bacteria were always separated from the host cytoplasm by the plasmalemma (Fig. 2 and 3). Wild-type- and mutant-induced infection threads differed greatly in structure and composition, however. Wild-type-induced infection threads were narrow, tubular structures with a thin, fibrillar wall (Fig. 3). In contrast, the thick infection thread walls in root hairs infected by CE109 contained abundant amorphous material and microfibrils with random orientation (Fig. 2). A copious, fibrillar matrix surrounded the bacteria within the thread. Although abnormal morphology was the rule, in short portions distal to the site of original infection, the CE109-induced infection threads resembled tubular wild-type-induced infection threads.

Nodule differentiation. In mutant-induced nodules sampled 3 weeks after inoculation, no bacteroids (27) were present (Fig. 4), although bacteria were occasionally observed in intercellular spaces (Fig. 5). An aborted infection thread was sometimes detected within a root hair at the nodule periphery. Vascular bundles, frequently observed to be branched, were located centrally, rather than peripherally as in a normal nodule. Mutant-induced nodules lacked the enlarged peroxisomes and proliferation of tubular endoplasmic reticulum that is associated with ureide metabolism in normal nodules (26); small microbodies typical of undifferentiated plant tissues were present, however (Fig. 5).

SDS gel electrophoresis was performed on the soluble portion of nodules 21 days after inoculation by the mutants.



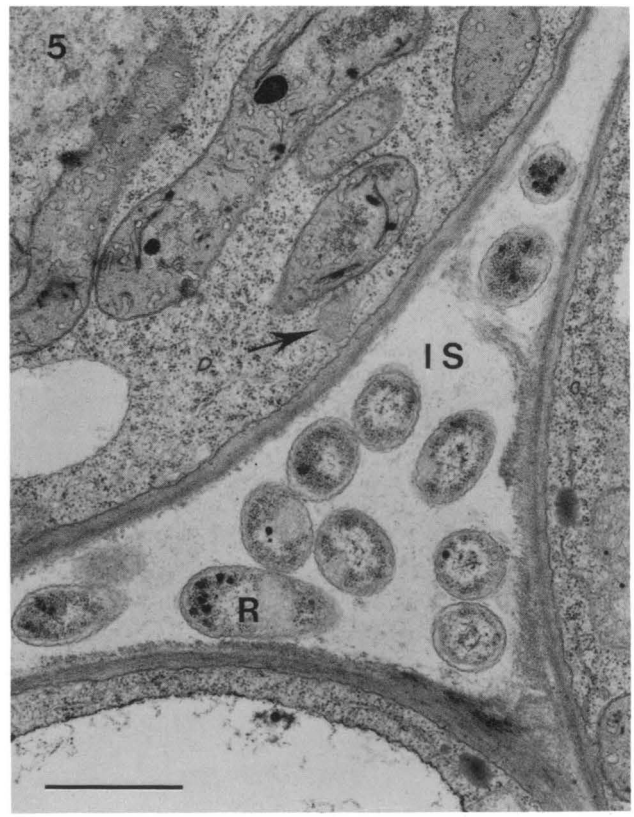


FIG. 4. Light micrograph of nodule, 21 days after inoculation with *R. phaseoli* strain CE109. No bacteria were observed in the nodule. Two vascular bundles (V) are visible in the central tissue. Bar, 100 μm.

FIG. 5. Rhizobia (R) present in an intercellular space (IS) of a nodule. A small microbody (arrow) is visible within the host cells. Bar, 1 μm.

The pattern of protein bands was indistinguishable from that of nodules induced by noninfective mutants, as documented by VandenBosch et al. (38). These nodules lacked leghemoglobin and resembled mature roots more than normal nodules in protein content (38).

Culture characteristics. Growth of the mutants in minimal broth and minimal agar media was identical to that of the wild type. Colonies on agar were stained normally with Calcofluor. However, in tryptone-yeast extract broth, which suppresses slime formation (38), both mutant strains autoagglutinated. Colonies on TY agar appeared "rough" (CE113) or somewhat rough (CE109) in comparison with the smooth wild-type colonies.

Gel electrophoresis and phenol-water extraction. Washed cultures of strains CE3 (wild type), CE109, and CE113 were subjected to SDS-polyacrylamide gel electrophoresis (Fig. 6). Protein staining with Coomassie blue was identical for each strain except for a region in which wild-type bands were distorted. By varying sample or acrylamide concentrations, it was found that even in this region the mutants and the wild type had the same protein bands. Under different sample or acrylamide concentrations, different wild-type bands were distorted. The basis of the anomalous Coomassie patterns became apparent when the gels were stained for carbohydrate by the PA-Schiff procedure. An intense carbohydrate band (designated as saccharide I [Fig. 6]) from the wild type migrated in this region of the gel, but was absent from mutants CE109 and CE113. The PA-Schiff staining was not affected by protease digestions that eliminated Coomassie blue staining or by growth of the strains in minimal, instead of rich, medium. With PA-silver, widely used to stain lipopolysaccharides on SDS gels (19), saccharide I was imbued with a deep red color.

Saccharide I was observed after SDS gel electrophoresis of the aqueous phase of hot phenol-water extracts of wild-type cells (Fig. 6 and 7). In phenol-water extracts of each mutant, the absence of saccharide I was coincident with more intense PA-silver or PA-Schiff staining at the position of the wild-type band of greater mobility in SDS gel electrophoresis (Fig. 6 and 7).

SDS gels of total cellular contents exhibited two bands migrating faster than saccharide I (Fig. 6). The band with greater mobility was released from the wild-type cells in variable amounts. The phenol-water extracts of Fig. 6 contained this band, but those of Fig. 7, which were dialyzed much more extensively, did not.

Gel chromatography and mild hydrolysis. Sepharose 4B column chromatography of the mutant CE109 and wild-type phenol-water extracts yielded two major peaks of carbohydrate (Fig. 8), as seen in other *Rhizobium* strains (8, 9). KDO, a common constituent of LPS, was associated with the first peak and a shoulder that emerged somewhat earlier (Fig. 8). On SDS gel electrophoresis each fraction in this peak yielded the PA-Schiff (and PA-silver) bands of the phenol-water extracts, including saccharide I in the case of the wild type (Fig. 7). The intensities of these bands increased and decreased in concert with the apparent KDO content of the fractions. By assay of slices of an acrylamide

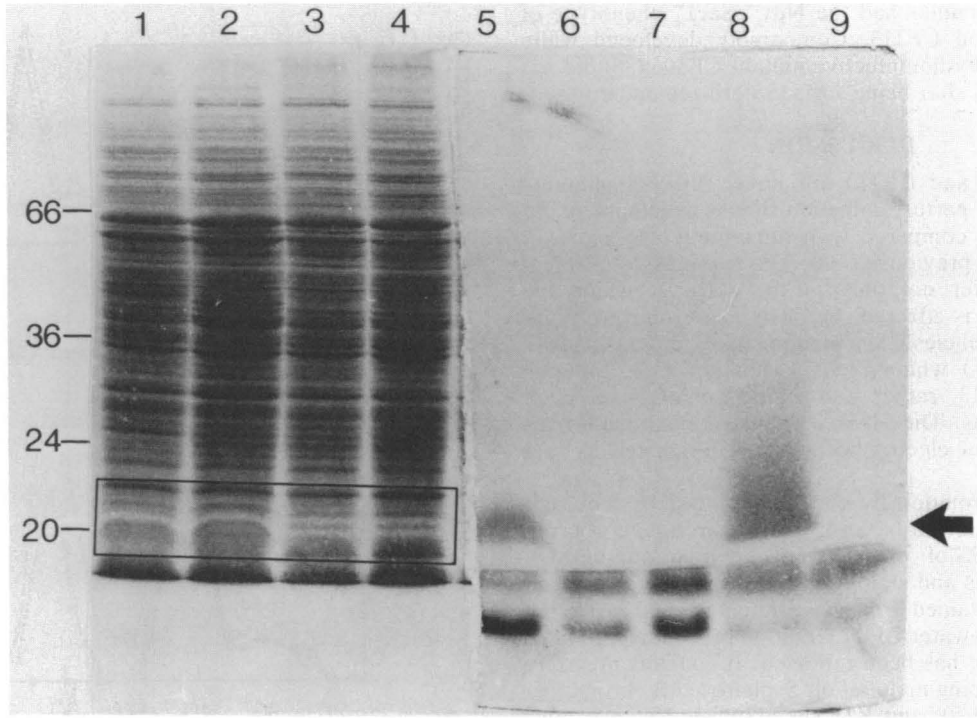


FIG. 6. SDS-polyacrylamide gel electrophoresis of extracts of cultures grown in TY medium. Washed cells of strains CE3, CE106, CE109, CE113, CE3, CE109, and CE113 (lanes 1 to 7, respectively) were extracted directly into SDS sample buffer at 100°C. The samples of lanes 8 and 9 were from the aqueous phases of hot phenol-water extracts of strains CE3 (lane 8) and CE109 (lane 9). The gel (11.5% acrylamide) was stained with Coomassie blue (lanes 1 to 4) or PA-Schiff (lanes 5 to 9). The arrow indicates the position of saccharide I (the PA-Schiff band missing from mutants CE109 and CE113). The box outlines the distortion of wild-type protein bands that comigrated with saccharide I. The lowest PA-Schiff band was variably released from cells and was lost during saccharide I purification. The migration of standard proteins with the given molecular weights (10^3) is depicted on the left.

gel (see Materials and Methods), 60% of the KDO in the Sepharose peak was found associated with saccharide I, and 9% was recovered in the other wild-type band.

The relative carbohydrate content, particularly the apparent hexose, of this first Sepharose 4B peak was much less from mutant CE109 than from the wild type (Fig. 8). The ratio of hexose/KDO was even lower in the corresponding peak of material from mutant CE113 (data not shown).

The contents of the KDO-containing Sepharose 4B peaks of mutant CE109 and the wild type were treated under mild conditions known to cleave LPS at labile bonds involving KDO and to release lipid A and a polysaccharide or oligosaccharides (7). After this treatment neither saccharide I nor the other PA-silver bands were detected upon SDS gel electrophoresis (Fig. 9A). A precipitate that formed during the reaction was extracted completely into chloroform, and the water-soluble portion was analyzed by Sephadex G-50 chromatography (Fig. 9B). The wild-type G-50 profile contained a peak not found in the mutant. This peak corresponded to the oligosaccharide(s) of higher molecular weight. Before the mild hydrolytic treatment the carbohydrate content of both mutant and wild-type samples was found only in the void volume of Sephadex G-50 chromatography (emerging before fraction 24 of Fig. 9B). The aqueous hydrolysate of the mutant was subject to low yields and variable G-50 elution profiles. Often, a peak in the void volume was detected where unhydrolyzed material runs. These results may have been due to the obviously low solubility in 2% acetic acid of the Sepharose peak material from the mutant.

Genetic analysis and nodule bacterial sampling. Experiments reported previously (29) had indicated that the chromosomal Tn*5* insertion of strain CE109 was the cause of its Ndv^- phenotype or was linked >98% to the responsible mutation. Similar experiments were carried out to probe whether the absence of saccharide I ($SacI^-$) likewise was due to the Tn*5* insertion. Strain CE109 (Km^r [due to the Tn*5*] Str^r) was crossed with an erythromycin-resistant (Ery^r) symbiotically proficient strain (CE8 [Table 1]), and transfer of Tn*5* to this recipient was selected on agar with erythromycin and kanamycin. Ery^r Km^r Str^s transconjugants were tested for the presence of saccharide I and for the ability to nodulate bean plants. All of the 41 tested were Ndv^- $SacI^-$, as expected if Tn*5* insertion were the cause of both phenotypes.

Analogous transconjugants carrying the Tn*5* insertion from CE113 were always Ndv^+ $SacI^+$. Thus, the Ndv^- and $SacI^-$ phenotypes did not seem linked to the Tn*5* insertion. Whether Ndv^- and $SacI^-$ were due to the same mutation was tested by reversion analysis. Occasionally, among the small white nodules induced by strain CE113, a large red one appeared. Bacteria from such nodules were Km^r Str^r Ndv^+ $SacI^+$, as expected if Ndv^- and $SacI^-$ were due to the same mutation and unlinked to the Tn*5* insertion. Of 20 mutants having various symbiotic or auxotrophic phenotypes (29) that have been analyzed genetically after Tn*5* mutagenesis of *R. phaseoli* CFN42, this is the only one in which the Tn*5* insertion was not responsible for the observed phenotype.

When surface-sterilized, 3-week-old, white nodules induced by either mutant CE109 or CE113 were crushed, 10^3 to 10^5 colony-forming bacteria were released. Red nodules carrying the wild type or revertants of strain CE113 yielded 10^8 colony-forming bacteria. Each bacterial colony tested

from the white nodules had the Ndv$^-$ SacI$^-$ phenotype of strains CE109 and CE113. Comparably developed white nodules induced by noninfective mutant CE106 (38) did not yield any bacteria after being surface sterilized and crushed.

DISCUSSION

Strains CE109 and CE113 are novel rhizobial mutants whose properties perturb infection thread development. In Table 2 they are compared with noninfective *R. phaseoli* mutants reported previously (38). The two classes of infection mutants differ not only in the stage at which the infection process is affected, but also in presumptive polysaccharide deficiencies. The noninfective mutants lack a cellular product(s) which binds Calcofluor (38). Mutants CE109 and CE113, rather than being noninfective, elicit abortive infections. They lack a cellular constituent(s) detected by SDS gel electrophoresis and designated as saccharide I.

Although confirmation by chemical analysis is required, saccharide I appears to be an LPS. It shares several properties with the LPS of other bacteria. Its migration on SDS gel electrophoresis and staining properties are typical of an LPS (7). It is obtained from a sedimented culture by the traditional phenol-water LPS extraction procedure (39) after extracellular slime has been removed. It coelutes precisely with KDO-containing material on Sepharose 4B chromatography (Fig. 7 and 8), and KDO is associated with it after electrophoresis on SDS gels. It appears to be susceptible to a mild treatment that cleaves typical LPS into lipid A and oligosaccharide/polysaccharide components (7), for it cannot be detected on SDS electrophoresis after this treatment (Fig. 9A).

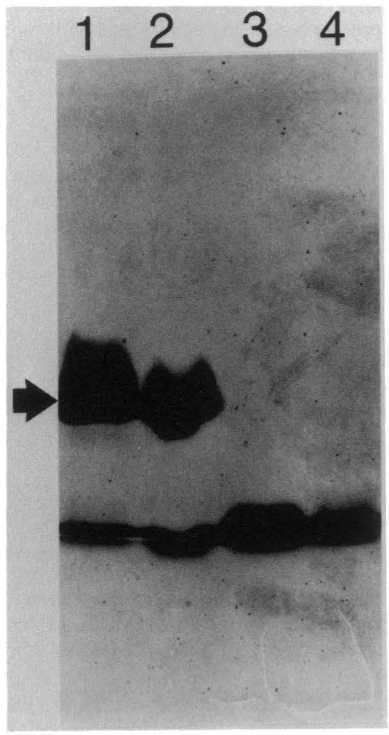


FIG. 7. Phenol-water extracts and pooled Sepharose 4B fractions with KDO content (Fig. 8) compared by SDS gel electrophoresis stained with PA-silver. For lanes 1 and 2 the phenol-water and the Sepharose samples, respectively, from strain CE3 were applied. For lanes 3 and 4 the phenol-water and Sepharose samples from strain CE109 were applied. PA-Schiff (not shown) revealed the same bands as PA-silver in each case. Saccharide I (arrow) and the lower band from the wild type were separated more greatly than in Fig. 6 because the gel was polymerized from 15% acrylamide.

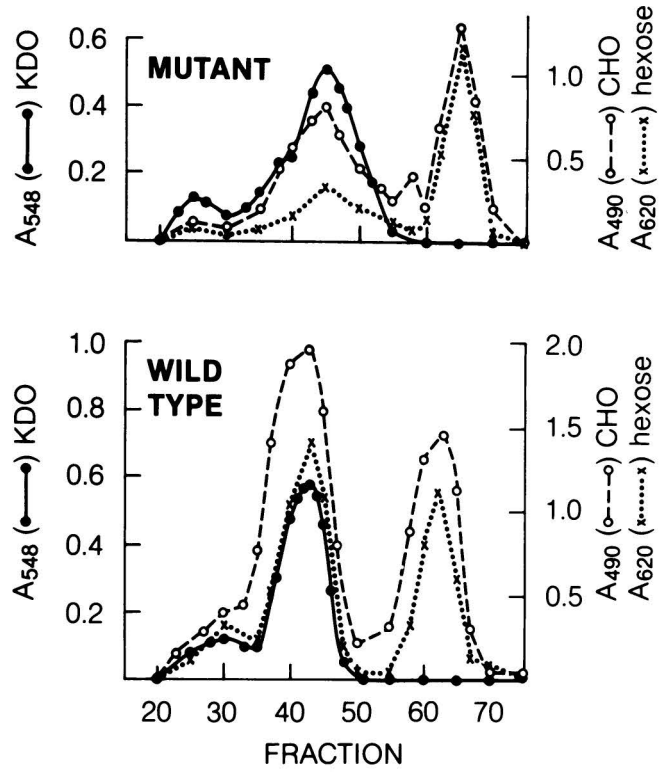


FIG. 8. Sepharose 4B chromatography of hot phenol-water extracts of the mutant (strain CE109) or the wild type (strain CE3). The extract from 3 liters of culture (40 to 60 mg of lyophilized powder) was applied to a column (2.5 by 42 cm) in 100 mM EDTA–300 mM triethylamine. Fractions of 3 ml were collected, and equal portions were assayed for KDO, hexose, and total carbohydrate (CHO) content. Fractions 39 to 47 from each column were pooled for the experiments of Fig. 7 and 9.

The analysis of phenol-water extracts in this study was based on the methods used by Carlson et al. in studies of the LPS of *R. leguminosarum*, *R. trifolii*, and *R. phaseoli* strains (7, 8). The results corroborate his finding of at least two bands of carbohydrate material when the LPS fraction purified by Sepharose 4B chromatography is separated by SDS gel electrophoresis (7). Saccharide I corresponds to what he has termed the higher-molecular-weight heterogeneous banding region.

The LPS structures of *Escherichia coli* and *Salmonella* spp. are understood in terms of the O-antigen polysaccharide-core oligosaccharide-lipid A concept (24). The two carbohydrate bands of wild-type *R. phaseoli* strain CE3 on SDS gels and the structural defects of mutants CE109 and CE113 can be interpreted in terms of this concept, with some modifications unique to *Rhizobium* spp. (7). The faster-migrating band of the wild type may be core oligosaccharide attached to lipid A, and saccharide I may consist of an O antigen attached to this structure by a very labile bond (7). The mutants, in turn, may have truncated versions of saccharide I, perhaps lacking the O antigen altogether. The larger carbohydrate species generated by mild hydrolysis of putative wild-type LPS (Fig. 9B) might represent the portion

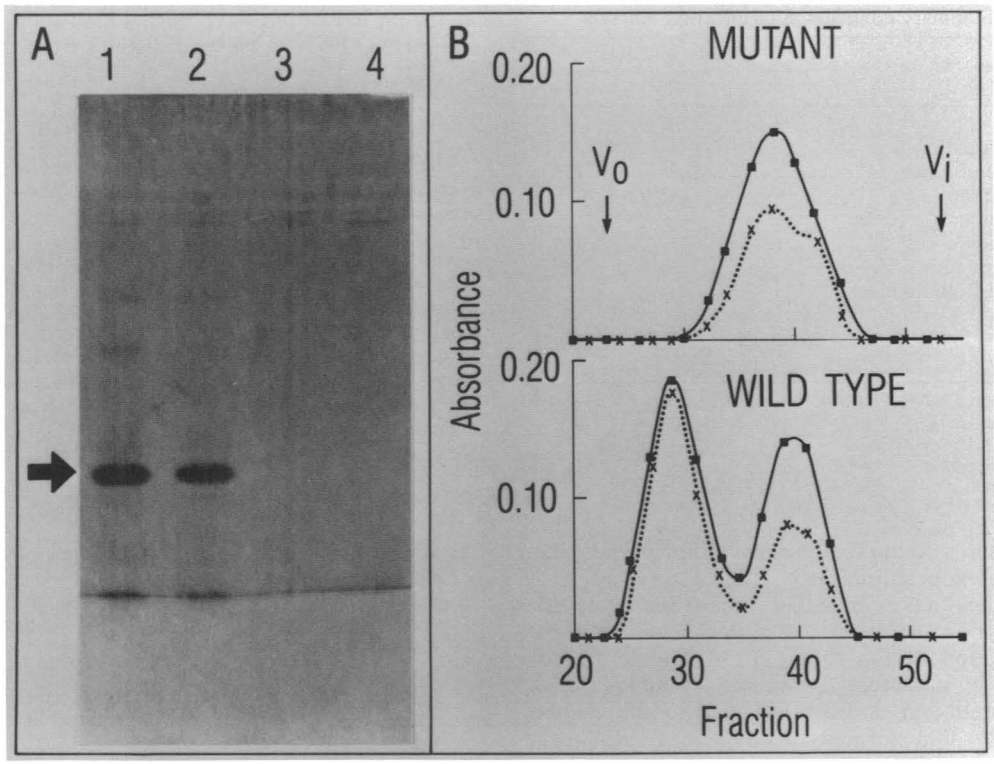


FIG. 9. Mild acid hydrolysis of Sepharose 4B-purified fractions of the phenol-water extracts. (A) SDS gel electrophoresis of the wild-type sample before acetic acid treatment (lane 1), the water-soluble portion after hydrolysis and lyophilization (lane 3), and the chloroform-extracted material after hydrolysis (lane 4). For lane 2 the samples of lanes 1 and 3 were mixed. The samples of lanes 3 and 4 represented the yields from 100-fold more material than that applied to lane 1. The gel (15% acrylamide) was stained by PA-silver. The arrow points to saccharide I. (B) Sephadex G-50 chromatography of the water-soluble portions of the mutant (CE109) and wild-type (CE3) hydrolysates. Each was run on a 28-ml column (1.0 by 35 cm). Fractions eluted with water were assayed for hexose (×) and uronic acid (■) content. Before the acetic acid treatment the carbohydrate of both the mutant and the wild type emerged before fraction 24. The void volume (V_0) and the included volume (V_i) were estimated by the migration of blue dextran and glucose, respectively.

truncated or missing in the mutants. This hypothesis is consistent with the existing data; however, further work is required to prove that saccharide I is LPS. Moreover, it is unclear whether or to what degree rhizobial LPS (1, 6, 7, 20, 41) conforms to the *E. coli-Salmonella* model.

Genetic evidence suggests that both the absence of saccharide I and the abnormality in root hair infection are due to a single mutation in each mutant. One interpretation is that the infection phenotype is a consequence of the polysaccharide alteration. Another is that abortive infection and the absence of saccharide I are independent consequences of a lesion in carbohydrate metabolism (as a hypothetical example, the inability to synthesize fucose). The first interpretation is bolstered by having two mutants that seem to have different degrees of structural alteration. However, to support this idea more firmly, the metabolic defects of these two mutants must be analyzed and additional mutants must be isolated. LPS defects sometimes cause deficiencies in outer membrane proteins (24). That is a possible factor in the mutant phenotype, but it does not appear that outer membrane structure has been grossly altered, for the major proteins stain identically after SDS gel electrophoresis of mutants and the wild type (Fig. 6).

A few other rhizobial mutants have been reported to be defective in both nodulation and material extracted by the phenol-water procedure. One such *Bradyrhizobium japonicum* mutant induces no visible nodulation at all (35). Another (25) induces slow development of nodules that eventually have nitrogenase activity (34). Loss of a plasmid from an *R. trifolii* strain results in inability to nodulate and different LPS composition (32). However, in none of these cases have the nodulation phenotype and the polysaccharide alterations been shown to result from a single mutation.

Some early interactions between the host plant and strains CE109 and CE113 seem unimpaired. The mutants are capable of host-specific interactions that lead to the onset of cell divisions in the root cortex. The nodules first appear on the portion of the root first nodulated after wild-type inoculation (1). Root hair curling occurs. The bacteria breach the root hair wall and induce a structure with elements of the normal infection thread. However, the thread differs in morphology and composition from a normal infection thread. It rarely invades host cells produced by meristematic activity in the cortex, but rather remains confined to the root hair.

Other studies have described similar infection phenotypes. *Agrobacterium* strains, carrying either the Sym plasmid of *R. meliloti* or fragments of this plasmid, induce the formation of white ineffective nodules on alfalfa (18, 36, 40). Occasionally, infection threads penetrate root hairs (18, 40), but infection thread growth is distorted or of limited duration. Bacteria are also found in intercellular spaces of the nodules (18, 36, 40). Bacterial release from infection threads is not observed, nor are living host cells found to be infected. A plant mutation may have a similar effect. A non-nodulating cultivar of pea, *Pisum sativum* cv. *Afghanistan*, develops small white swellings when inoculated with *R. leguminosar-*

TABLE 2. Summary comparison of infection mutants

Property	Phenotypic class		
	I[a]	II[b]	WT[c]
Root hair curling	+	+	+
Infection thread formation	−	+	+
Infection thread persistence	−	−	+
Meristematic induction	+	+	+
Nodule differentiation	−	−	+
Bacteroid production	−	−	+
Symbiotic C_2H_2 reduction	−	−	+
Bacteria recovered from nodules	−	+	++
Calcofluor staining[d]	−	+	+
Colony texture[e]	Smooth	Rough	Smooth
Saccharide I	+	−	+

[a] Noninfective strains CE106, CE107, CE110, and CE115.
[b] Strains CE109 and CE113.
[c] Wild-type strains CE3 and CE8.
[d] On minimal agar medium.
[e] On TY agar medium.

um. These structures do not fix nitrogen, although root hairs appear to become infected normally (10).

Previous studies have indicated that a bean nodule meristematic region can be induced by *R. phaseoli* without bacterial penetration of the root (38). Although the cell division induced by noninfective mutants resembles that of normal nodule initiation, it does not lead to the cellular differentiation (26) that normally accompanies subsequent nodule development. The present results indicate that mere initiation of infection threads is also insufficient to bring about the differentiation characteristic of mature nodules. Anatomy, ultrastructure, and protein content of mutant-induced nodules are identical whether infection is arrested within the root hair or does not occur at all.

Under normal circumstances, the development of nodules in one region of the root inhibits the emergence of nodules in younger regions of the root (1, 5). Since infection threads still form in large numbers in these younger regions, the regulatory phenomenon has been called abortion of infection (1). However, the findings with mutants CE109 and CE113 suggest that if infection thread development per se were the only focus of inhibition, nodule structures such as that of Fig. 4 should result. Such is not normally the case. This reasoning and the careful observations of Calvert et al. (5) of this phenomenon in soybean imply that cortical cell division is also a target, or perhaps the sole target, of the regulation. In addition, since nodules with these mutants were distributed in equivalent density throughout the root system, nodule development beyond the initial stages allowed by the mutations must be required to trigger the inhibition of nodulation in younger regions of the root.

Although the onset of meristematic activity in the root cortex and infection thread initiation and growth are usually coincident, they may be regarded as two separable processes. Meristem formation and nodule growth may occur without infection, or with only a limited infection, as demonstrated by a growing number of studies with mutant rhizobia. Even with wild-type inoculation, limited cortical cell division can be induced by the bacteria without concomitant infection threads (5). These two processes are effected by distinct sets of bacterial determinants, although the sets may overlap. The bacterial genes governing the onset of cortical cell division, including the so-called *nod* genes (11, 12, 15, 31), have been found in fast-growing rhizobia only on the Sym plasmid (28, 29a). Bacterial genes influencing infection thread initiation or persistence, but unnecessary for meristem induction, have been found only on the chromosome (13a, 29) or a plasmid distinct from the Sym plasmid (17, 23).

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