Cytological Evidence for a Host Defense Response That Reduces Cell and Tissue Invasion in Pea Nodules by Lipopolysaccharide-Defective Mutants of *Rhizobium leguminosarum* Strain 3841

S. Perotto,[1] N. J. Brewin,[2] and E. L. Kannenberg[3]

[1]Università di Torino, Dipartimento di Biologia Vegetale, Viale P. A. Mattioli 25, I-10125 Torino, Italy, [2]John Innes Institute, Colney Lane, Norwich NR4 7UH, Great Britain, and [3]Universität Tübingen, Mikrobiologie I / Botanik, Auf der Morgenstelle 1, D-7400 Tübingen, Germany

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Mutants of *Rhizobium leguminosarum* bv. *viciae* with modifications in the structure of their lipopolysaccharide (LPS) macromolecules induce ineffective pea root nodules showing little or no capacity for symbiotic nitrogen fixation. The development of these nodules was investigated by the use of cytochemical techniques and monoclonal antibodies to monitor the progress of plant-microbial cell surface interactions. LPS-defective mutants were only partially successful in colonizing the nodule tissue and were released into only a minority of the host cells. Tissue and cell invasion by *Rhizobium* was often associated with a host plant defense reaction characterized by the accumulation of intercellular matrix material and possibly callose, secondary cell wall modification, and sporadic cell death. The severity of the host defense response was correlated with the degree of LPS structural modification shown by the various mutants examined. Furthermore, LPS-defective mutants released into host plant cells differentiated into abnormally swollen and elongated bacteroids, apparently defective in bacteroid cell division and in synchronized division of the plant-derived peribacteroid membrane. These observations suggest an essential role for *Rhizobium* LPS in the avoidance of host cell defense reactions during the invasive phase of nodule development. The molecular structure of LPS also affects the nature of bacteroid development during the endosymbiotic phase of nitrogen-fixing bacteroids.

Additional keywords: infection thread; pathogenesis; *Pisum sativum*; plant-microbe interactions; symbiosis

The soil bacterium *Rhizobium leguminosarum* can infect legume roots and induce symbiotic root nodules, in which rhizobia fix atmospheric nitrogen and furnish it as ammonia to the host plant cells (Long 1989). Nodule formation is initiated by an exchange of pre-infection signals between the host plant and *Rhizobium*, which leads to root hair deformation and cortical cell division in the immature region of the legume root (Brewin 1993). While the dividing cells lay down the future nodule tissue, the bacterial cells interact with plant cell surfaces and gain access to host plant tissue. The rhizobia are guided to the emerging nodule tissue via a specialized tunnel, the infection thread, whose cylindrical wall is laid down by the plant. Bacteria are released into nodule tissue cells by endocytosis and come to occupy an organelle-like compartment, termed a symbiosome. The bacteria inside these structures continue to proliferate and develop into pleomorphic, nitrogen-fixing bacteroids (Brewin 1991). In nodules of pea and *Vicia* spp., there is an uninfected apical meristem, and this indeterminate meristem subsequently gives rise to all the tissues of the nodule. Proximal to the meristematic zone is an invasion zone where ramifying infection threads initiate a continual process of cell invasion and symbiosome development. Other legumes, such as French beans (*Phaseolus vulgaris*), have nodules with determinate meristems and a somewhat different morphology and ontogeny. In these spherical nodules, tissue invasion often depends on a relatively short phase of infection thread growth, and the central infected tissue of the nodule is formed mainly by continued proliferation of infected host cells and the bacteria within them (Rolfe and Gresshoff 1988; Rae *et al.* 1992). These delicately balanced plant-microbial interactions, which are normally sustained for several weeks, imply that rhizobia grow and divide within plant tissue and inside nodule cells without being restricted by plant defense mechanisms.

Bacterial surface polysaccharides are crucial for many commensal and pathogenic relationships involving animals (Costerton and Irvin 1981; Nikaido and Vaara 1985; Raetz 1990) and plants (Coplin and Cook 1990; Long and Staskawicz 1993). Genetic analysis has also shown that bacterial surface polysaccharides are required for normal development of the *Rhizobium*-legume symbiosis (Sobral *et al.* 1991), but no clear function has yet been described for any of the different classes of surface polysaccharides (Sobral *et al.* 1991; Noel 1992). Lipopolysaccharide (LPS) comprises a class of abundant macromolecules that are firmly integrated in the outer leaflet of the outer membrane through their lipid A component (Carlson *et al.* 1992). Conceptually, LPS is subdivided into two classes of macromolecules. LPS-1 molecules are built from lipid A, a core oligosaccharide, and the O-

Corresponding author: N. J. Brewin. Telephone +44-603-52571. Fax +44-603-56844

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antigen polysaccharide, which comprises variable numbers of a strain-specific oligosaccharide repeating unit. LPS-2 molecules, on the other hand, lack the strain-specific O-antigen (Carlson *et al.* 1992). An important role for *Rhizobium* lipopolysaccharide in legume symbiosis is suggested by the fact that mutants with major modifications in the structure of LPS macromolecules cannot establish normal nitrogen-fixing nodules (de Maagd *et al.* 1989; Priefer 1989; Kannenberg *et al.* 1992). The phenotype of LPS-defective *Rhizobium* mutants depends to some extent on whether the host legume forms indeterminate or determinate nodules. In the case of *R. leguminosarum* bv. *trifolii* and bv. *viciae*, which form indeterminate nodules on clover and pea, respectively, the mutants proceed through infection threads and are eventually released into host cells, so that gross nodule morphology is relatively normal (Priefer 1989). However, in the case of *Bradyrhizobium japonicum* and *R. leguminosarum* bv. *phaseoli*, which form determinate nodules on soybean and *Phaseolus* bean, respectively, the mutants fail in the formation of proper infection threads at an early stage of nodule initiation (Noel *et al.* 1986; Stacey *et al.* 1991): in these cases the meristematic cells of these induced nodule-like structures are therefore not invaded by rhizobia. These morphological differences are caused by differences in ontogeny between determinate and indeterminate nodules, and they may also reflect differences in the size, structure, and development of infection threads in these different classes of legumes (Rae *et al.* 1992).

In order to relate structure to function of rhizobial LPS in symbiosis, several chemically induced and transposon-induced LPS mutants were characterized biochemically and immunochemically in this laboratory (Wood *et al.* 1989; Kannenberg *et al.* 1992). Here, we attempt to analyze the role of *Rhizobium* LPS in sustaining a symbiotic rather than a pathogenic form of plant-microbe interaction by describing the development and the cytological features of ineffective pea nodules induced by representative classes of the LPS-defective mutants. Our results indicate that the development and ramification of infection threads is severely affected by modifications in the LPS macromolecules of invading rhizobia. Nodules induced by these LPS-defective mutants showed evidence of a relatively strong host defense response at all stages of tissue and cell invasion. Furthermore, the intracellular bacteroids showed gross abnormalities in size and shape, compared with those of the wild-type strain.

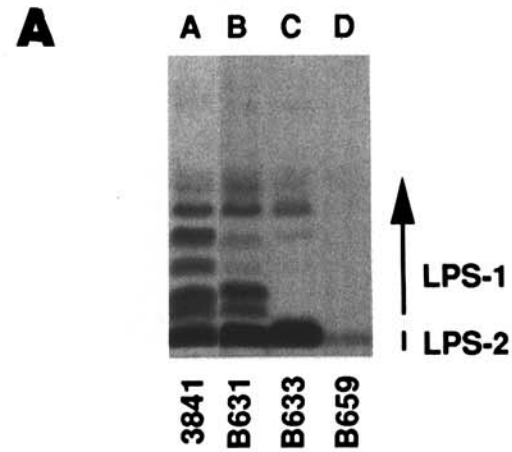
Table 1. *Rhizobium leguminosarum* strain 3841 and mutant derivatives

Strain number and mutant class	Derivation	Phenotype	Ref.[a]
3841, wild type	300 *str*, biovar *viciae*	Fix^+	(1)
B631, class I	3841 *lps* mutant (NTG)	Fix^-	(2)
B661, class I	3841 *lps*::Tn*5*	Fix^-	(2)
B633, class II	3841 *lps* mutant (NTG)	Fix^-	(2)
B660, class II	3841 *lps*::Tn*5*	Fix^-	(2)
B659, class III	3841 *lps*::Tn*5*	Nod^+, Inv^-	(3)

[a] (1) Wood *et al.* 1989; (2) Kannenberg *et al.* 1992; (3) Rae *et al.* 1991. NTG refers to mutants isolated after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

RESULTS

Three classes of symbiotically defective LPS mutants were examined in this study (Table 1), and their LPS compositions were compared after electrophoresis of bacterial samples on polyacrylamide gels (Fig. 1A). Compared with *R. l.* bv. *viciae* strain 3841, which carries wild-type LPS, mutants B631, B658, B665, and B661 (representatives of class I mutants)



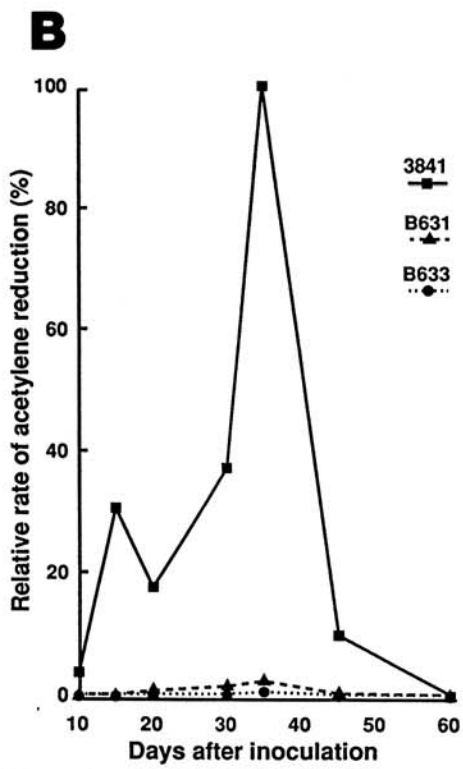


Fig. 1. Comparison of wild-type *Rhizobium leguminosarum* bv. *viciae* strain 3841 and representative lipopolysaccharide-mutant derivatives. **A**, Analysis of lipopolysaccharides following polyacrylamide gel electrophoresis and carbohydrate-specific periodate silver staining. (Strain B661 gave a gel profile identical to that of strain B631, and strain B660 was indistinguishable from strain B633). **B**, Rates of acetylene reduction activity of the wild-type strain 3841 and of mutants B631 and B633. Each value is the mean of four determinations expressed as a percentage of the maximum value observed in the wild-type strain 3841 (equivalent to 2.6 μmole of reduced C_2H_2 per plant and hour).

showed a defective form of the slow-migrating species LPS-1; class II mutants, such as B633, B654, B657, and B660, largely or completely lacked the LPS-1 species; class III mutant B659 lacked the major LPS-1 bands and also showed abnormalities in the fast-migrating species LPS-2. Further characterization of LPS of many of these mutants was given in a previous study (Kannenberg *et al.* 1992). Plant inoculation tests revealed that all the class I and class II mutants were basically similar, resulting in nodules that developed slowly and never attained a level of nitrogenase activity (acetylene reduction activity) greater than 5% of that of the wild-type strain (Fig. 1B). A cytological survey was conducted on several pea nodules infected with each of these nine LPS-defective mutants. Because all the class I and class II mutants were basically similar, strain B631 was taken as representative of this group and is used here for detailed cytological illustration. The phenotype of the class III mutant, B659, however, was completely different, with very small nodule-like structures and no evidence of tissue invasion by the inoculant strain.

A longitudinal section of a pea root nodule induced by strain 3841 (carrying wild-type LPS) was stained with toluidine blue to reveal the anatomy of a normal nodule (Fig. 2A). Three weeks after inoculation, most cells of the central nodule tissue were colonized by bacteria. Proximal to the uninfected apical meristem, a zone of cell invasion was succeeded by a zone where infected cells differentiated and developed the capacity for symbiotic nitrogen fixation. A longitudinal section of a 4-week-old nodule induced by mutant strain B631 and stained with toluidine blue is shown for comparison (Fig. 2B) to illustrate the altered morphology of nodules caused by LPS mutants of classes I and II (Table 1 and Fig. 1). In these nodules, only a few cells were colonized by bacteria, and an uninfected parenchyma occupied a considerable part of the central nodule tissue. Also, starch was abundant in both infected and uninfected cells of the central nodule tissue and in the nodule parenchyma (data not shown). In contrast to nodules formed by the wild-type strain, those formed by LPS mutant strains lacked a clear sequence of developmental zones arising from the apical meristem. Nodules formed by all the class I and class II LPS mutant strains tested showed the phenotype illustrated here at some stage during their development, although the time-course of nodule development proceeded at different rates, being slower for the class II mutants.

In order to study the process of tissue invasion by bacteria, nodule sections were immunolabeled with monoclonal antibody MAC 265, which recognizes an intercellular plant matrix glycoprotein secreted by plant cells into infection threads, infection droplets, and intercellular junctions (VandenBosch *et al.* 1989a; Rae *et al.* 1991). A light micrograph of the apical region of a wild-type nodule immunolabeled with MAC 265 (Fig. 3A) shows the normal appearance of infection threads and droplets formed during invasion by wild-type rhizobia. The presence of rhizobial cells within these structures was verified either by immunolabeling with the LPS-specific monoclonal antibody MAC 57 or by ultrastructural analysis of an adjacent serial section. When a longitudinal section from a nodule induced by the class II mutant B633 was immunostained with MAC 265, large intrusion structures were observed in the subapical region of the nodule tissue (Fig. 3B). These structures were apparently filled with matrix material recognized by MAC 265, and closer examination at the ultrastructural level revealed invading bacteria, embedded in abundant amounts of matrix material, either in expanded intercellular spaces or in large intracellular walled infection threads and membrane-bound infection droplets. A similar overproduction of MAC 265 matrix glycoprotein was observed in the nodules formed by all other class I and class II LPS-defective strains examined, although these structures were larger and more frequent in mutants lacking the LPS-1 forms altogether (i.e., in class II rather than class I mutants). The presence of bacteria in association with the plant matrix glycoprotein was demonstrated either by examining adjacent

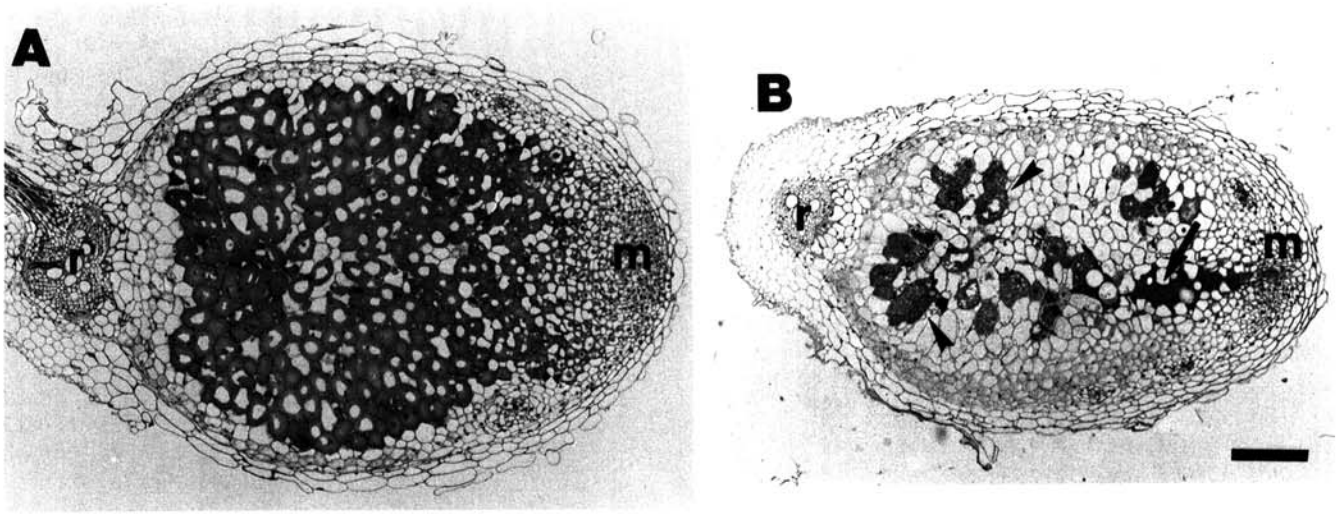


Fig. 2. General morphology of pea root nodules (longitudinal sections stained with toluidine blue). **A,** Nodule induced by the wild-type *Rhizobium leguminosarum* bv. *viciae* strain 3841 illustrates the normal phenotype 3 weeks after infection. The nodule is connected to the root (r), seen in transverse section in these micrographs. There is a clear progression of cell colonization from the uninfected apical meristem (m) to the symbiotic (nitrogen-fixing) zone. **B,** Nodule induced by lipopolysaccharide-defective strain B631, 4 weeks after infection. There are few infected cells containing bacteroids (arrowheads), large numbers of uninvaded cells, and a large invasion structure (arrow). Sections from the same pea nodule are analyzed further in Figures 4 and 5B. Scale bar = 0.5 mm.

sections under the electron microscope or, where possible, by immunostaining with MAC 57, which recognizes bacterial LPS (Fig. 4A).

To examine nodule anatomy in more detail for LPS mutants, adjacent longitudinal sections of nodule tissue were treated with different antibodies that monitor the distribution of individual bacterial or plant components. Following inoculation with LPS mutant B631, all bacteria present on a nodule longitudinal section could be visualized by immunostaining with MAC 57, an antibody that recognizes a constitutive LPS-1 epitope (Fig. 4A). Evidence that the bacteria were not reverting as a result of selection pressures inside the nodule came from the absence of staining with MAC 203 (Fig. 4B), which was consistent with the mutant LPS phenotype of strain B631. Bacteria in infection threads or related structures could be identified in nodule sections by their association with the intercellular plant matrix glycoprotein recognized by MAC 265 antibody (Fig. 4D). Similarly, bacteria that had been released into the cytoplasmic domain of nodule cells were revealed by light microscopy using immunolabeling with MAC 206 (Fig. 4E), a monoclonal antibody that recognizes a plant component on the peribacteroid membrane (Bradley *et al.* 1988; Perotto *et al.* 1991). Thus, by treating adjacent tissue sections from the same nodule with a range of antibody probes, all the bacteria present in the nodule tissue could be assigned to extracellular or cytoplasmic locations (Fig. 4). The degree of symbiotic integration between bacterial LPS mutants and host plant cells was further studied by using specific polyclonal antisera to analyze the accumulation of nodule-specific bacterial or plant products (nitrogenase or leghemoglobin, respectively) in adjacent nodule sections (Fig. 4C and F). Both proteins were expressed to some extent in the nodules induced by bacterial LPS mutants, although acetylene reduction activity for these nodules was always less than 5% of the values observed for nodules induced by the wild-type strain (Fig. 1B).

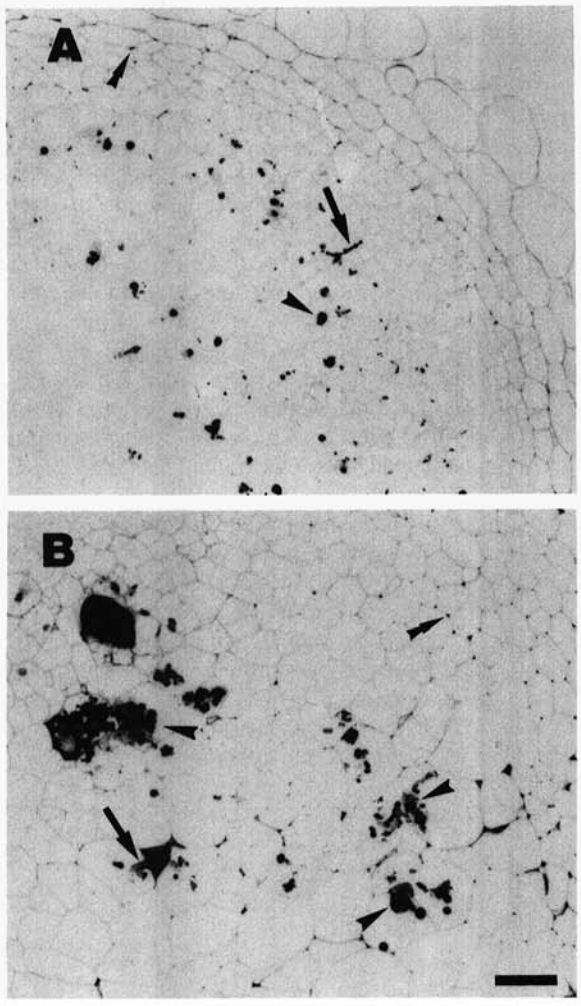


Fig. 3. Pea nodule sections stained in an immunogold silver enhancement procedure with MAC 265 antibody to illustrate the different amounts of plant matrix material accumulated in nodule tissues in response to infection by wild-type or mutant rhizobia. The tissue sections have not been counterstained, so that the black-and-white image represents the distribution of MAC 265 antigen, i.e., the plant matrix glycoprotein that is present in intercellular junctions and infection structures. The sections were taken from the subapical "invasion" zone of pea nodules containing wild-type *Rhizobium leguminosarum* bv. *viciae* strain 3841 (**A**) and lipopolysaccharide-defective strain B633 (**B**). The accumulation of MAC 265 antigen was identified in several nodule structures in **A**: infection threads (arrows), infection droplets (arrowheads), and uninfected intercellular junctions in the nodule cortex (double arrowheads). The nodule section containing strain B633 reveals several very large intercellular and intracellular deposits of MAC 265 matrix antigen (arrowheads). The base of a large infection thread is also visible (arrow), adjacent to a large intercellular space filled with MAC 265 antigen. Double arrowheads indicate antigen in intercellular spaces between the uninfected cells of the nodule cortex. Scale bar = 100 μm.

When serial semi–thin sections from numerous nodule samples were examined, the large amorphous invasion structure was frequently found traversing large areas of the central nodule tissue: this appeared to be a common feature of all nodules formed by class I and II LPS-defective strains (Figs. 5A and 6). Ultrastructural analysis of this invasion structure revealed the presence of matrix material embedding numerous bacteria (Fig. 5B). This material, which reacts with MAC 265 (Fig. 5C), was very similar in texture to the matrix material embedding bacteria inside infection threads. The analysis of nodules by serial sections also revealed that the large invasion structures extend outward to the surface of the nodule, either on the side or in the apical region (Figs. 5A and 6). This result was obtained repeatedly in several nodules formed by each of the different LPS-defective mutant strains. In this sequence of serial sections, it was often possible to trace the infection structure to a point on the nodule epidermis where a root hair cell shaped like a shepherd's crook contained a large infection thread (Fig. 6). These observations indicate that the large invasion structure is probably derived from the primary infection thread that conveys bacterial invasion into the root cortex from the infected root hair.

To test whether the unusual features of these infection structures are related to a plant defense response, nodule cryosections were stained with berberine and aniline blue to reveal callose or secondary metabolites in the walls of nodule cells. When nodules formed by the LPS mutants were observed under UV light, the cell walls surrounding the large invasion structures were brightly fluorescent, suggesting the presence of lignin-like compounds or suberin (Fig. 7). Some material, presumably callose, could be detected in the matrix filling these large threads, as detected by its UV fluorescence following treatment of tissue sections with aniline blue. No reactivity with berberine and aniline blue was found in the

infected tissue of nodules formed by the wild-type bacterial strain, apart from the suberized nodule endodermis. In particular, the absence of callose deposition in spreading infection threads formed by wild-type rhizobia is in agreement with previous findings on clover, where callose formation in response to wild-type rhizobia was stimulated only on the root hair surface but not along the subsequent route of infection (Kumarasinghe and Nutman 1977).

Ultrastructural analysis of infected nodule cells revealed clear differences between cell colonization by wild-type bacteria and that by LPS-defective bacteria (Fig. 8). From infection droplets that were very often fragmented, LPS mutant bacteria were released into the plant cytoplasm surrounded by the peribacteroid membrane. These released bacteria only partially filled the space within the nodule cell, the remaining space being occupied by a fragmented cell vacuole. These invaded host cells frequently showed clear signs of cytoplasmic disorganization, and the nuclei were often pleomorphic (Fig. 8B).

In contrast to wild-type bacteria, which are usually found in pea nodules as Y-shaped bacteroids individually enclosed by peribacteroid membrane (Fig. 9A), the bacteroids formed by LPS mutant bacteria were a more pleomorphic, highly branched shape and were larger (Fig. 9B). This finding was

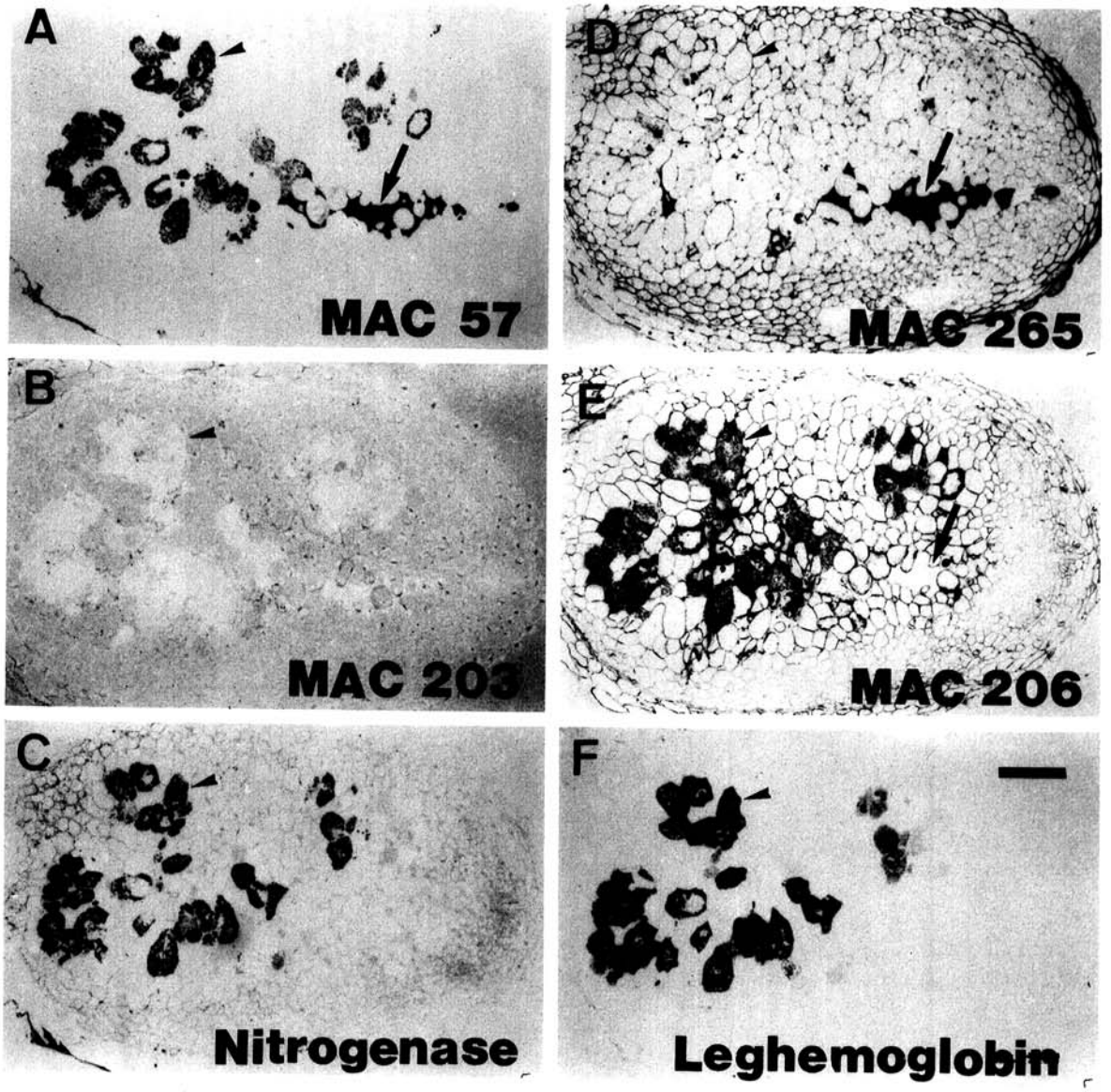


Fig. 4. Serial adjacent sections of a pea nodule containing lipopolysaccharide (LPS)–defective *Rhizobium leguminosarum* bv. *viciae* strain B631, taken from the nodule illustrated in Figure 2B. Each section was treated with a different antibody and stained in an immunogold silver enhancement procedure without counterstain to reveal the progress of symbiotic development with reference to three bacterial antigens (**A–C**) and three plant antigens (**D–F**): **A,** MAC 57 (recognizing constitutive LPS antigen); **B,** MAC 203 (LPS epitope, not expressed by mutant B631); **C,** nitrogenase; **D,** MAC 265 (intercellular matrix glycoprotein); **E,** MAC 206 (peribacteroid membrane and plasma membrane glycocalyx); and **F,** leghemoglobin. All bacteria present in **A** are visualized by MAC 57. An arrow indicates the presence of bacteria associated with a large invasion structure containing MAC 265 antigen (**D**). Bacteria released into the cytoplasm (i.e., bacteroids) are identified in adjacent serial sections (arrowheads) by their associated peribacteroid membrane (MAC 206 antigen in **E**), by the induction of nitrogenase (in **C**), and by the associated induction of leghemoglobin in the host cytoplasm (in **F**). Scale bar = 0.5 mm.

confirmed in squashed nodule preparations of the various mutants: the preparations contained, besides undifferentiated rod-shaped cells, a considerable proportion of abnormally swollen and elongated bacteroid forms. The usual Y-shaped bacteroid form was found rarely or not at all. To investigate the origin of this abnormal bacteroid morphology, earlier stages of bacteroid differentiation were examined in host cells in which endocytosis had only recently taken place: several bacteroids could be identified inside the same peribacteroid membrane envelope, even during the stage of active bacteroid cell division (Fig. 9D). The multiplicity of LPS-defective mutant bacteroids in each peribacteroid envelope was confirmed by the analysis of numerous samples of different LPS-defective mutants and by serial thin sectioning through infected nodule cells.

A comparison of the various nodule cells invaded by bacterial LPS mutants showed that they were very heterogeneous. Next to viable infected cells containing bacteroids, other cells were found with clear signs of cytoplasmic disorganization and collapse (Fig. 8B). Some rare instances of cell degeneration were also observed in nodules induced by wild-type rhizobia, but the proportion of infected cells that degenerated inside nodules formed by LPS-defective bacteria was much higher. Some of the degenerated cells observed in these nodules could be the result of early senescence, but degeneration was also found to occur in very early stages of nodule

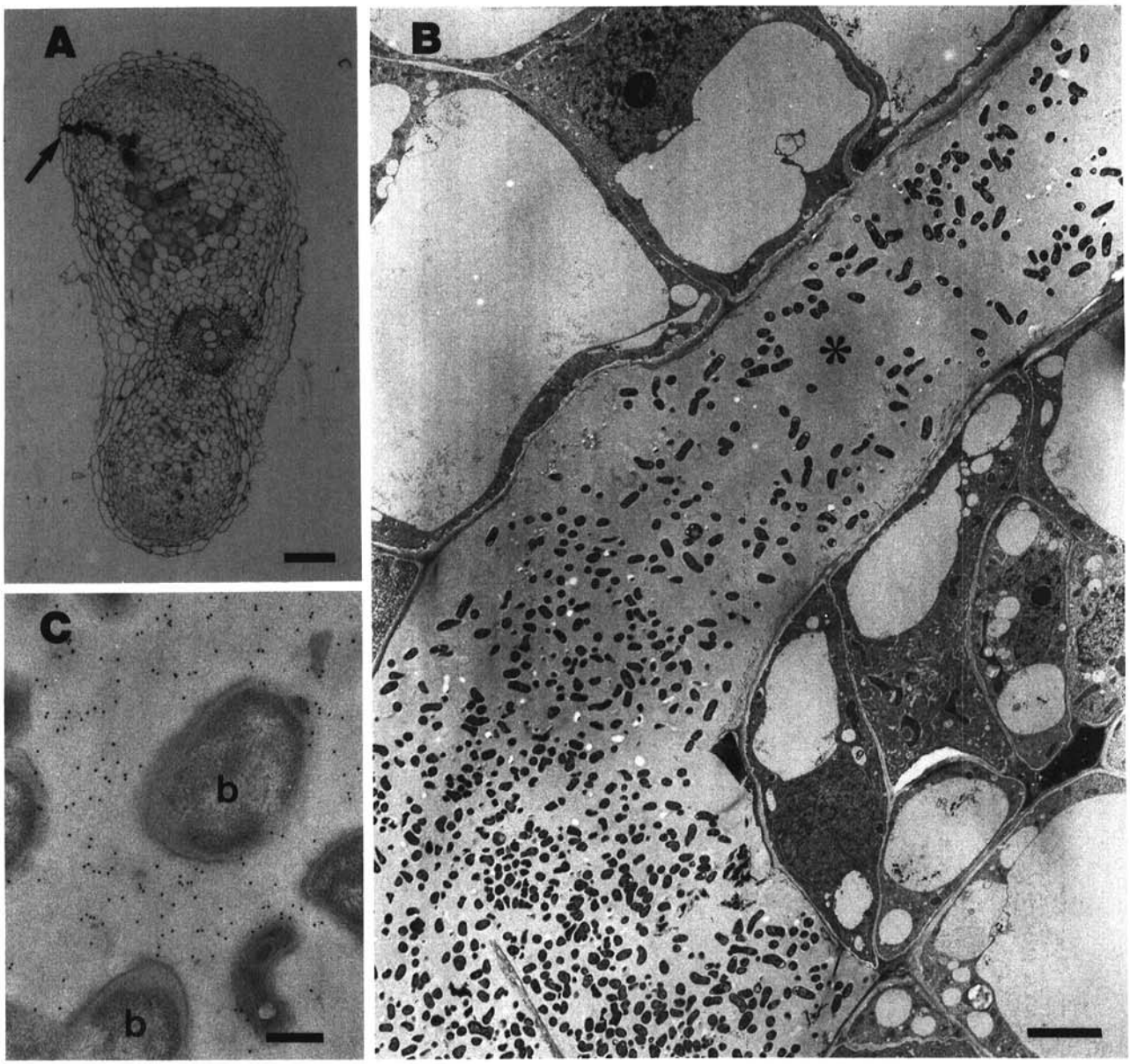


Fig. 5. A, B, Light and electron micrographs showing the large invasion structure formed in response to colonization by lipopolysaccharide-defective *Rhizobium leguminosarum* bv. *viciae* strain B631. **A,** Low-power light micrograph of a longitudinal section of a nodule: the arrow shows the point where the infection structure originates at the nodule epidermis (see also Fig. 6). **B,** Electron micrograph of the same structure, revealing an abundant amorphous matrix (asterisk) that embeds numerous bacteria. **C,** High-power electron micrograph of part of the matrix material from the invasion structure shown in **B,** following immunogold staining with MAC 265. Colloidal gold particles are associated with the embedding matrix, but not with the bacterial cells (b). Scale bars = 0.5 mm (**A**), 5 μm (**B**), and 0.2 μm (**C**).

development (less than 10 days from inoculation). The proportion of degenerated cells found in nodules formed by LPS mutants was related to the severity of the alterations in the LPS macromolecular structure: mutants with more profound modifications usually provoked more severe symptoms.

When nodules formed by LPS-defective strains were cryosectioned and observed directly under UV light, some of the infected nodule cells showed a bright autofluorescence (Fig. 10), indicating the presence of aromatic compounds in the host cytoplasm. This autofluorescence was not seen in comparable infected nodule tissue colonized by wild-type bacteria. Even for nodules containing LPS-defective bacteria, examination of the same nodule section with bright-field illumination showed that not all the infected cells were autofluorescent, again indicating a heterogeneity in the host response to cell invasion.

Inoculation of pea roots with the class III LPS-defective strain B659 (Table 1) resulted in the development of only a few small tissue proliferations. As previously described (Rae *et al.* 1991), the anatomy of these tissue proliferations was consistent with that of uninvaded nodule-like structures (Truchet *et al.* 1989). The protuberances induced by strain B659 were further analyzed for their morphological structure and for the presence of invading bacteria. Repeated attempts to stain bacteria with LPS-specific monoclonal antibody MAC 57 failed, despite the fact that this antibody reacts with B659 in free-living culture under all conditions tested (M. M. Lucas, unpublished observations). It was also not possible to detect infection threads by immunostaining the nodule sections with MAC 265 antibody, which is specific for a component of the infection thread matrix (VandenBosch *et al.* 1989a), although the matrix glycoprotein identified by this antibody was abundant in intercellular spaces (Rae *et al.* 1991). This study was further extended by the examination of serial semi–thin sections through the entirety of one of these tissue proliferations, but again no evidence was found for tissue or cell invasion by mutant B659.

DISCUSSION

The results indicate that mutations affecting the structure of the LPS macromolecule of *R. l.* bv. *viciae* strain 3841 have a

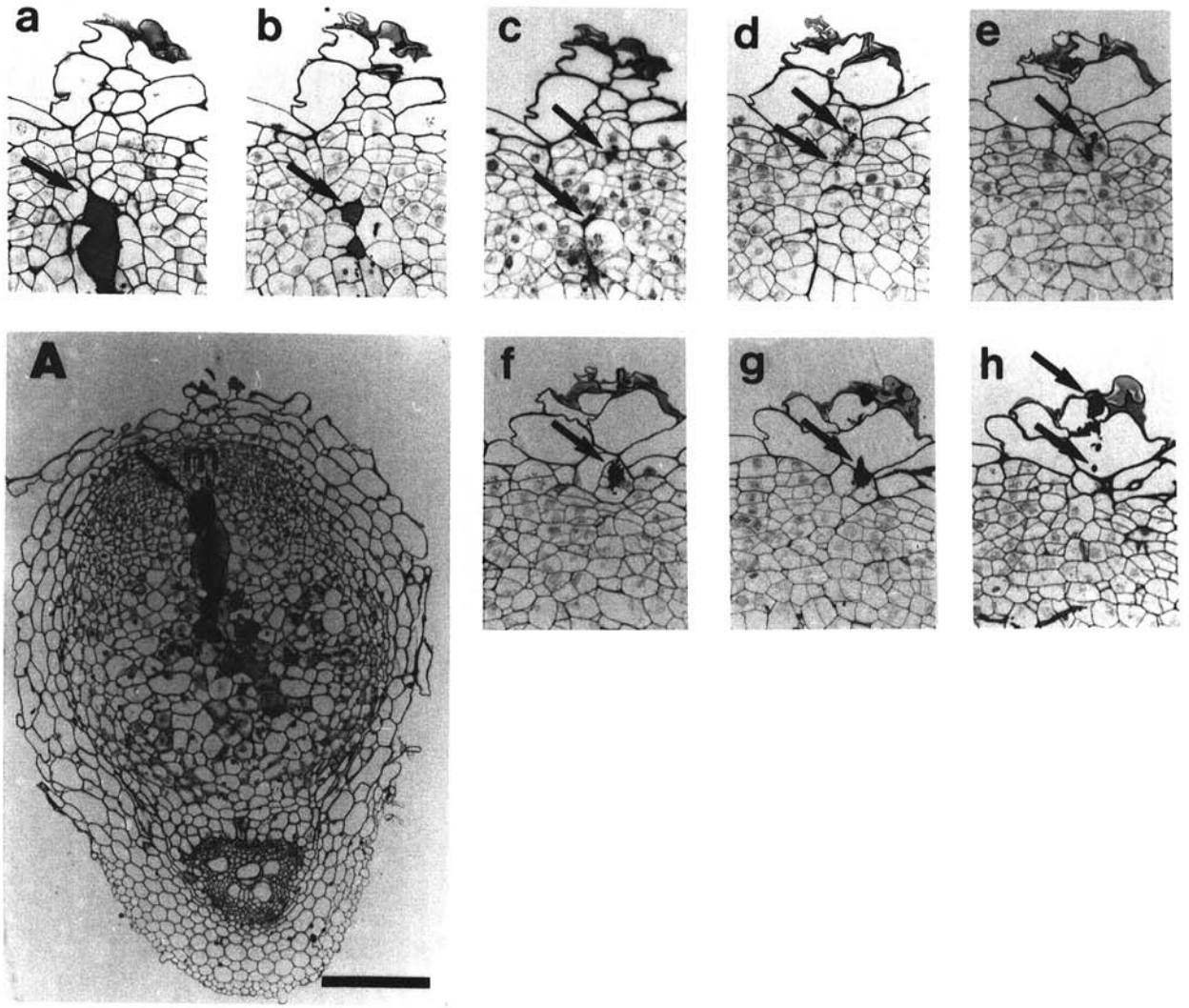


Fig. 6. Serial sections (**a–h**) derived from a pea nodule (**A**) colonized by lipopolysaccharide-defective *Rhizobium leguminosarum* bv. *viciae* strain B631, showing the path of an invasion structure (arrows) in the nodule tissue. Basic fuchsin was used for counterstaining, because it strongly stains the matrix material contained in the invasion structure. A curled root hair containing a large thread (arrow) is clearly visible in **h**. Scale bar = 0.5 mm.

severe impact on the progress of nodule cell colonization by rhizobia in pea plants (Fig. 2). This agrees with previous studies (Priefer 1989; de Maagd *et al.* 1989) describing the phenotype of *Vicia* nodules formed by some LPS-defective mutants: it was always observed that a smaller number of infected host cells was present, with only a few bacteria released from the infection threads. In the present study, plant tissue invasion by LPS mutants of class I and II (Table 1) induced a range of phenotypes that indicated the presence of a host cell defense response. The main features are the overproduction of intercellular plant matrix material (Figs. 3 and 4) and probably also callose (Fig. 7), the incorporation of aromatic compounds into the cell walls surrounding sites of bacterial invasion (signifying lignification or suberization of these walls), the production of aromatic metabolites within host cells, and sporadic plant cell death (Figs. 8–10). In pea root nodules, the colonization of plant cells by rhizobia relies on the active growth and branching of transcellular infection threads, which deliver the bacteria to the newly formed cells derived from the apical meristem. If the plasticity of the infection thread wall is decreased by the apposition of secondary metabolites such as lignins, both growth and branching

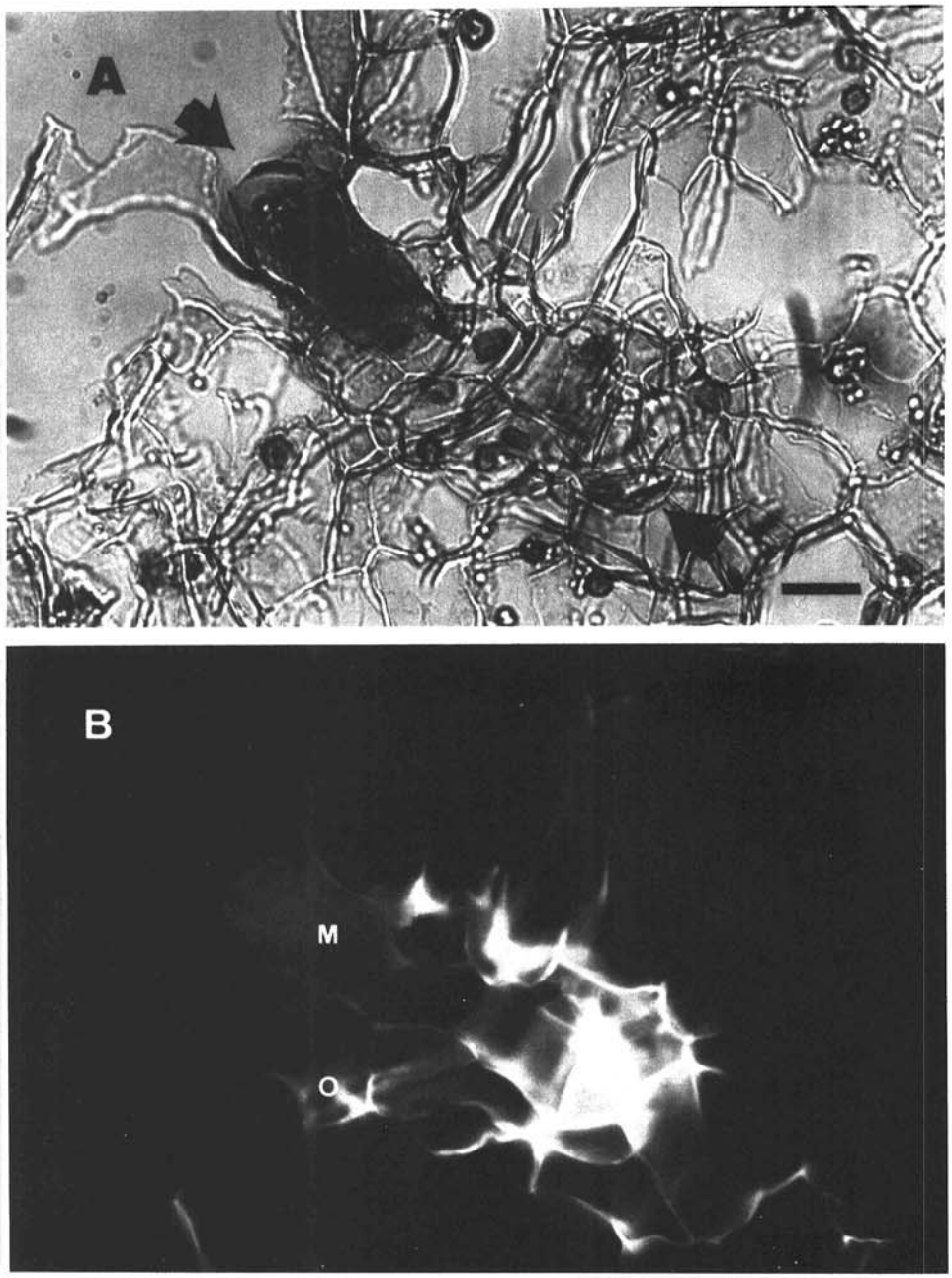


Fig. 7. Cryosection of part of a pea nodule containing lipopolysaccharide-defective *Rhizobium leguminosarum* bv. *viciae* mutant strain B633 stained with berberine and aniline blue. **A,** Bright-field image showing the position of a large invasion structure (between arrows). The nuclei of cells surrounding this structure are stained with aniline blue dye. **B,** The same section observed under UV light. A blue fluorescence, possibly representing callose, is visible on the amorphous matrix (m) of the invasion structure. There is also a yellow fluorescence (o), indicating the presence of lignin or suberin (or both) on the cell walls of nodule cells surrounding the large invasion structure. Scale bar = 100 μm.

would be profoundly affected, and the number of nodule cells reached by infection threads would be less than normal (Fig. 2). Therefore, the abnormal infection threads and the impaired tissue invasion in nodules induced by bacterial LPS-defective mutants may be the consequence of a plant defense response triggered by a mutant with defective LPS macromolecules.

The involvement of LPS mutations in provoking host defense responses could be explained in several ways. One possibility is that the region of the LPS macromolecule that is modified or absent in these LPS-defective mutants may normally contain a positive signal needed for recognition by the plant to promote the development of a symbiotic rather than a pathogenic interaction (Raetz 1993). Another possibility is that the O-antigen polysaccharide of the LPS-1 molecule may be needed to shield the core region of the bacterial LPS macromolecule, which may otherwise be a determinant for pathogenicity and cause a defense response when it comes into contact with the plant cell surface. This hypothesis is supported by the observation that LPS mutants that retain the ability to polymerize a long O-antigen polysaccharide chain induce nodules with a normal phenotype (Kannenberg *et al.* 1992) and also by the fact that mutants unable to synthesize an extracellular polysaccharide fail to establish infection threads (Borthakur *et al.* 1986; Leigh and Coplin 1992), suggesting a possible masking effect of a capsular sheath that might prevent contact between LPS core components and plant components that respond to invading pathogens by eliciting a host defense response. An alternative class of possibility to explain the fact that LPS-defective mutants induce a host defense response is that the effect of altered LPS structure could act indirectly to release or expose a metabolite that elicits the host defense response. On this model, the LPS-defective cells might become leaky or otherwise physiologically stressed because of impaired cell wall integrity (Nikaido and Vaara 1985).

In order that LPS can function as a signal molecule that controls the nature of the host cell response to microbial invasion, it must be detachable from the bacterial cell surface and capable of diffusion to host cell membranes. LPS could then act as a signal molecule either (positively) promoting symbiotic interaction or perhaps (negatively) eliciting a host defense response under certain conditions. Such a model is possible for several reasons. 1) LPS is released into the supernatant fluid by rhizobial cells in culture (Carlson and Lee 1983; Dazzo *et al.* 1991; M. M. Lucas, personal communication). 2) LPS was identified immunologically in the luminal matrix of infection threads in *Vicia* nodules (Goosen–de Roo *et al.* 1991), and this is confirmed for pea nodules by our own unpublished observations. 3) The molecular structure and amphiphilic nature of LPS is somewhat similar to that of the well-described lipooligosaccharide signal molecules secreted by *Rhizobium* (Lerouge *et al.* 1990). 4) Lipopolysaccharide components (such as endotoxin) have long been recognized as elicitors of host defense reactions in animal cells (Raetz 1990).

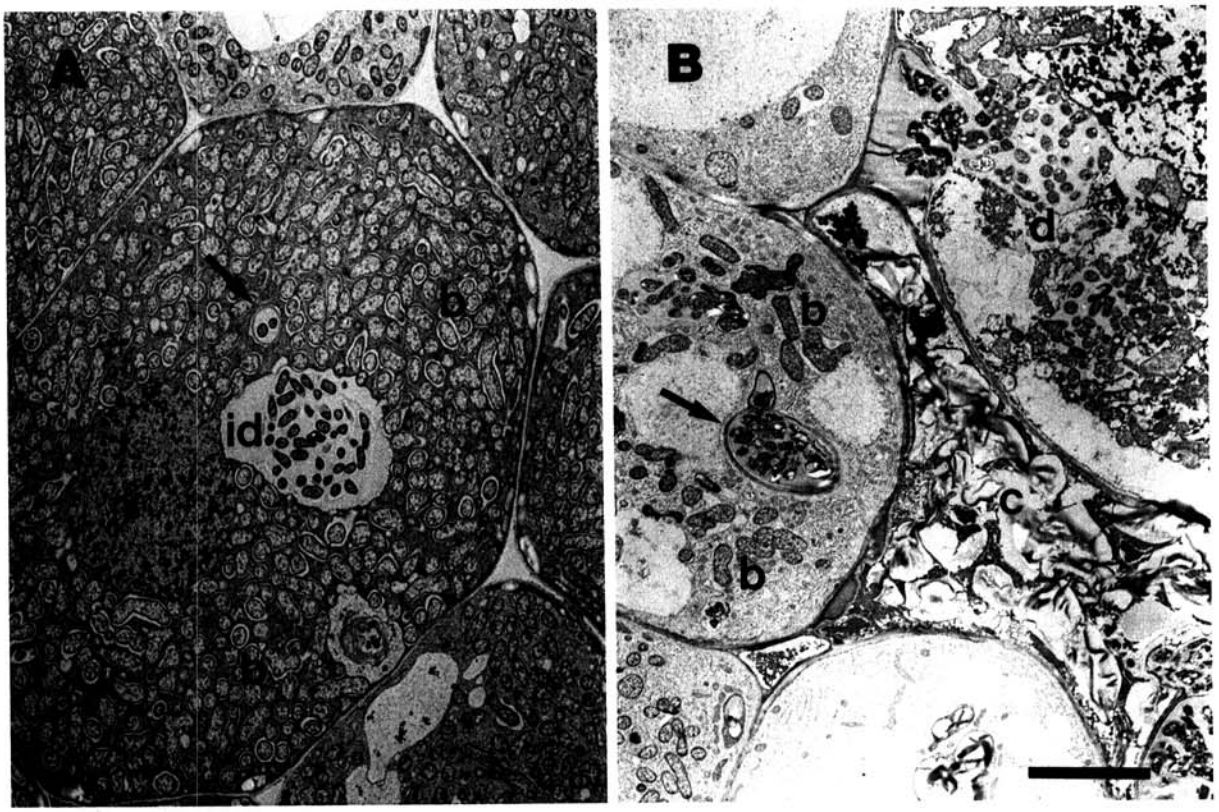


Fig. 8. Ultrastructure of nodule cells invaded by wild-type *Rhizobium leguminosarum* bv. *viciae* strain 3841 **(A)** and lipopolysaccharide-defective mutant strain B631 **(B)**. Cells with clear signs of cytoplasmic disorganization (d) and cell collapse (c) are often visible in nodules colonized by a lipopolysaccharide-defective strain. Arrows indicate infection threads surrounded by a sheath of plant cell wall material. Also visible are bacteroids (b) and an infection droplet (id). Scale bar = 5 μm.

It has recently been demonstrated by *in situ* hybridization that pea nodules invaded by LPS-defective mutants express genes encoding chalcone synthase (Yang *et al.* 1992), an enzyme involved in the production of flavonoids. Flavonoids play a double role in plant-microbe interactions in legumes (Wingender *et al.* 1989; Peters and Verma 1990). They are part of the early signals that induce in rhizobia the expression of the nodulation genes, but they are also phytoalexins produced in response to pathogens. The observation that LPS-defective mutants trigger chalcone synthase expression would therefore support the interpretation of the plant phenotype described here as being the product of a form of host defense response. Similarly, induction of chalcone synthase activity is sometimes observed with other classes of symbiotically defective mutant (Grosskopf *et al.* 1993).

The most severely LPS-defective mutant, strain B659 (class III) (Table 1), could induce small nodule-like structures on pea roots but apparently could not invade the newly formed nodule tissue (Rae *et al.* 1991). This phenotype of empty (uninvaded) nodules has not been observed previously for LPS mutants of pea or any other legume giving indeterminate nodules. Mutant B659 was induced as a result of a single transposon-derived mutation that is suppressed by the introduction of a cosmid clone carrying chromosomal DNA (E. L. Kannenberg, unpublished results). On the basis of immunological analysis and banding pattern on polyacrylamide gels, B659 has been described as lacking the LPS-1 forms of the LPS macromolecules and showing defects in the LPS-2 forms (Fig. 1B) (M. M. Lucas, personal communication). The failure to invade pea root tissues has previously been recog-

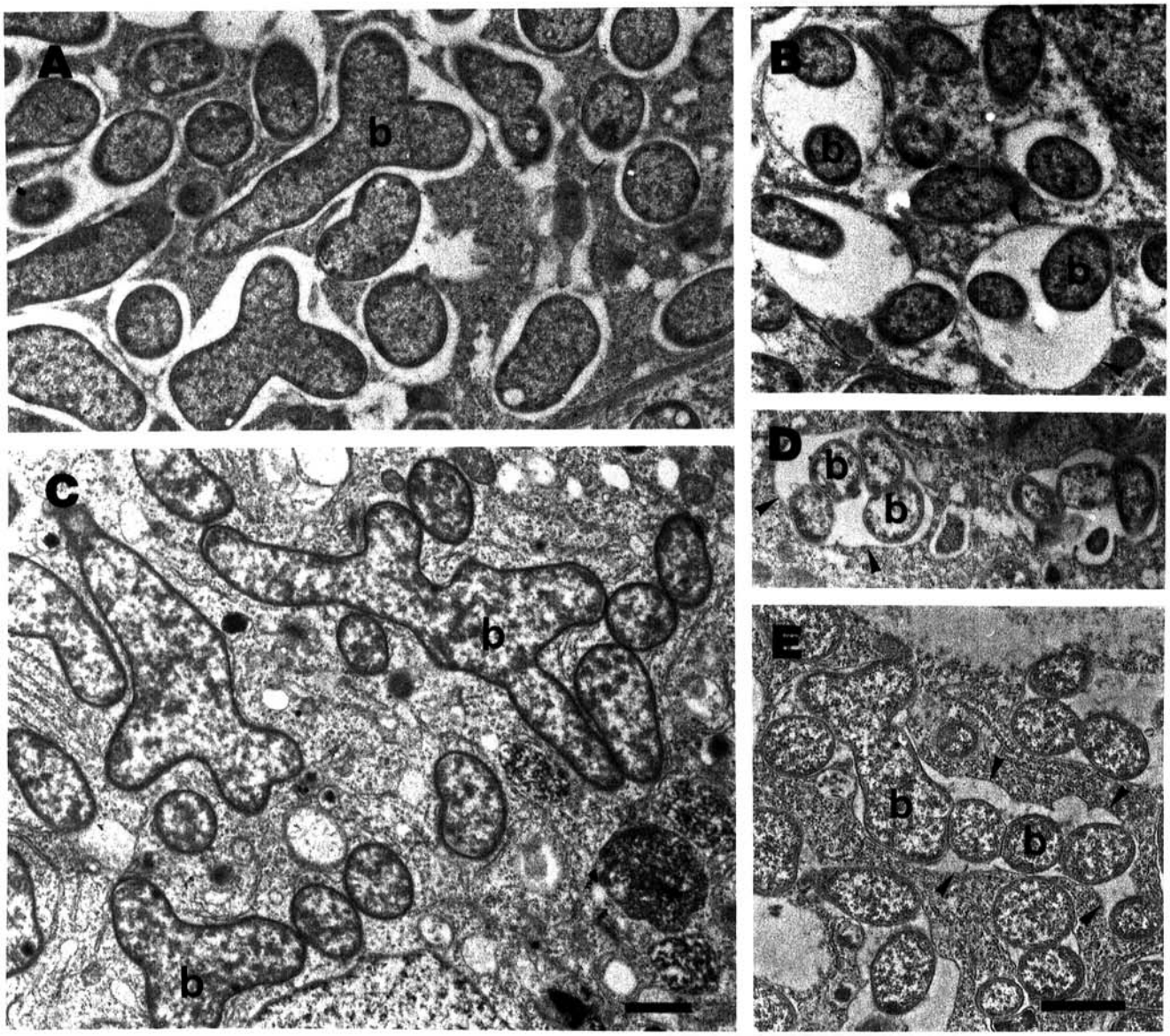


Fig. 9. Electron micrographs showing the development of bacteroids in wild-type *Rhizobium leguminosarum* bv. *viciae* strain 3841 (**A, C**) and lipopolysaccharide (LPS)–defective strain B631 (**B, D, E**). **A,** Mature wild-type bacteroids (b). **B,** Mature LPS-defective bacteroids: enlarged and highly branched bacteroids formed by strain B631. **C,** Early stage of wild-type bacteroid development, showing no more than two wild-type bacteroids per symbiosome for strain 3841; **D, E,** Early stage of LPS-defective bacteroid development, showing greater numbers of bacteroids enclosed within peribacteroid membranes. Serial thin sections (data not shown) revealed that the LPS-defective bacteria form chains of unseparated cells enclosed in a single symbiosome compartment. Arrowheads indicate the position of the peribacteroid membrane. Scale bars = 100 μm.

nized as a characteristic of *R. leguminosarum* mutants lacking acidic extracellular polysaccharides (Borthakur *et al.* 1986), but preliminary studies indicate that this compound and cyclic β-1,2-glucans are still synthesized by B659 (data not shown). Moreover, the inability of exopolysaccharide (EPS) mutants of rhizobia to invade the plant tissues can often be rescued by the external addition of purified EPS (Djordjevic *et al.* 1987) or by co-inoculation with nonnodulating bacterial mutants lacking the symbiotic plasmid but producing normal EPS (Muller *et al.* 1988). However, the co-inoculation of strain B659 with nonnodulating strains of *R. l.* bv. *viciae* did not significantly facilitate tissue invasion by strain B659 (data not shown). One class of explanation for these observations would be that strain B659 could fail to colonize plant tissue as a result of a pleiotropic effect of the mutation on cell fitness, somewhat similar to the effect of mutations affecting β-1,2-glucan synthesis of *R. meliloti; ndv* mutants of that species were reported osmotically unstable and unable to invade alfalfa nodules (Quandt *et al.* 1992). Alternatively, these observations suggest that strain B659, because of its LPS defect, may provoke a host defense response that prevents any significant degree of tissue and cell invasion. Therefore, further analysis of the LPS of strain B659 could help to distinguish the molecular basis of a pathogenic rather than a symbiotic form of host-microbial interaction. It seems noteworthy that recently plant defense reactions were also suggested to prevent EPS-deficient rhizobia from entering alfalfa nodules (Niehaus *et al.* 1993), indicating that EPS and LPS may have somewhat similar roles in preventing host defense reactions.

At the stage in nodule development when rhizobia are released into host cells, the composition of the lipopolysaccharide changes (Kannenberg and Brewin 1989; Sindhu *et al.* 1990; Tao *et al.* 1992) and the expression of genes involved in the production of EPS ceases (Latchford *et al.* 1991). Moreover, because the peribacteroid membrane does not synthesize an associated plant cell wall, LPS macromolecules apparently come into more direct contact with the plant cell surface membrane (Rae *et al.* 1992). Wild-type bacteria normally multiply in phase with divisions of the plant membrane envelope and, even during the stage of most active division of bacteroids, no more than two bacteria are found in the same envelope in clover (Robertson and Lyttleton 1984) and pea (Fig. 9C). In contrast, the division of LPS mutants after their release leads to the accumulation of several bacteria packaged in a single symbiosome compartment (Fig. 9D). These bacteria develop into very swollen and abnormally branched bacteroids (Figs. 9B and D). The presence of multiple bacteria in a single symbiosome is not normal for wild-type pea nodules, although it has been described for a Fix^- mutant pea line (Borisov *et al.* 1993). The mechanisms that drive synchronous division of bacteria and the plant membrane during the multiplication of symbiosomes in pea nodules are still unknown. However, attachment of the bacterial surface to the plant membrane is often observed within pea symbiosomes containing wild-type bacteria. This physical adhesion has been postulated to be involved both in the internalization of bacteria and in the synchrony between bacterial division and the peribacteroid membrane division (Robertson and Lyttleton 1984; Bradley *et al.* 1986; Brewin 1990). If LPS macromolecules are involved in the attachment of the bacterial outer membrane to the plant membrane, a modification in the LPS structure may weaken this interaction, leading to the uncoupling of bacterial division from the segregation of bacteria into separate symbiotic compartments.

The abnormal morphology of bacteroids derived from LPS-defective mutants suggests that structural modifications of the LPS macromolecule may have a direct effect on the stability of the outer membrane, which in turn might affect the survival and viability of bacteroids in the intracellular environment. This hypothesis is consistent with previous observations that nodule bacteria modify the structure of their LPS macromolecules during their life cycle within the host plant (Kannenberg and Brewin 1989; Sindhu *et al.* 1990; Tao *et al.* 1992). Thus, if bacterial LPS-defective mutants cannot adjust their LPS structure to the microenvironmental conditions of the symbiosome compartment, this might lead to a less stable bacteroid envelope and might be the cause of the abnormal morphology observed.

Finally, it should be emphasized that although the *Rhizobium*-legume interaction is often described as being symbiotic, there are many instances in which this is not the case and

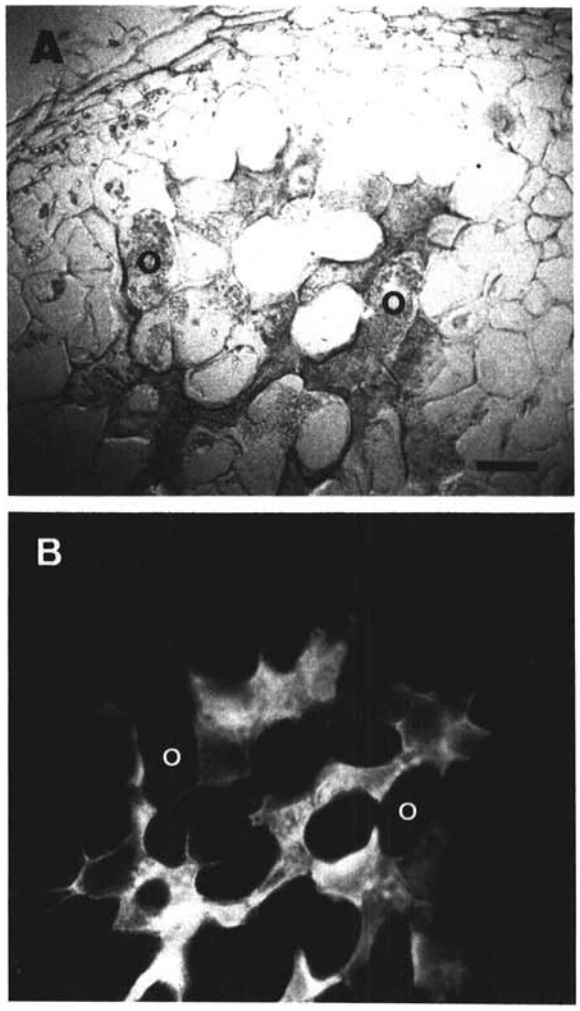


Fig. 10. A, Bright-field micrograph of a cryosection from a nodule containing *Rhizobium leguminosarum* bv. *viciae* strain B633, illustrating the distribution of infected cells. **B,** The same section photographed under UV light shows autofluorescence of many infected cells, which indicates the presence of aromatic compounds. Some of the infected cells (o) are not autofluorescent. Scale bar = 100 μm.

in which host cell responses are observed that are similar to those observed in pathogenic interactions: 1) There are cases in which the host-*Rhizobium* interaction fails to develop into nitrogen-fixing symbiosis because of mutational modifications in the symbiotic partners involved, as in symbiotically defective plant mutants, in which premature nodule senescence is often observed (Kneen *et al.* 1990). Similarly, symbiotically defective *Rhizobium* mutants, e.g., polysaccharide-defective or nitrogenase-defective mutants associated with wild-type hosts, induce nodules with aberrant invasion (Noel 1992) or premature bacteroid senescence (Ma *et al.* 1982, Parniske *et al.* 1991). 2) There are also cases in which the host-*Rhizobium* interaction does not lead to nitrogen-fixing bacteroids despite the fact that the symbiotic partners are wild-type. This is the case when wild-type rhizobia associate with an inappropriate host; for example, *R. l.* bv. *viciae* (which nodulates peas) forms ineffective nodules on *Phaseolus* spp. and *Trifolium subterraneum* (Hrabak *et al.* 1985; Huang *et al.* 1993; Salzwedel and Dazzo 1993). Furthermore, during the colonization of alfalfa roots by wild-type strains of *R. meliloti*, a high percentage of infection threads are arrested in the subepidermal layers of the root cortex, because of a process resembling a localized host cell defense response (Vasse *et al.* 1993), and tissue invasion by *Rhizobium* is further restricted if the host plants are grown with an adequate supply of nitrate or are already abundantly nodulated (Rolfe and Gresshoff 1988). Thus, the activation of host defense responses is already occurring with wild-type rhizobia, and its increased activation by bacteria carrying altered LPS macromolecules may represent an accentuation of this phenomenon (Djordjevic *et al.* 1987), in which the LPS defect merely serves to change the balance between a symbiotic and a pathogenic interaction.

MATERIALS AND METHODS

Plant inoculations.

Pea seedlings (*Pisum sativum* L. cv. Wisconsin Perfection) were grown as previously described (Sindhu *et al.* 1990) and inoculated with strain 3841, a Str^r derivative of field isolate *R. l.* bv. *viciae* strain 300 (Brewin *et al.* 1985), or with a mutant derivative of strain 3841. Nodules were harvested for cytological analysis 1–4 weeks after inoculation. Other nodules from the same plants were surface-sterilized in sodium hypochlorite (bleach) for 1 min and used for the reisolation of nodule bacteria to confirm strain identity (Brewin *et al.* 1986): the reisolated clones were tested for antibiotic resistance markers and also used to re-inoculate pea seedlings to test for continued expression of the mutant phenotype. For measurements of acetylene reduction activity, nodulated roots were taken from four plants per time point and tested as described (Johnston and Beringer 1975). The relative amount of acetylene reduction was established as a percentage of the maximum amount of acetylene reducing activity observed in the wild-type strain.

Table 2. Rat monoclonal antibodies used in this study

Designation	Isotype	Antigen recognized[a]	Ref.[b]
AFRC MAC 57	Rat IgM	LPS-1 of *Rhizobium leguminosarum* strain 3841	(1)
AFRC MAC 203	Rat IgM	LPS-1 of *R. leguminosarum* strain 3841	(2)
AFRC MAC 206	Rat IgG_{2c}	Plant membrane component	(3)
AFRC MAC 265	Rat IgG_{2a}	Matrix glycoprotein of infection thread	(4)

[a] LPS = lipopolysaccharide.
[b] (1) Brewin *et al.* 1986; (2) Bradley *et al.* 1988; (3) Perotto *et al.* 1991; (4) VandenBosch *et al.* 1989.

Rhizobium LPS-defective mutants.

The main LPS-defective bacterial mutants are listed in Table 1. Strain B660 is another derivative of wild-type strain 3841 isolated following transposon mutagenesis as described by Kannenberg *et al.* (1992): although its LPS composition and symbiotic phenotype was indistinguishable from those of other mutants identified in that study, e.g., strain B633, the relevant transposon insertion mapped to a different position on the genome, and the mutant phenotype was suppressed by the introduction of a different cosmid, termed pIJ1797. Several other mutants, e.g., B654, B657, B658, and B665 (Kannenberg *et al.* 1992), were also examined with respect to their effects on pea nodule development, but the results obtained were indistinguishable from those described here for class I and class II mutants. Mutant B659 has the most severe structural defect in LPS and is noninvasive (Inv^-), inducing empty uninvaded nodules on pea roots (Rae *et al.* 1991). All these mutants were previously characterized immunochemically with respect to a panel of monoclonal antibodies reacting with different LPS epitopes expressed by strain 3841, which has wild-type LPS (Kannenberg *et al.* 1992): relevant information is summarized in Table 1 and Figure 1.

Antibodies.

Rat monoclonal antibodies are listed in Table 2. MAC 265 is a rat monoclonal antibody that recognizes an intercellular plant glycoprotein abundant in the infection thread (VandenBosch *et al.* 1989a) and also present in intercellular spaces in uninfected tissue (Rae *et al.* 1991, 1992). MAC 206 is a rat monoclonal antibody that recognizes a component of the plant membrane glycocalyx, both on host cell plasma membranes and on the peribacteroid membrane (Perotto *et al.* 1991). MAC 57 and MAC 203 are rat monoclonal antibodies that recognize (respectively) a constitutive and a nodule-inducible epitope associated with the LPS-1 component of strain 3841 LPS (Kannenberg *et al.* 1992). The MAC 203 epitope is not expressed by any of the LPS-defective mutants used in the present study. Rabbit antisera reacting with purified pea leghemoglobin (Lbc_1) and nitrogenase component 1 (Rr1) of *Rhodospirillum rubrum* were generously provided by T. Bisseling and P. Ludden, respectively.

Microscopy on nodule sections.

Basic methods of immunogold staining and silver enhancement were described previously (VandenBosch *et al.* 1989b; Perotto *et al.* 1991). To obtain serial sections for electron microscopy, thin sections of resin-embedded nodule tissue (silver-gold in color) were collected as a ribbon onto single-slot grids and counterstained, before being placed on a carbon-coated film of pyroxilin, as described by Wells (1974). For electron microscopy, 10-nm colloidal gold conjugates were used. For light microscopy, 5-nm colloidal gold was used with silver enhancement. To exclude the possibility

that a bacterial LPS mutant used as inoculum had reverted to a wild-type phenotype within the nodule, adjacent sections from the same nodule were tested with the panel of monoclonal antibodies listed in Table 2. In most cases the bacteria in the nodule proved to be immunologically indistinguishable from the original inoculum strain (although a few cases of reversion were noted when strain B633 was used as inoculum, and these nodules were dismissed from further analysis.)

A fluorescent staining procedure (Brundett *et al.* 1988) was used to detect suberin, lignin, and callose in nodule tissues. Cryosections of wild-type and mutant nodules (15–20 μm) were stained with 0.1% (w/v) berberine hemisulfate (Sigma, London) in water for 1 hr. After four or five rinses in distilled water, sections were transferred to 0.5% (w/v) aniline blue WS (Polysciences, Warrington, PA) in distilled water for 30 min and rinsed as above. Before the sections were mounted on microscope slides, the samples were soaked for several minutes in a solution of 0.1% (w/v) $FeCl_3$ in 50% (v/v) glycerol. Following this procedure, hydrophobic components of cell walls (i.e., lignified or suberized walls) are revealed as a fluorescent yellow staining, while callose material produces a blue fluorescence under UV illumination.

Analysis of bacterial LPS.

Bacterial LPS was separated by electrophoresis on 15% polyacrylamide gels, following the protocol of Kannenberg *et al.* (1992). According to this method, the detergent sodium dodecyl sulfate was substituted in all buffers with an equivalent amount of taurodeoxycholate. LPS on the acrylamide gels was revealed by oxidation of the carbohydrate residues with sodium metaperiodate followed by a staining procedure with silver nitrate, as described by Wood *et al.* (1989).

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