Structural Studies of Alfalfa Roots Infected with Nodulation Mutants of *Rhizobium meliloti*

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Alfalfa roots infected with four nodulation defective (Nod⁻) mutants of *Rhizobium meliloti* which were generated by transposon Tn5 mutagenesis were examined by light and electron microscopy. In one class of Nod⁻ mutants, which we call nonreactive, the bacteria did not induce root hair curling or penetrate host cells. In a second class of Nod⁻ mutants, which we call reactive, the bacteria induced some root hair curling and entered root epidermal cells, although no infection threads were formed. In addition, reactive Nod⁻ mutants induced extensive root hair proliferation and hypertrophied roots. This study presents the details of the phenotype of the association between each mutant strain and alfalfa roots.

Nitrogen-fixing root nodules are produced by the symbiotic association of bacteria of the genus *Rhizobium* with plant hosts of the legume family. These complex structures are the result of a multi-step process requiring the specific gene products of both partners (40). The characteristic host-range specificity of different *Rhizobium* species is presumably related to species-specific surface interactions between the two partners. The earliest visible signs of a successful infection are the presence of curled root hairs and the development of an infection thread which grows through the infected root hair and into the main body of the root. As the infection thread penetrates the host, it forms branches within which the bacteria proliferate. At about this stage, the cells of the inner root cortex initiate new division. Eventually, branches of the infection thread invade some host cells and inoculate them with rhizobia, which are then called bacteroids to distinguish them as rhizobial cells living within the central tissue of legume nodules (28). The bacterial cells undergo morphological and biochemical differentiation; in their mature symbiotic state they fix nitrogen. The host cells show changes also in protein composition (17) and ultrastructure (13, 24).

Among the most critical stages of the developmental sequence leading to nodulation are the early steps of colonization, recognition, and infection of the host by the bacterium. Recent biochemical studies have focused on the importance of cell-cell recognition between bacterium

and host (for reviews, see references 2 and 8). One approach to this problem is to study the early stages of nodulation with characterized bacterial mutants. Previous publications from this laboratory (18, 20, 33, 34) described general techniques for the generation and manipulation of *Rhizobium* mutants. We now offer phenotypic descriptions of four *Rhizobium meliloti* mutants which are unable to form nodules on their legume host, alfalfa (*Medicago sativa* L.).

MATERIALS AND METHODS

Bacterial strains. *R. meliloti* 1021 is a symbiotically effective, streptomycin-resistant derivative of strain SU47 (20) and was the parent strain used to obtain symbiotic mutants. The symbiotic mutants used in this study fail to stimulate the formation of effective nodules and were isolated during a large-scale screening for symbiotically defective mutants (20). Strains 1027 and 1126 appeared to be blocked at a very early step in the recognition process, because they did not cause root hair curling. In contrast, strains 1145 and 1028 induced abnormal root hair deformations, but nodules were induced in only 20% of the inoculated plants. These nodules were small and ineffective.

Plant material. Seeds of alfalfa (*M. sativa* cv. Iroquois) were germinated on nitrogen-free agar slants in 18- by 150-mm test tubes and handled as previously described (20).

Transmission electron microscopy. Nodules 21 to 28 days old were fixed overnight in either 4% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) or in a mixture of 3.5% glutaraldehyde and 1% formaldehyde in 0.1 M cacodylate buffer (pH 7.4) at 0°C. After two rinses in the appropriate buffer, the nodules were postfixed in unbuffered 1% aqueous OsO₄ at 0°C for 4 to 6 h. After removal of the OsO₄ and two rinses in distilled water, the samples were dehydrated at 0°C in a graded

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acetone series. They were infiltrated and embedded in either Spurr standard low viscosity medium (37) (Polysciences, Inc., Warrington, Pa.) or in the mixture described as Quetol 651 (Ted Pella Co., Tustin, Ca.).

Thick sections (0.5 μm) were cut with glass knives, placed on slides, and stained with toluidine blue (0.25% in 0.25% borate buffer). Ultrathin sections were obtained with either glass or diamond knives, collected on clean 300-mesh copper grids, and stained with uranyl acetate-lead citrate (30). Alternatively, grids were stained with 1% $KMnO_4$ plus lead citrate (3). Although the latter procedure improved the contrast of the specimens, stain contamination was greater. The best results were obtained by use of Quetol 651 followed by conventional uranyl acetate-lead citrate. Grids were examined in a Zeiss 9A electron microscope operated at 60 kV.

Scanning electron microscopy. Nodules induced by wild-type *R. meliloti* 1021 were cut in half after fixation as described above. Swollen roots induced by the mutants 1145 and 1028 were left intact and cut after critical-point drying. Because specimens for transmission and scanning electron microscopy were prepared simultaneously, all material was postfixed in OsO_4 as described above. After complete dehydration in acetone, the specimens were critical-point dried, mounted on stubs, coated with gold-palladium, and examined with an AMR 1000 scanning electron microscope.

Root hair examination. The early stages of the symbiotic sequence were examined with a Fahraeus slide assembly (11). Alfalfa seeds were treated with 70% ethanol for 1 h, rinsed in sterile water, and then treated with sodium hypochlorite (5%, wt/vol) for 20 min. After several rinses with sterile water, the seeds were allowed to soak and germinate in sterile water. When radicles were 2 to 4 mm long, two seedlings were placed in each apparatus. The medium used was that described by Nutman (26), except that the iron concentration was 0.05 instead of 0.005 mg/liter. Bacteria were suspended in liquid and added to the slide chamber along with the emerging root.

RESULTS

Effective nodules: wild-type *R. meliloti*. Effective nodules induced by wild-type *R. meliloti* have been described previously (15, 16, 39). Because some variations may exist depending on the strain of *R. meliloti* and the variety of alfalfa used, and as a prelude to descriptions of mutants with defects at several symbiotic stages (Hirsch et al., manuscript in preparation), we include a brief description of overall nodule development induced by strain 1021 on *M. sativa* 'Iroquois.'

Nodules of *M. sativa* 'Iroquois' are elongate and cylindrical, with a distinct apical meristem at the distal end. Figure 1A shows a 25-day-old nodule with four characteristic histological zones: the meristematic zone, the early symbiotic or thread invasion zone, the late symbiotic or mature bacteroid zone, and the senescent zone.

Cells of the meristematic zone are typically small and isodiametric and contain numerous small vacuoles, mitochondria, and other organ-elles and a single large nucleus. This region is devoid of rhizobia.

The early symbiotic zone consists of cells which have increased significantly in size. Infection threads are common in this region, and rhizobia within the infection threads store poly-β-hydroxybutyrate granules (Fig. 1B). Small (1- to 1.5-μm-long), rod-shaped bacteroids are released from the threads via an unwalled droplet into the host cytoplasm (23). Upon endocytosis from the unwalled droplet, each bacteroid becomes surrounded by a peribacteroid membrane (Fig. 1B; reference 31), which isolates it from the host cytoplasm. Released rhizobia are devoid of poly-β-hydroxybutyrate.

In the late symbiotic zone the host cells mature. The host cell organelles are displaced to a peripheral position as the centrally located vacuole enlarges. The bacteroids elongate and are also found in the peripheral cytoplasm (Fig. 1C and D).

The senescent zone of the nodule is located at its proximal end. The transition from the late symbiotic to the senescent region occurs abruptly (Fig. 1A). Elongate bacteroids and degenerating rhizobia may be found adjacent to one another in these two sections. In the senescent zone, the degenerating rhizobia appear to break apart within the confines of the peribacteroid membrane. Different degrees of deterioration are observed within this zone. The organelles of the host cell appear to remain intact until the bacteroids are completely disorganized (Fig. 1E).

Ineffective nodules: nonreactive mutants. Mutants 1027 and 1126 did not induce curling of alfalfa root hairs at any time during the culture period. Root hairs continued to elongate after inoculation and were usually straight (Fig. 2A and C) in contrast to the typical "shepherd's crook" morphology of root hairs infected by wild-type *R. meliloti* 1021 (Fig. 2B).

Uninoculated plants showed root hair branching at a low frequency, as did plants infected with mutant 1027. In plants inoculated with mutant 1126, branching occurred more frequently and several days to 1 week earlier than it did in uninoculated controls or with strain 1027. Examination of root hairs 24 to 48 h after inoculation showed that both the 1126 and 1027 mutant bacteria appeared to grow near to and closely associate with the root hair surface. Infection threads were never observed in root hairs infected by either strain. The growth rates of each of these two strains in culture was identical to the parental wild-type strain 1021.

Ineffective nodules: reactive mutants. Both mutant strains 1145 and 1028 brought about root hair curling (Figs. 2D and 3B). In addition, various root hair deformations, such as hyper-
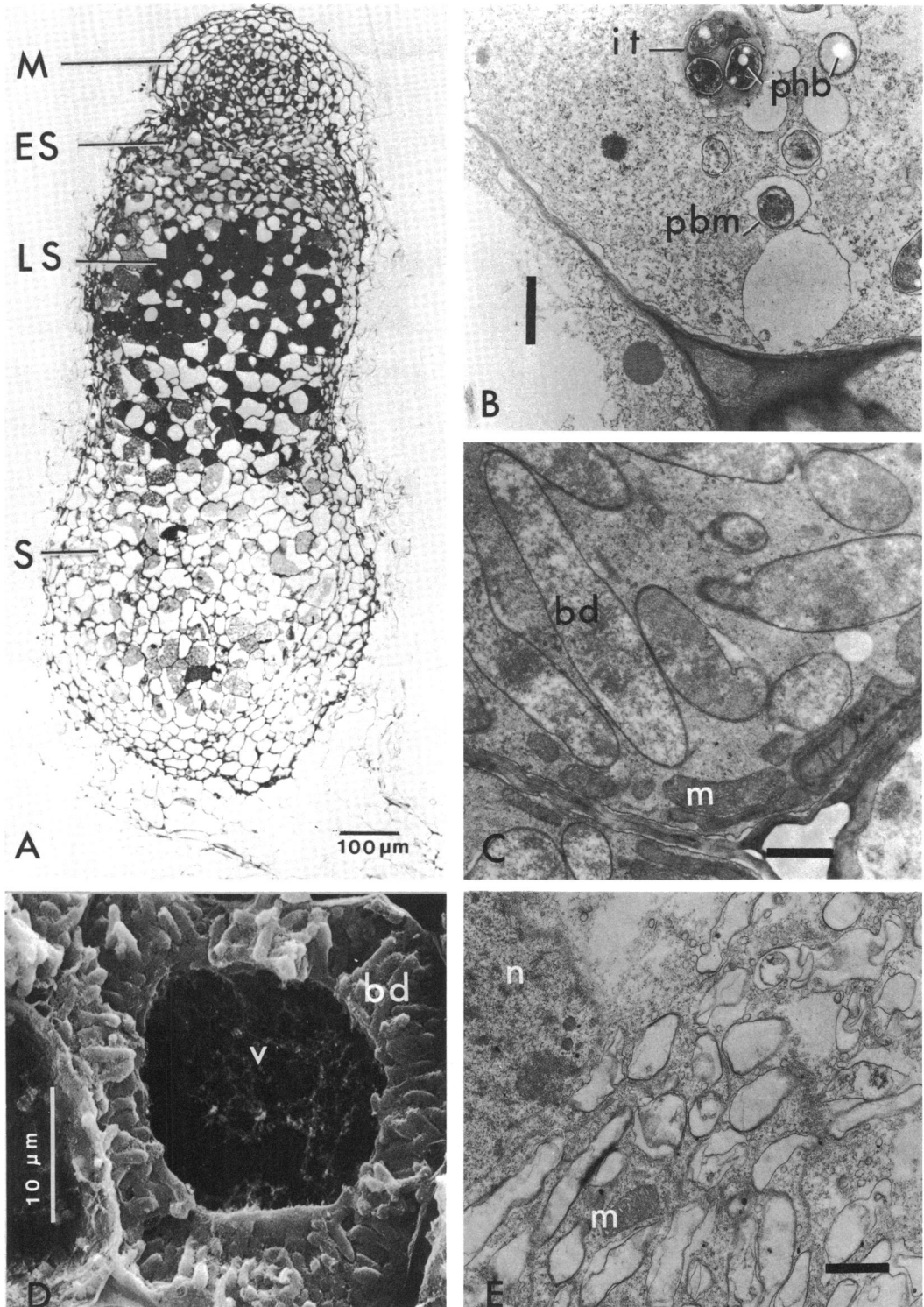
FIG. 1. Sections of nodules infected by *R. meliloti* 1021. (A) Non-median longitudinal section of a nodule showing four zones differing in the degree of bacterial invasion. M, Meristematic or noninvasive zone; ES, early symbiotic or thread invasion zone; LS, late symbiotic or bacteroid zone; S, senescent zone (×100). (B) Cell of ES zone. Infection thread (it) contains rhizobia with poly-β-hydroxybutyrate (phb) granules. The bacteria are enclosed by a peribacteroid membrane (pbm) (×9,800). (C) Cell of LS zone containing bacteroids (bd); mitochondria (m) and other host organelles which are displaced to the cell periphery (×9,800). (D) Scanning electron micrograph of cell from LS zone displaying central vacuole (v) and peripherally positioned bacteroids (×2,000). (E) Senescing cell (S zone) with degenerating bacteroids. Host nucleus (n) and mitochondria (m) are still intact (×9,800). (B, C, E) Bar, 1 μm.

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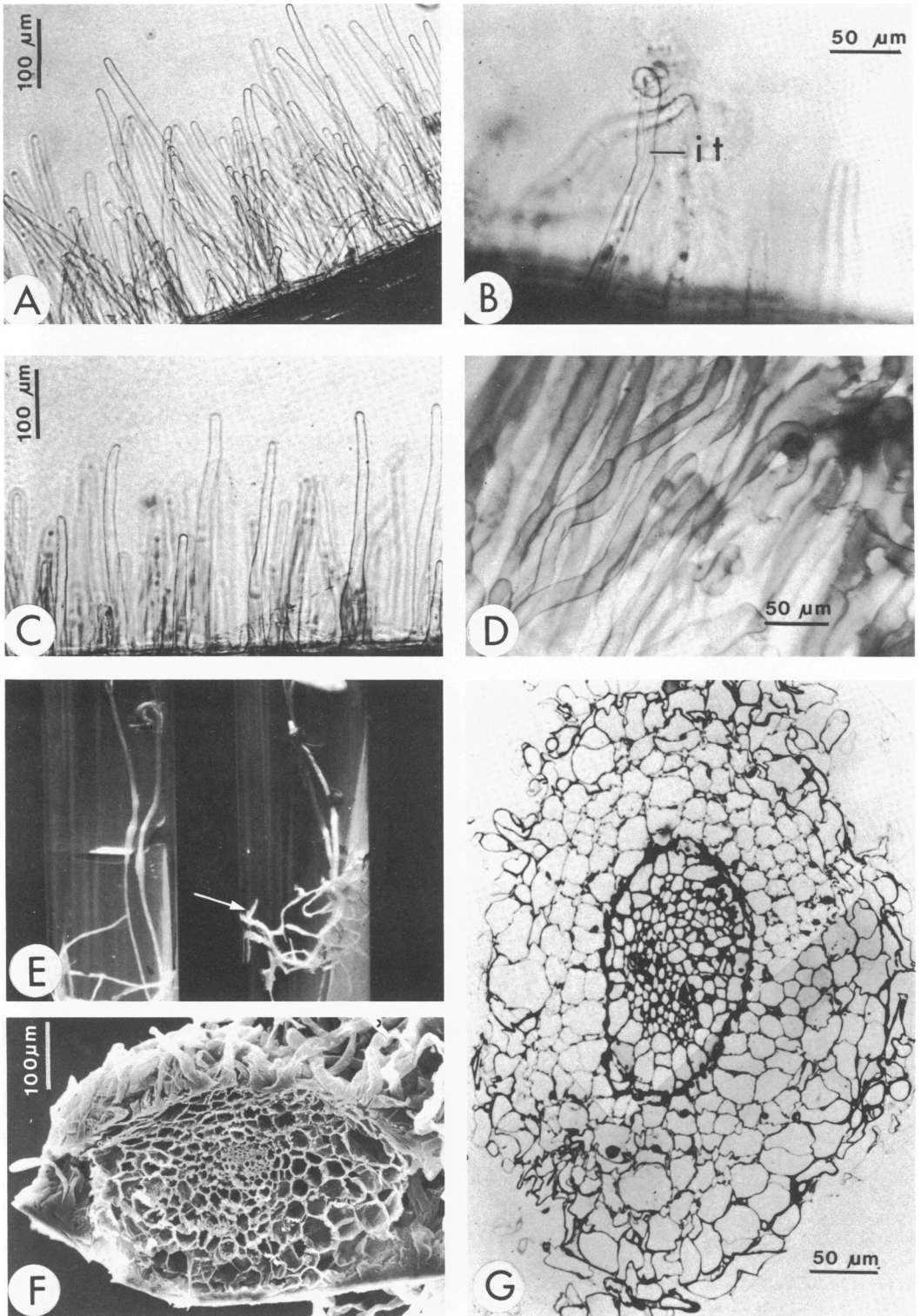
FIG. 2. (A–D) Views of root hairs. (A) Root hairs infected with nonreactive mutant 1027 (×100). (B) Curled root hair infected with wild-type *R. meliloti* showing infection thread (it) formation (×200). (C) Root hairs infected with nonreactive mutant 1126 (×100). (D) Root hairs infected with reactive mutant 1145 showing loose curling, stained with toluidine blue (×200). (E–G) Swollen roots infected with reactive mutant 1145. (E) Arrow points to hairy, hypertrophied roots (×1.2). (F) Scanning electron micrograph of transverse section of swollen root (×100). (G) Transverse light microscopic section of root showing swollen and empty root cortical and epidermal cells (×200).

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trophy, branching, and plasmolysis, were observed (Fig. 3B), especially after inoculation with mutant 1028. Examination of root hairs within 24 h after infection indicated that localized aggregation and adhesion of the bacterial mutants took place on the root surfaces. Polar attachment to the root hairs by mutant 1145, but not by mutant 1028, was obvious at the light microscopic level.

After infection with either mutant 1028 or 1145, the peripheral cells of the alfalfa roots became distended and swollen (Fig. 2G and 3C). These root swellings, or galls, were often marked by the presence of numerous elongate root hairs (Fig. 2E and F). Both scanning electron and light microscopic observation showed that the cells of the root were empty of rhizobia (Fig. 2F and G, 3A, and 4C). Cells of the gall were highly vacuolate, and the peripheral cytoplasm contained amyloplasts, mitochondria, and microbodies. No meristem comparable to that observed in wild-type nodules was initiated.

Some root hairs of the swollen root contained rhizobia (Fig. 4D). This was more commonly observed in roots infected with mutant 1145 than in those infected with mutant 1028. Polar attachment of mutant 1145 to the root hair surfaces was preserved at the electron microscopic level (Fig. 4B and D). In some micrographs, slender fibrils were observed at the point of attachment (Fig. 4B). Additionally, mutant 1145 often contained one or more electron-dense inclusions which may be polyphosphate bodies (Fig. 4B and E). These were not observed in mutant 1028. Epidermal cells containing mutant rhizobia often appeared to be devoid of cytoplasmic contents (Fig. 3D and F, 4C and E). Examination of a large number of electron micrographs confirmed this observation. Occasional amyloplasts were observed in these cells, as well as remnants of other host organelles and membranes. Rhizobia of both strains were found in both intra- and intercellular locations. Unlike wild-type bacteroids, no peribacteroid membrane was observed surrounding the intracellular mutant rhizobia (Fig. 3D and F, 4D and E). These naked rhizobia, especially strain 1145 (Fig. 4E), were plasmolyzed as a result. Cultured bacteria fixed under the same conditions did not exhibit this symptom (unpublished data).

In more than 20 galls sectioned, including several that were sectioned serially, infection threads were not observed in the root swellings induced by either mutant.

Occasionally, in about 20% of the infected plants, mutants 1028 and 1145 formed a limited number of small, ineffective nodules. In these cases, infection threads were apparent, and rhizobia were released into the cytoplasm but did not differentiate to form elongate bacteroids.

The fact that strains 1028 and 1145 formed occasional nodules could not be correlated with changes in medium composition or inoculation conditions and may reflect unknown differences in the host plants themselves or genetic or developmental "leakiness" of the mutations.

DISCUSSION

The goal of this study was to compare microscopically four nodulation-deficient mutants of *R. meliloti* with the effective nitrogen-fixing nodule-forming wild-type *R. meliloti* in their interaction with alfalfa roots. In the following discussion, we use a phenotypic code described by Vincent (40) and expanded upon by Rolfe et al. (32) to refer to various stages in the nodulation process.

Our light microscopic analyses showed that mutant 1027 was almost totally nonreactive, although root colonization and adhesion were observed. Root hair curling did not occur, and root hair branching was observed at levels similar to those found in uninoculated controls. In contrast to the root hair response to mutant 1027, hairs inoculated with mutant 1126 branched more frequently but the majority of hairs remained straight and elongated.

Unlike the nonreactive strains, the reactive Nod⁻ mutants 1145 and 1028 induced extensive root hair branching and deformation and a limited number of ineffective nodules. The shepherd's crook morphology characteristic of hairs inoculated with wild-type rhizobia was occasionally induced by mutant 1145, but the root hairs were not as tightly coiled. Mutant 1028 frequently caused extreme root hair distortion and hypertrophy. Inoculation with either reactive mutant not only stimulated the cells of the infected root to become hypertrophied but also brought about the expansion of numerous root hairs. Localized mitotic activity within the interior of the root, resulting in the onset of nodule development as reported for peas and soybeans (25), was not observed.

At the electron microscopic level, the polar attachment of strain 1145 bacteria to the root hairs was similar to that described by previous workers for wild-type strains (9, 10, 19, 21, 22, 29, 35). Slender fibrils were observed at the points of attachment, but we did not determine whether these were cellulosic as was reported by Napoli et al. (21). Although we did not observe polar attachment of strain 1028 to root hairs at either the electron or light microscopic level, examination of living roots in the Fahraeus slide assemblies showed localized aggregations of numerous bacteria to the deformed root hairs. We could not determine whether there was true bacterial attachment to plant surfaces for strain 1028. Paau et al. (27) reported
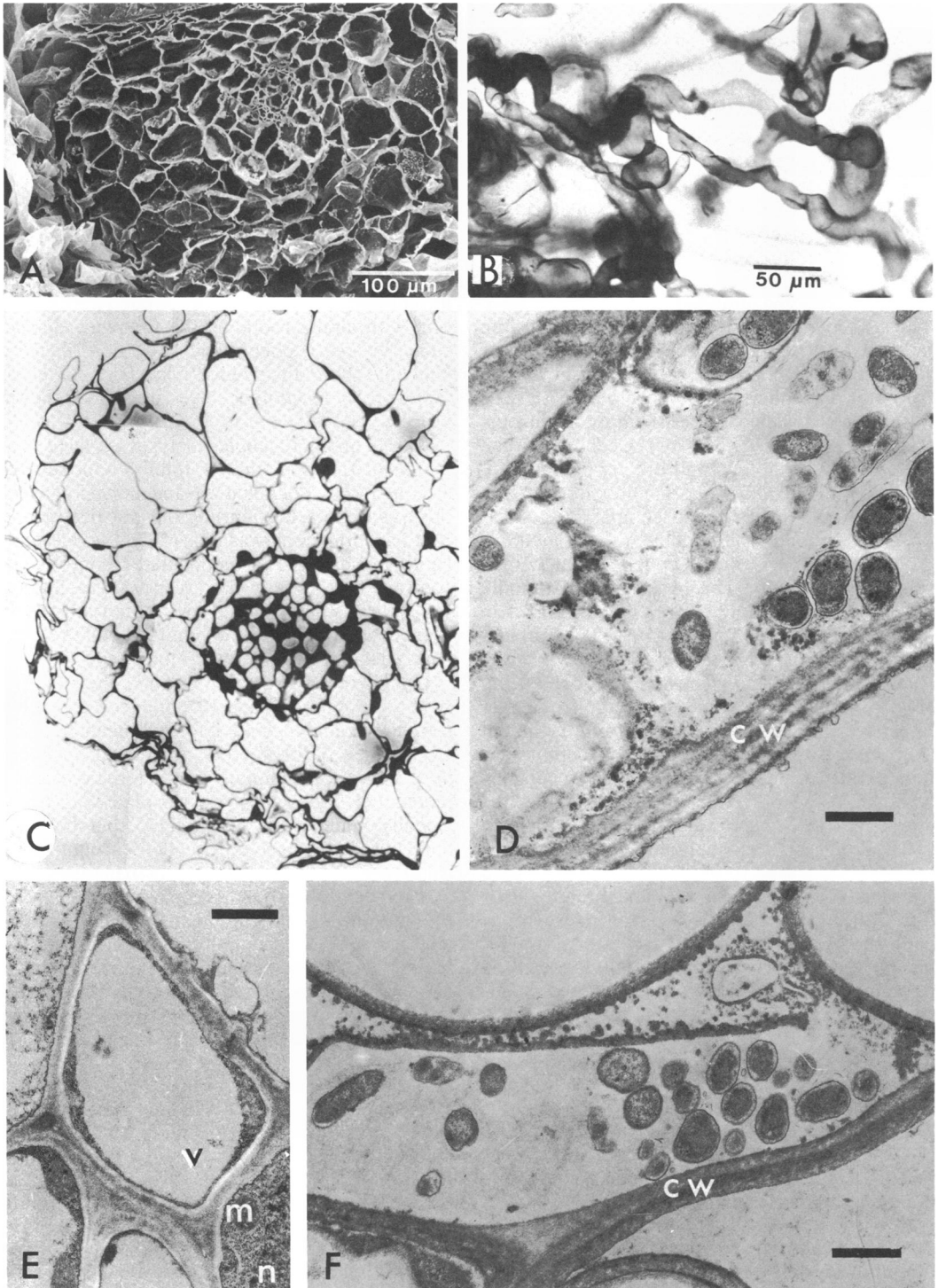
FIG. 3. Interaction of alfalfa root cells with reactive mutant 1028. (A) Scanning electron micrograph of transverse section of swollen root (×150). (B) Deformed root hairs after inoculation with reactive mutant 1028 (×200). (C) Transverse light microscopic section of infected root showing hypertrophied and empty epidermal cells (×250). (D) Infected epidermal cell containing mutant 1028. The host cytoplasm is obliterated. cw, cell wall (×9,800). (E) Interior cell with peripheral cytoplasm around a central vacuole (v). Mitochondria (m) and other organelles are visible in adjacent cell (×9,800). (F) Epidermal or subepidermal cell containing mutant rhizobia 1028. No peribacteroid membranes are observed (×9,800). (D, E, F) Bar, 1 μm.
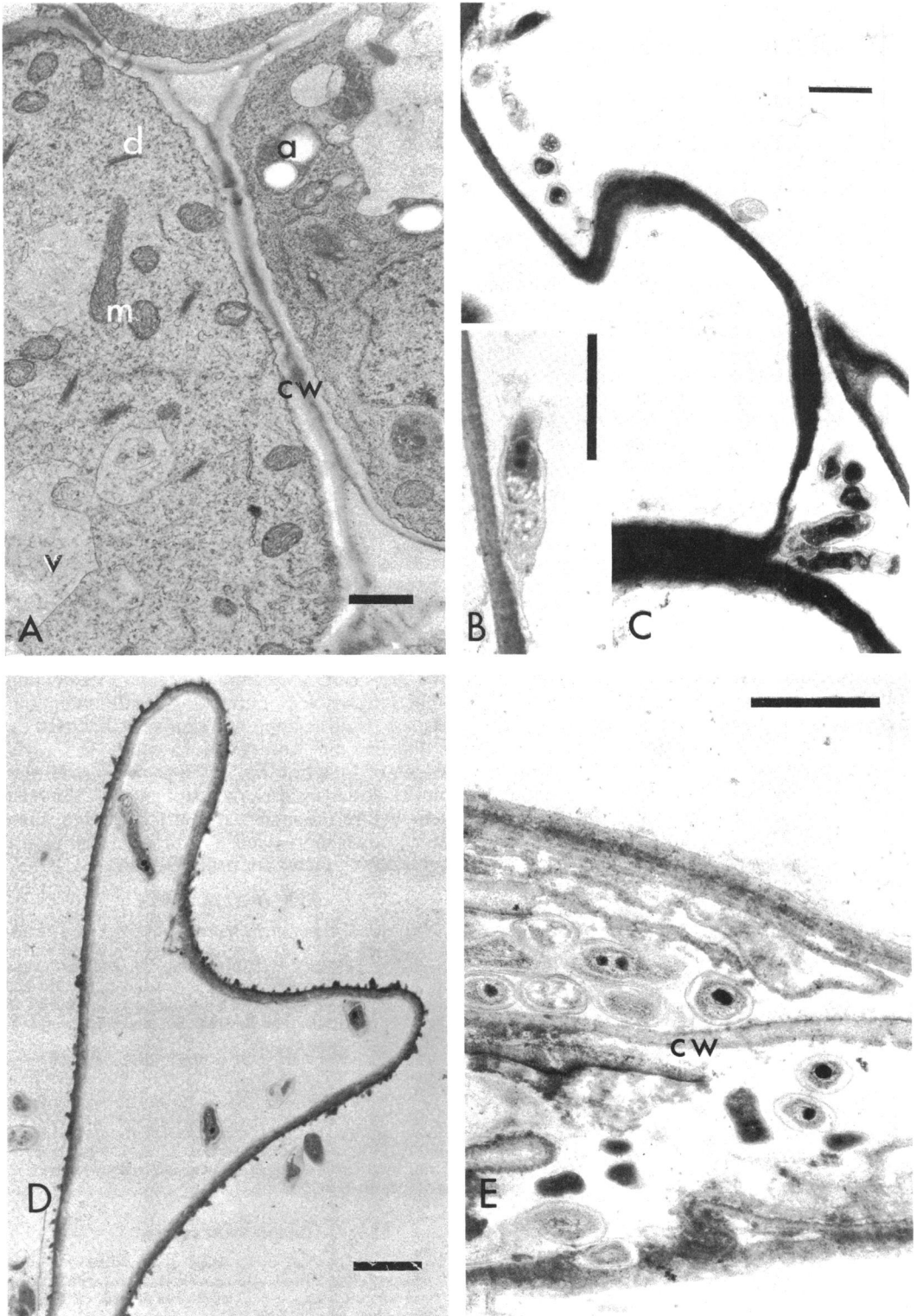
FIG. 4. Transmission electron micrographs of swollen cells of roots infected by reactive mutant 1145. (A) Interior cells showing typical plant cell cytoplasm containing mitochondria (m), dictyosomes (d), amyloplasts (a), vacuoles (v), and other organelles. cw, Cell wall. Fixation damage is not apparent (×9,800). (B) Reactive mutant 1145 attached to epidermal cell by slender fibrils (×19,000). (C) Outer root epidermis with polarly attached rhizobia (×9,800). (D) Root hair with mutant 1145 rhizobia inside and outside (×9,800). (E) Epidermal cell with included rhizobia. The host cell cytoplasm is no longer intact. The electron-dense structures within the 1145 rhizobia are presumed to be polyphosphate bodies (×19,000). Bar, 1 μm.

the failure of mutant 1028 to attach to root hairs or to bind to alfalfa agglutinin. Unlike mutant 1028 and wild-type *R. meliloti*, mutant 1145 contains a large number of electron-dense bodies (presumably polyphosphate bodies) associated with the nuclear material (7, 38).

Although rhizobia were seen in the proximal part of the root hair, we found no infection threads in the hypertrophied roots or in numerous root hairs. Infection by *Rhizobium* without thread formation can occur in other plant species, notably in peanuts and lupines (1, 5, 6). In peanuts, root cells separate at the middle lamella and become filled with rhizobia (5), giving rise to an intercellular region of infection. Rhizobia enter the cortical cells through a structurally altered cell wall and become surrounded by a membrane, presumably the now naked host plasma membrane (5).

There are both intriguing similarities and important differences between this mode of entry and the apparent entry of the reactive mutants. Like the rhizobia which infect peanuts, mutants 1145 and 1028 frequently were observed between cells, separating the middle lamella, and in intercellular spaces. In addition, rhizobia were found within the host cells, but electron microscopic observation showed that, unlike peanut rhizobia, the mutants lacked the surrounding peribacteroid membrane. We have not determined the exact mechanism of entry of these mutants into the host cell, but we hypothesize that the cell wall is altered in some way to permit their passage. The electron micrographs showed that the integrity of the plasma membrane was not maintained, with the result that mutant rhizobia were released naked into the host cell.

There are at least two explanations to account for the disruption of the host cell membrane and the entry of strains 1145 and 1028. Infection with these mutants may affect the structure of the host cell so that the plasma membrane and cytoplasm do not retain their integrity after fixation. The fact that the interior cells (Fig. 3E and 4A) were well preserved in the same preparations suggests that necrosis of the epidermal cells may be an immediate or indirect consequence of the bacterial infection and not a result of poor fixation. Moreover, we observed various degrees of host cell degradation, pointing to a possible induction of necrotic symptoms after infection with mutants 1028 and 1145.

An alternative explanation may be that the host cells senesce before the entry of the mutant rhizobia. A similar situation was reported (12) for the entry of bacteria from the surrounding rhizosphere into wheat roots. The death of the outermost cells after water stress leads to a loss of the production of inhibitors or suppressors of bacterial infection by these cells. The net result is that once the outermost cells die the bacteria of the rhizosphere are able to lyse the cell walls and enter the roots.

If mutants 1145 and 1028 caused the loss of cytoplasmic integrity upon inoculation, it may be that these bacteria provoke a pathogenic or parasitic rather than symbiotic host response which is suppressed or not elicited by wild-type rhizobia. The ultrastructural symptoms seen in our electron micrographs are reminiscent of those observed in micrographs of infection by bacteria causing a hypersensitive reaction (14, 36). However, we did not observe any host-generated pellicles or other structures for immobilizing bacteria outside of the host epidermal cells. Recognition and attachment to hairs (at least with mutant 1145) appeared to proceed after inoculation with the reactive Nod⁻ mutants, but internally redirected growth of the root hair cell plasma membrane and formation of the infection thread did not occur.

One way to differentiate between the above-mentioned possibilities is to study the infection before host cell senescence becomes apparent. We are currently in the process of examining infections by Nod⁻ mutants soon after infection. We are using an approach similar to that used by Callaham and Torrey (4) to study the early stages of infection by *Trifolium repens*. If mutant rhizobia are observed to be within the host cells before the membranes are disrupted, then disruption is more likely a result of, rather than a contributing factor to, bacterial entry.

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