Morphology of Root Nodules and Nodule-like Structures Formed by Rhizobium and Agrobacterium Strains Containing a Rhizobium meliloti Megaplasmid

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ABSTRACT We examined expression of the megaplasmid pRme41b of Rhizobium meliloti in two different Rhizobium sp. Strains and in Agrobacterium tumefaciens. Transfer of pRme41b into these bacteria was facilitated by insertion of a recombinant plasmid coding for mobilization functions of RP4 into the nil region (Kondorosi, A., E. Kondorosi, C. E. Pankhurst, W. J. Broughton, and Z. Banfalvi, 1982, Mol. Gen. Genet., 188:433-439). In all cases, transconjugants formed nodule-like structures on the roots of Medicago sativa. These structures were largely composed of meristematic cells but they were not invaded by bacteria. Bacteria were found only within infection threads in root hairs, and within intercellular spaces of the outermost cells of the structures.

The donor strain of R. meliloti containing pAK11 or pAK12 in pRme41b initially produced nodules on M. sativa that did not fix nitrogen (Fix−). In these nodules, bacteria were released from infection threads into the host cells but they did not multiply appreciably. Any bacteroids formed degenerated prematurely. In some cases, however, reversion to a Fix+ phenotype occurred after 4 to 6 wk. Bacteria released into newly infected cells in these nodules showed normal development into bacteroids.

The soil bacterium Rhizobium meliloti fixes molecular nitrogen in symbiotic association with the legume Medicago sativa. Development of this nitrogen-fixing symbiosis is a multistage process and is probably controlled by a large number of bacterial and plant genes.

R. meliloti strains harbor very large plasmids (megaplasmids). Some of the genes required for nodulation (nod) and for nitrogen fixation (fix) are carried by these plasmids (1, 16). Evidence for the presence of nod and fix genes on the megaplasmid pRme41b of R. meliloti strains 41 comes from mapping mutations in symbiotically defective (Nod− and Fix−) mutants on pRme41b (1, 5) and from hybridization of cloned R. meliloti nif genes to pRme41b (1). More recently, pRme41b was made susceptible to mobilization by inserting a recombinant plasmid (pAK11 or pAK12) carrying RP4 mobilization and Tn5 functions into the nif region of pRme41b (9). Using another plasmid (pJB3J1) as a helper plasmid, and selecting for the kanamycin resistance marker of Tn5, pRme41b was transferred into other R. meliloti strains, into other Rhizobium sp. and into Agrobacterium tumefaciens. Upon transfer, pRme41b was able to restore nodulating ability to a nod nif deletion mutant of R. meliloti and enabled the other Rhizobium sp. as well as A. tumefaciens to form nodule-like structures on the roots of Medicago sativa. Not only does this confirm the presence of nod genes on pRme41b, it also demonstrates that some of these genes can be expressed in other bacteria (9).

In this paper, we assess the extent to which nod genes carried by pRme41b are phenotypically expressed in two other distantly related Rhizobium strains and in Agrobacterium by...
examining the structure of the nodules formed by the trans-conjugants on M. sativa. As comparisons, we used nodules induced by the wild-type strain (AK631) and the wild-type carrying the RP4-mob region (AK5000).

MATERIALS AND METHODS

Bacterial Strains: All strains used are listed in Table I. Details of growth, construction, etc., are given in Kondorosi et al. (9). Basically, the approach used to mobilize the megaplasmid was to introduce by in vitro means the mobilization functions of RP, in pACYC184 together with a selectable marker (KmR of Tn5) into a cloned fragment of the megaplasmid (pID1, containing nifR and nifD genes in pBR322). Hybrid plasmids so formed (pAK11 and pAK12) were then introduced into R. meliloti wild type strain AK631 by conjugation. Due to the homology between the cloned megaplasmid AK5001, and AK5016 after surface sterilization with ethanol/H2O2 (1:1) for 2 min, in 96% ethanol for 1 min, and washed with several changes of sterile water. Nodule contents were streaked out on yeast-extract mannitol agar and were fixed in 4% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C for 14-20 h and postfixed in 1% osmium tetroxide in the same buffer at 4°C for 2 h. Dehydration was progressed from early to late symbiotic development. Ultimately, bacteroids filled the cytoplasm of the nodule cells.

Development of Nodules Formed by AK631

The wild-type R. meliloti strain AK631 formed nitrogen-fixing nodules on the roots of M. sativa. The nodules, which began to appear 6-7 d after inoculation, were elongate, cylindrical and, in gross structure, similar to nitrogen-fixing nodules formed by other R. meliloti strains on M. sativa (see Discussion). Essential features included an apical meristem, a zone of rhizobial release from infection threads, and a zone of nodule cell enlargement and bacteroid development (Fig. 1a). Cells of the nodule meristem were uniform in shape and contained a centrally located nucleus with at least one prominent nucleolus. The cytoplasm surrounding the nucleus contained several small vacuoles, numerous mitochondria, and a few plastids. Infection threads that originated from epidermal root hair cells were found in cells immediately below the meristem. Rhizobia were released from the infection thread and became enclosed within a membrane (the peribacteroid membrane) as they entered the host cell cytoplasm (Fig. 1b). Host cells containing released bacteria increased in size and contained many small vacuoles, vesicles, and mitochondria (Fig. 1c). After release from the infection thread, the bacteria increased the number and size (Fig. 1d). Bacteroids were enclosed singularly within the peribacteroid membrane and had a dispersed nucleoid, few ribosomes, and few internal membranes (Fig. 1d). Both the bacteroids and the host cells containing them continued to increase in size as they progressed from early to late symbiotic development. Ultimately, bacteroids filled the cytoplasm of the nodule cells.

Table I

| Bacterial strains | Relevant characteristics | Source or reference |
|-------------------|--------------------------|---------------------|
| R. meliloti        |                          |                     |
| AK631             | Nod*Fix*, wild type variant with compact colony morphology | 9                   |
| AK5000            | AK631 (pRme41b::pAK11)  | 9                   |
| AK5001            | AK631 (pRme41b::pAK12)  | 9                   |
| AK5016            | AK631 (pRme41b::pAK12)  | 9                   |
|                   |                          |                     |
| Rhizobium sp.     |                          |                     |
| PN4003            | Nod*Fix*Str*Rif* derivative of strain N2P2037 which nodulates Lotus pedunculatus | 13                  |
| PN4015            | PN4003 (pRme41b::pAK11)  | C. E. Pankhurst     |
| MHIK3030          | Nod*Fix*Str*Rif* derivative strain NCR234 which nodulates several tropical legumes | 18                  |
| CP100             | MHIK3030 (pRme41b::pAK11)  | C. E. Pankhurst     |
| A. tumefaciens    |                          |                     |
| GV3101            | Ti-plasmid cured, Rif* derivative of strain C58 | 25                  |
| AK5026            | GV3101 (pRme41b::pAK11)  | C. E. Pankhurst     |

Table II

| Phenotype of Bacteria Isolated from M. sativa Nodules Formed by R. meliloti Strain AK631 and Its Transconjugants |
|--------------------------------------------------|
| Rhizobium strain* | Nodule phenotype at 6 wk | No. of nodules containing kanamycin/ neomycin sensitive bacteria |
|--------------------|--------------------------|-----------------------------|
| AK631              | Nod*Fix*                 | 25                          |
| AK5000             | Nod*Fix*                 | 59                          |
| AK5016             | Nod*Fix*                 | 38                          |
|                   |                          | 23                          |

* Refer to Table I for relevant characteristics of these strains.
Fix*: some pink acetylene-reducing nodules present among white (non-acytelyne-reducing) ones.

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FIGURE 1 Light and electron micrographs of nodule tissues produced by wild type R. meliloti strain AK631. (a) Light micrograph of a median longitudinal section of a 2-wk-old nodule. Zones illustrated are: meristem (M), thread invasion (TI) area, early symbiotic (ES) and late symbiotic (LS) areas. The nodule has vascular traces (VT) outside the central mass of cells containing bacteroids and is enclosed by a layer of thick-walled sclerenchyma cells (SC) and a nodule cortex (NC). Bar, 100 μm. × 83. (b) Electron micrograph of the unwalled tip of an infection thread (IT) showing release of rhizobia into the host cell cytoplasm. The bacteria (B) became enclosed within the peribacteroid membrane (PBM) as they moved into the cytoplasm. MT, mitochondria; NC, host cell nucleus. Bar, 1 μm. × 6,900. (c) Electron micrograph of part of an infected nodule cell in the early symbiotic zone showing many developing bacteroids (BR) within the host cell cytoplasm. The cytoplasm contained many vesicles (VS) and mitochondria (MT). CW, cell wall. Bar, 1 μm. × 4,900. (d) Electron micrograph of part of an infected nodule cell in the late symbiotic zone showing mature bacteroids (BR). Bar, 1 μm. × 4,900.
of the pink region coincided with development of acetylene-reducing activity, we suggest that this region contained bacteria which had reverted to a Fix' phenotype.

A median longitudinal section of a Fix' nodule is shown in Fig. 2a. The nodule cells distal to the nodule meristem were enlarged but appeared to contain few bacteria (Fig. 2a). Electron microscopic examination of these cells indicated that the rhizobia were released from infection threads into the host cell cytoplasm in the normal fashion. After their release, however, there appeared to be very little increase in the number of bacteria per cell (Fig. 2b, cf. Fig. 1c). Bacteria which were released appeared to develop into bacteroids (Fig. 2b) but many showed signs of premature degeneration within the enclosing peribacteroid membrane. The host cell cytoplasm contained few ribosomes but had large quantities of endoplasmic reticulum and associated vesicles as well as large numbers of mitochondria and plastids containing starch deposits (Fig. 2b). A cell in which pronounced degeneration of bacteroids has occurred is shown in Fig. 2c. Significant endoplasmic reticulum is again a feature of the host cell cytoplasm.

A variable proportion of the bacteria re-isolated from nod-

**Figure 2** Light and electron micrographs of 7-wk-old Fix' nodule tissues formed by *R. meliloti* strain AK5000. (a) Light micrograph of a median longitudinal section of a Fix' nodule. Zones illustrated are meristem (M), thread infection (TI) area, and a symbiotic zone (SYM). Cells in the symbiotic zone were enlarged but contained few bacteroids. Bar, 100 μm. × 78. (b) Electron micrograph of part of an infected nodule cell located in the upper region of the symbiotic zone. A few bacteroids (BR) were present in the cell cytoplasm which contained many mitochondria (MT) and starch-filled plastids (S). Bar, 1 μm. × 5,300. (c) Electron micrograph of part of an infected nodule cell located in the lower region of the symbiotic zone. The cell cytoplasm contained degenerating bacteroids (DBR) and a few intact bacteroids (BR), mitochondria (MT), starch-filled plastids (S), and extensive profiles of endoplasmic reticulum (ER). Bar, 2 μm. × 3,200.
ules produced AK5000, AK5001, and AK5016 were sensitive to kanamycin and neomycin (Table II). The presence of these rhizobia in some of the nodules examined suggests that the recombinant plasmids pAK11 and pAK12 may be spontaneously excised from pRme41b. Excision of pAK11 or pAK12 from the nif gene of pRme41b would permit the "reverted" rhizobia to proceed with normal symbiotic development in the nodules. We examined 10 nodules of this type under the electron microscope. Of these, 20% were Fix-, the rest were in the full Fix+ condition (i.e. identical in appearance to those of the wild-type R. meliloti, AK631; Fig. 1). A median longitudinal section of a nodule that reverted to a Fix+ phenotype is shown in Fig. 3a. Infected nodule cells below the newly developing Fix+ region are similar in structure to those described for the Fix- nodule (Fig. 3a, cf. Fig. 2a). Transition between presumably Fix- and Fix+ regions of the nodules is apparently abrupt (Fig. 3, a and b). In Fig. 3b a cell containing swollen bacteroids in the Fix+ region is seen adjacent to cells containing only a few bacteroids in the Fix- region.

Nodulation of M. sativa by Rhizobium sp. and A. tumefaciens Transconjugants Containing pRme41b

Rhizobium sp. strains PN4003 and MPIK3030 and A. tumefaciens strain GV3101 did not induce nodule formation of M. sativa (in one or two instances, MPIK3030 induced tumor-line protrusions on the roots of M. sativa). All transconjugants of these bacteria containing pRme41b (strains PN4015, CP100 and AK5026, Table I) produced nodule-like structures (in 60–80% of more than 100 plants examined), however. These structures were of variable appearance (Figs. 4a and 5a and b). AK5026 transconjugants formed distinctive swellings at the junction of primary and lateral roots, and often a significant portion of a lateral root became swollen and misshapen (Fig. 4a). Strains PN4015 and CP100 formed structures that were nodule-like (Fig. 5a). In some instances these structures became considerably enlarged (2–3-mm diam) and tumor-like (Fig. 5b).

Light microscope sections of these structures showed that in general there was more extensive meristematic activity in the PN4015 and CP100 structures than in the AK5026 transconjugant structures. In every case the mass of meristematic cells appeared to have been initiated from cells of the inner cortex (Figs. 4b and 5c). These cells had prominent nuclei, many mitochondria, and starch-filled plastids, with no evidence of bacterial invasion. Bacteria were commonly found only among the root hairs and within the intercellular spaces of the epidermal and some large outer cortical cells (Figs. 4d and 5, e and f). Within the intercellular spaces the bacteria were generally surrounded by copious deposits of an electron-dense material that was probably polysaccharide. Infection thread development within root hair cells was seen on the outer surface of the nodule-like structures formed by all three transconjugants (Figs. 4c and 5d).

DISCUSSION

Studies of root nodule structure are an important adjunct to understanding the role of individual Rhizobium and plant genes in nodule development and nitrogen fixation. Mutations in nod and nif genes (5, 6, 19–21) demonstrate at what
stage of nodule development these genes are important and provide clues as to their possible functions. Ability to transfer nod and nif genes (contained on mobilizable Rhizobium plasmids) into other bacteria further defines the extent to which these genes can be expressed in other chromosomal backgrounds.

In the present study, root nodules produced by the wild-type R. meliloti strain AK631 were similar to those reported by other workers (6, 15, 19, 20, 22, 23). A consequence of insertion of the recombinant plasmids pAK11 or pAK12 into the nif region of the megaplasmid pRme41b of strain AK631, however, was that the derivatives so obtained (strains AK5000, AK5001, and AK5016) formed root nodules that were initially Fix-. At some stage during nodule growth, however, the recombinant plasmid was occasionally excised from pRme41b, leading to full restoration of nif gene functions and a consequent reversion of the nodules to a Fix+ phenotype (9). This sequence of events has been followed by examining the structure of AK5000 Fix− nodules and the structure of AK5000 nodules that reverted to Fix+. Rhizobia were released normally into the host cells of Fix− nodules but failed to multiply. They appeared to enlarge into bacteroids but showed signs of premature degeneration.

Reversion of nodules from a Fix− to a Fix+ phenotype was apparent ~4 wk after the nodules had appeared and occurred in different nodules at different times. The transition zone between presumptive Fix− and Fix+ regions of the nodules was well defined. Other examples of Fix− nodule development by Rhizobium mutants containing lesions in the nif gene cluster are known (4, 11, 17) but structural studies of these nodules have not been reported. Reversion of Fix− via excision of pAK11 or pAK12 is supported by the observation that

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**Figure 4** Light and electron micrographs of the nodule-like structures formed by transconjugants GV3101 of AK5026 (a) A lateral root of a M. sativa seedling 4 wk after inoculation with a AK5026. The root was grossly misshapen and showed regions of swelling (small arrows) and development of more distinctive nodule-like structures (large arrows). Bar, 1 mm. x 7.4. (b) A median longitudinal section through a nodule-like structure. A compact mass of small meristematic cells (MC) has developed on each side of the lateral root (LR). No bacteria were found in these cells. RC, root cortex. Bar, 100 μm. x 73. (c) Light micrograph showing root cortex (RC) and root hair cells (RH). An infection thread (IT) is visible within one of the root hairs (degenerating bacteria were present in some of the cells in the subepidermal layers (SE) of the root cortex). Bar, 10 μm. x 695. (d) Electron micrograph of an intercellular space of outer cortical cells filled with bacteria. The bacteria were surrounded by electron-dense material that was probably polysaccharide. Granules of poly-hydroxybutyrate (PHB) were visible in some bacteria. CW, cell wall. Bar, 1 μm. x 5,500.
Figure 5 Light and electron micrographs of nodule-like structures formed by PN4003 and MPIK3030 transconjugants containing pRme41b. (a) Discrete nodule-like structures developed by the PN4003 transconjugant PN4015 (arrows). Bar, 1 mm. × 6. (b) An example of a large tumor-like structure formed by the MPIK3030 transconjugant CP100. Bar 1 mm. × 6. (c) A median longitudinal section through a nodule-like structure formed by PN4015. As in Fig. 4b, the structure was composed of meristematic cells (MC) surrounded by root cortex (RC). No bacteria were visible in the meristematic cells. LR, lateral root. Bar, 100 μm. × 74. (d) A light micrograph showing root cortical (RC) and root hair (RH) cells on the surface of a nodule-like structure formed by PN4015. Infection threads (IT) within root hair cells and bacteria (B) below the root hair cells were visible. Bar 10 μm. × 695. (e) A transverse section through a root hair cell containing an infection thread (IT) formed by CP100. Bacteria appear embedded in material on the surface of the root hair cell (see arrows). Bar, 2 μm. × 2,690. (f) PN4015 bacteria seen within an intercellular space. As with the AK5026 bacteria (Fig. 4d), the PN4015 bacteria were surrounded by a matrix of what was probably polysaccharide. PHB, polyhydroxybutyrate. CW, cell wall. Bar, 1 μm. × 5,100.
a high percentage of bacteria re-isolated from the nodules have lost the Km\(^r\) marker of Tn5. Moreover, the pRme41b::pAK11 and pRme41b::pAK12 exhibited some instability in A. tumefaciens cultured in free-living conditions. In two instances plasmid segregation was detected by DNA-DNA hybridization (data not shown). We suggest that this instability might be due to the duplication of the nif region in the cointegrates and precise excision of pAK11 or pAK12 may occur.

Transfer of pRme41b into Rhizobium strains PN4003, MlPK3030, and A. tumefaciens strain GV3101 resulted in all three bacteria acquiring the capacity to form nodule-like structures on the roots of M. sativa. The resultant nodule-like structures were analogous to the Rhizobium-induced tumour-like, ineffective nodules described previously for Medicago (10, 21, 24), Trifolium (2, 12), and Lotus (14). In all cases, these structures were composed of meristem cells with no intracellular invasion of bacteria. Despite the occurrence of infection threads within root hairs and the presence of bacteria within some differentiating subepidermal cells, bacterial invasion did not progress further. What prevents growth of infection threads and the normal release of bacteria into the meristem cells is not known. Perhaps some Rhizobium chromosomal genes are involved at this stage of nodule development, e.g., to overcome the plant defense mechanism against bacterial invasion. Obviously, if such genes exist, they are present on the R. meliloti chromosome but absent from the chromosomes of the other bacteria studied. Alternatively, it is possible that some genes on the transferred pRme41b megaplasmid are not expressed in the PN4003, MlPK3030, and GV3101 transconjugants.

In similar studies, Agrobacterium transconjugants containing the symbiotic (sym) plasmid of R. trifolii (7) or from R. leguminosarum (8, 23) formed ineffective nodules on Trifolium pratense and Vicia sativa, respectively. Bacterial release (though not nitrogen fixation) was only claimed in the first report, however (7). Thus the role of the host nucleus and/or cytoplasm in the expression of Rhizobium plasmid genes needs further clarification.

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