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

C. H. WONG, C. E. PANKHURST, A. KONDOROSI, and W. J. BROUGHTON
*Max-Planck-Institut für Züchtungsforschung, Abteilung Genetische Grundlagen der Pflanzenzuchtung, D-5000 Köln 30, Federal Republic of Germany. Dr. Wong's permanent address is School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. Dr. Pankhurst's permanent address is Applied Biochemistry Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand. Dr. Kondorosi's permanent address is Institute of Genetics, Biological Research Center, Hungarian Academy of Science, H-6701 Szeged, Hungary.*

ABSTRACT We examined expression of the megaplasmid pRme41*b* of *Rhizobium meliloti* in two different *Rhizobium* sp. Strains and in *Agrobacterium tumefaciens*. Transfer of pRme41*b* into these bacteria was facilitated by insertion of a recombinant plasmid coding for mobilization functions of $RP_4$ into the *nif* 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 pRme41*b* 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 occured after 4 to 6 wk. Bacteria released into newly infected cells in these nodules showed normal development into bacteriods.

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*)[1] and for nitrogen fixation (*fix*) are carried by these plasmids (1, 16). Evidence for the presence of *nod* and *fix* genes on the megaplasmid pRme41*b* of *R. meliloti* strains 41 comes from mapping mutations in symbiotically defective ($Nod^-$ and $Fix^-$) mutants on pRme41*b* (1, 5) and from hybridization of cloned *R. meliloti nif* genes to pRme41*b* (1). More recently, pRme41*b* was made susceptible to mobilization by inserting a recombinant plasmid (pAK11 or pAK12) carrying $RP_4$ mobilization and Tn5 functions into the *nif* region of pRme41*b* (9). Using another plasmid (pJB3JI) as a helper plasmid, and selecting for the kanamycin resistance marker of Tn5, pRme41*b* was transferred into other *R. meliloti* strains, into other *Rhizobium* sp. and into *Agrobacterium tumefaciens*. Upon transfer, pRme41*b* 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 pRme41*b*, 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 pRme41*b* are phenotypically expressed in two other distantly related *Rhizobium* strains and in *Agrobacterium* by

[1] *Abbreviations used in this paper:* *nod*, genes required for nodulation; *fix*, genes required for symbiotic nitrogen fixation; *nif*, a class of *fix* genes required for the enzyme nitrogenase synthesis; and *mob*, genes required for plasmid mobilisation.

THE JOURNAL OF CELL BIOLOGY · VOLUME 97 SEPTEMBER 1983 787–794

examining the structure of the nodules formed by the transconjugants on *M. sativa*. As comparisons, we used nodules induced by the wild-type strain (AK631) and the wild-type carrying the $RP_4$-*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 $RP_4$ (in pACYC184) together with a selectable marker ($Km^R$ of Tn5) into a cloned fragment of the megaplasmid (pID1, containing *nifH* 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 *nif* region and the megaplasmid *nif* region, co-integration of the two replicons readily occurred. This construction allowed the mobilization of pRme41*b*::pAK11 or pRme41*b*::pAK12 cointegrates into other *R. meliloti* strains and into *A. tumefaciens* by another P-1 type plasmid.

*Plant Tests:* *M. sativa* seedlings were grown in these tubes on slopes of nitrogen-free agar medium as described by Kondorosi et al. (9). For plant inoculation, bacteria were grown in TY-medium (3), sedimented, and washed with sterile nitrogen-free solution. 1 ml of a suspension of washed bacteria ($10^8$ bacteria $ml^{-1}$) was then added to 5-d-old seedlings.

Nitrogen-fixing activity was measured by acetylene reduction (9). Bacteria were re-isolated from 6-wk-old nodules produced by AK631, AK5000, AK5001, and AK5016 after surface sterilization with ethanol/$H_2O_2$ (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 LB agar containing kanamycin (200 $\mu g \cdot ml^{-1}$) and neomycin (200 $\mu g \cdot ml^{-1}$).

*Microscopy:* Root nodules and nodule-like structures were removed from the roots of individual plants 2, 4 and 8 wks after they were inoculated with the bacteria. The tissues 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% wt/vol osmium tetroxide in the same buffer at 4°C for 2 h. Dehydration was performed in a graded ethanol series and tissues were embedded in Araldite resin. For light microscopy, 1-$\mu$m sections were cut and stained for 1 min in 4% (wt/vol) basic fuchsin at 70°C, followed by 2% (wt/vol) aqueous methylene blue for 2 min. For electron microscopy, sections were stained for 30 min in 2% (wt/vol) uranyl acetate in 50% (vol/vol) ethanol followed by 15 min in Reynold's lead citrate solution. Sections were examined with a Siemens 101 electron microscope.

TABLE I

*Bacterial Strains Used in This Study*

| Bacterial strains | Relevant characteristics | Source or reference |
|---|---|---|
| *R. meliloti* | | |
| AK631 | $Nod^+Fix^+$, wild type variant with compact colony morphology | 9 |
| AK5000 | AK631 (pRme41*b*::pAK11) | 9 |
| AK5001 | AK631 (pRme41*b*::pAK12) | 9 |
| AK5016 | AK631 (pRme41*b*::pAK11) (pJB3JI) | 9 |
| *Rhizobium* sp. | | |
| PN4003 | $Nod^+Fix^+Str^RRif^R$ derivative of strain NZP2037 which nodulates *Lotus pedunculatus* | 13 |
| PN4015 | PN4003 (pRme41*b*::pAK11) (pJB3JI) | C. E. Pankhurst |
| MPIK3030 | $Nod^+Fix^+Str^R$ derivative strain NGR234 which nodulates several tropical legumes | 18 |
| CP100 | MPIK3030 (pRme41*b*::pAK11) (pJB3JI) | C. E. Pankhurst |
| *A. tumefaciens* | | |
| GV3101 | Ti-plasmid cured, $Rif^R$ derivative of strain C58 | 25 |
| AK5026 | GV3101 (pRme41*b*::pAK11) (pJB3JI) | 9 |

## RESULTS

### 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. 1 *a*). 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. 1 *b*). Host cells containing released bacteria increased in size and contained many small vacuoles, vesicles, and mitochondria (Fig. 1 *c*). After release from the infection thread, the bacteria increased the number and size (Fig. 1 *d*). Bacteroids were enclosed singularly within the peribacteroid membrane and had a dispersed nucleoid, few ribosomes, and few internal membranes (Fig. 1 *d*). 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.

### Development of Nodules Formed by AK5000, AK5001, and AK5016

Nodules formed by *R. meliloti* carrying the *mob* genes of $RP_4$ inserted into the *nif* region began to appear on the roots of *M. sativa* plants 7–8 d after inoculation. 90% of more than 100 plants examined were nodulated. 3 wk later, the cylindrical nodules were 1 to 1.5 mm long, were white, and did not reduce acetylene. The nodules continued to elongate and after 6 wk were 2.5–3 mm long. At about 4 wk, 30–60% of the nodules developed a pink region (indicating leghaemoglobin synthesis) just below the nodule meristem. Since appearance

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 examined | No. of nodules containing kanamycin/neomycin sensitive bacteria |
|---|---|---|---|
| AK631 | $Nod^+Fix^+$ | 25 | 25 |
| AK5000 | $Nod^+Fix^{\pm b}$ | 59 | 55 |
| AK5001 | $Nod^+Fix^{\pm}$ | 38 | 7 |
| AK5016 | $Nod^+Fix^{\pm}$ | 23 | 17 |

* Refer to Table I for relevant characteristics of these strains.

‡ $Fix^{\pm}$: some pink acetylene-reducing nodules present among white (nonacetylene-reducing) ones.

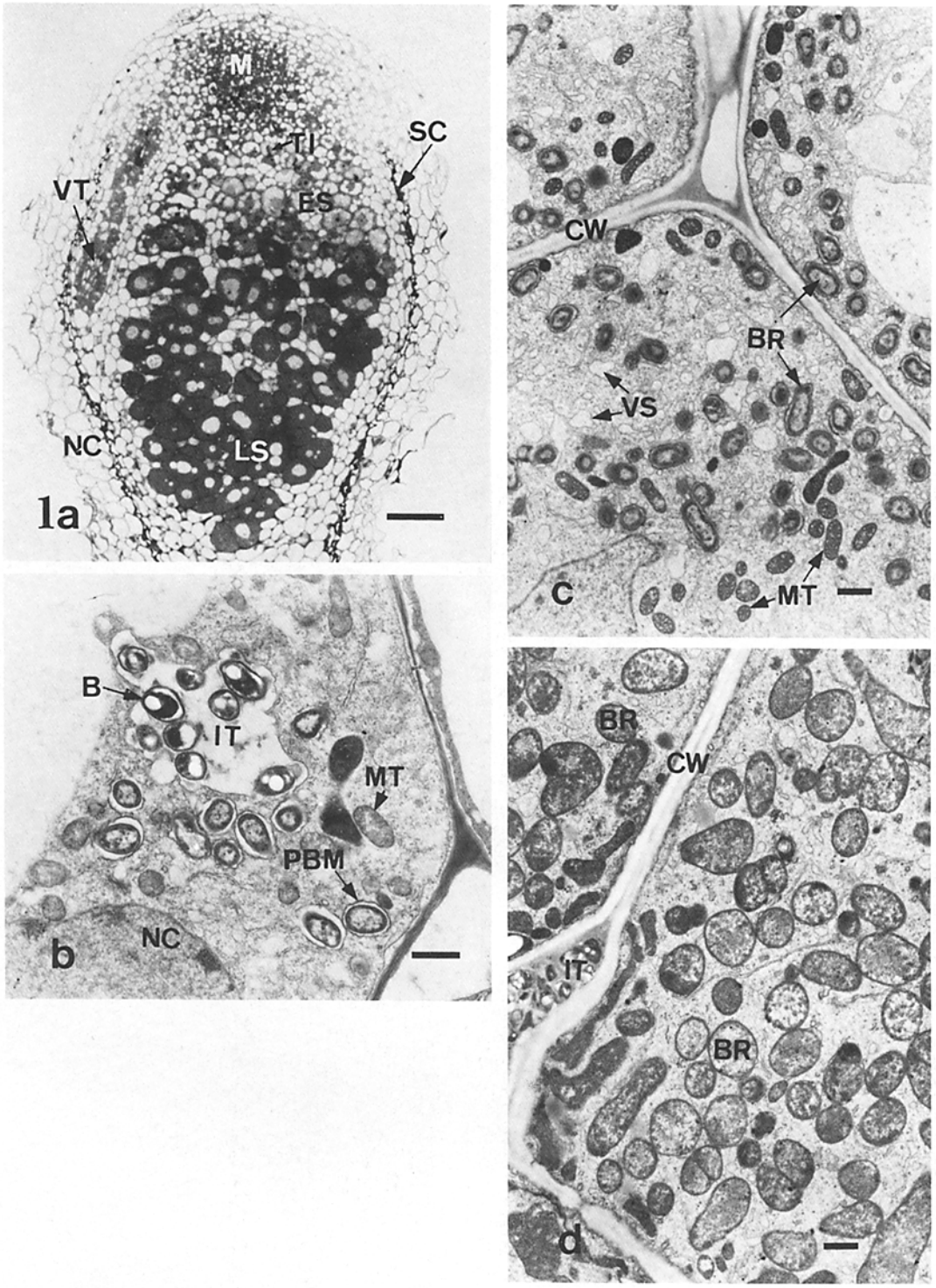


FIGURE 1 Light and electron micrographs of nodule tissues produced by wild type *R. meliloti* strain AK631. (a) Light micrograph of a median longitudinal section of a 2-wk-old nodule. Zones illustrated are: meristem (*M*), thread invasion (*TI*) area, early symbiotic (*ES*) and late symbiotic (*LS*) 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. 2*a*. The nodule cells distal to the nodule meristem were enlarged but appeared to contain few bacteria (Fig. 2*a*). 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. 2*b*, cf. Fig. 1*c*). Bacteria which were released appeared to develop into bacteroids (Fig. 2*b*) 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. 2*b*). A cell in which pronounced degeneration of bacteroids has occurred is shown in Fig. 2*c*. Significant endoplasmic reticulum is again a feature of the host cell cytoplasm.

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

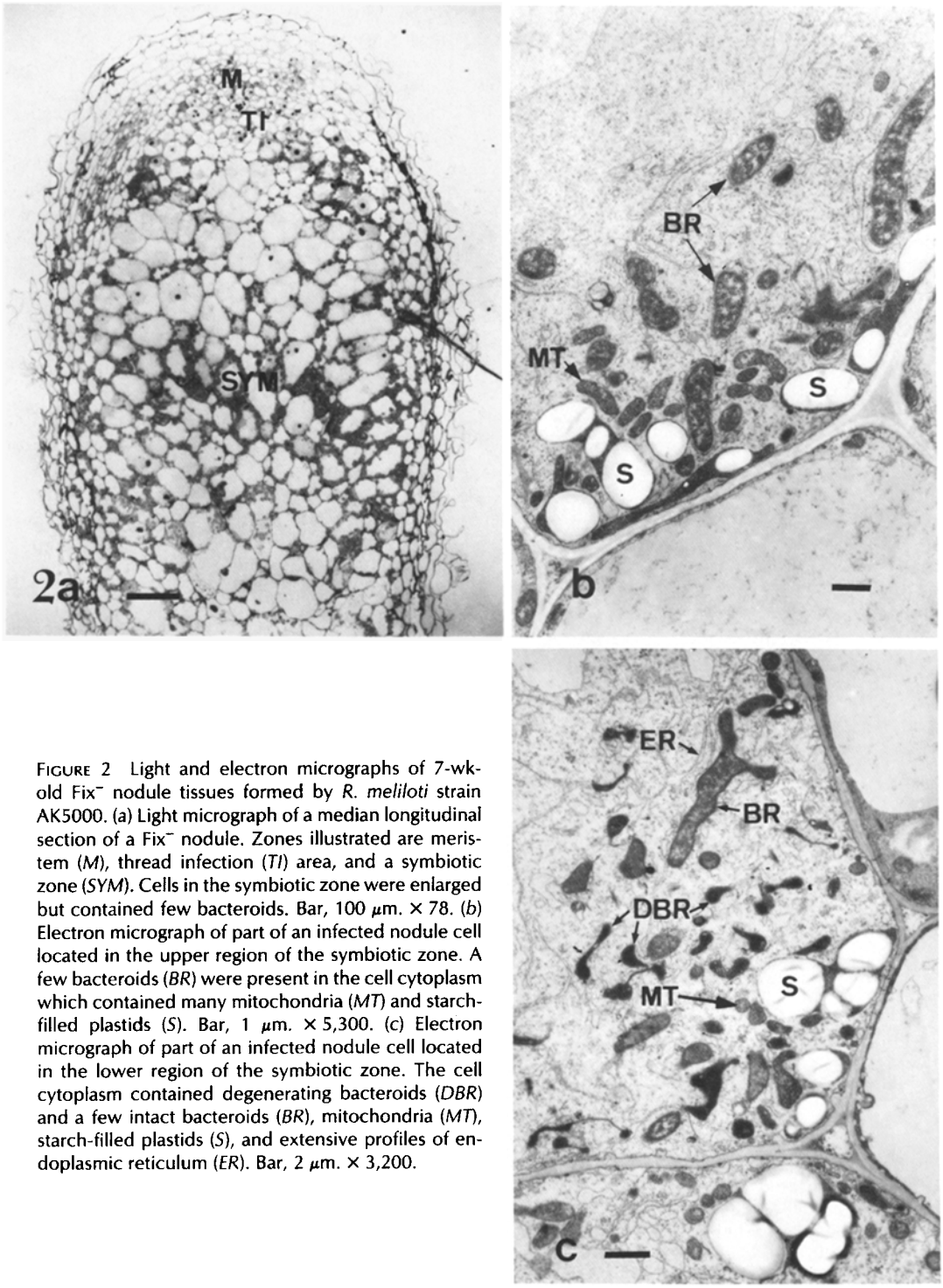


FIGURE 2 Light and electron micrographs of 7-wk-old Fix$^-$ nodule tissues formed by *R. meliloti* strain AK5000. (a) Light micrograph of a median longitudinal section of a Fix$^-$ nodule. Zones illustrated are meristem (*M*), thread infection (*TI*) area, and a symbiotic zone (*SYM*). Cells in the symbiotic 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 pRme41*b*. Excision of pAK11 or pAK12 from the *nif* gene of pRme41*b* 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 these 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. 3*a*. Infected nodule cells below the newly developing Fix$^+$ region are similar in structure to those described for the Fix$^-$ nodule (Fig. 3*a*, cf. Fig. 2*a*). Transition between presumably Fix$^-$ and Fix$^+$ regions of the nodules is apparently abrupt (Fig. 3, *a* and *b*). In Fig. 3*b* 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 pRme41*b

*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 pRme41*b* (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. 4*a* and 5, *a* 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. 4*a*). Strains PN4015 and CP100 formed structures that were nodule-like (Fig. 5*a*). In some instances these structures became considerably enlarged (2–3-mm diam) and tumor-like (Fig. 5*b*).

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. 4*b* and 5*c*). 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. 4*d* 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. 4*c* and 5*d*).

## 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

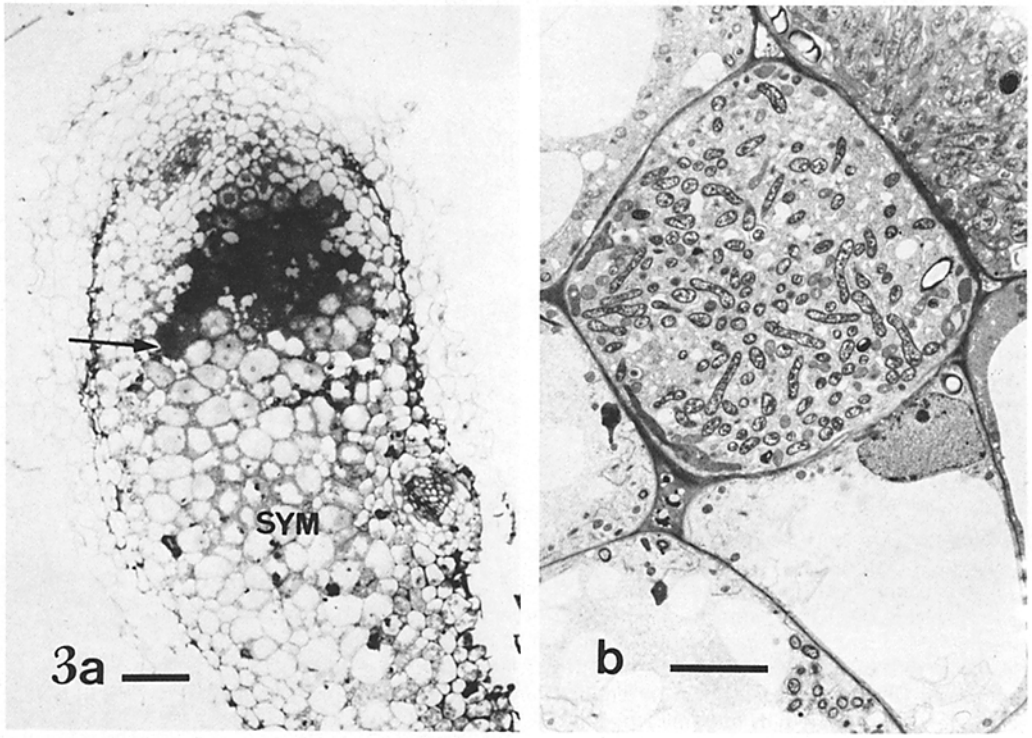


FIGURE 3 Light and electron micrographs of 7-wk-old Fix$^-$ nodule tissues formed by *R. meliloti* strain AK5000. (*a*) Light micrograph of a median longitudinal section of a nodule that reverted from a Fix$^-$ to a Fix$^+$ phenotype. The apical part of the nodule contained a region where bacteroid development was proceeding normally. The arrow indicates the point where normal development began. Below this region is a symbiotic zone (*SYM*) containing cells with only a few bacteroids. Bar, 100 μm. × 84. (*b*) Electron micrograph of infected cells from the region indicated by the arrow in *a*. A cell containing a large number of swollen bacteroids is seen adjacent to several cells containing only a few bacteroids. Bar, 5 μm. × 2,480.

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 pRme41*b* 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 pRme41*b*, 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

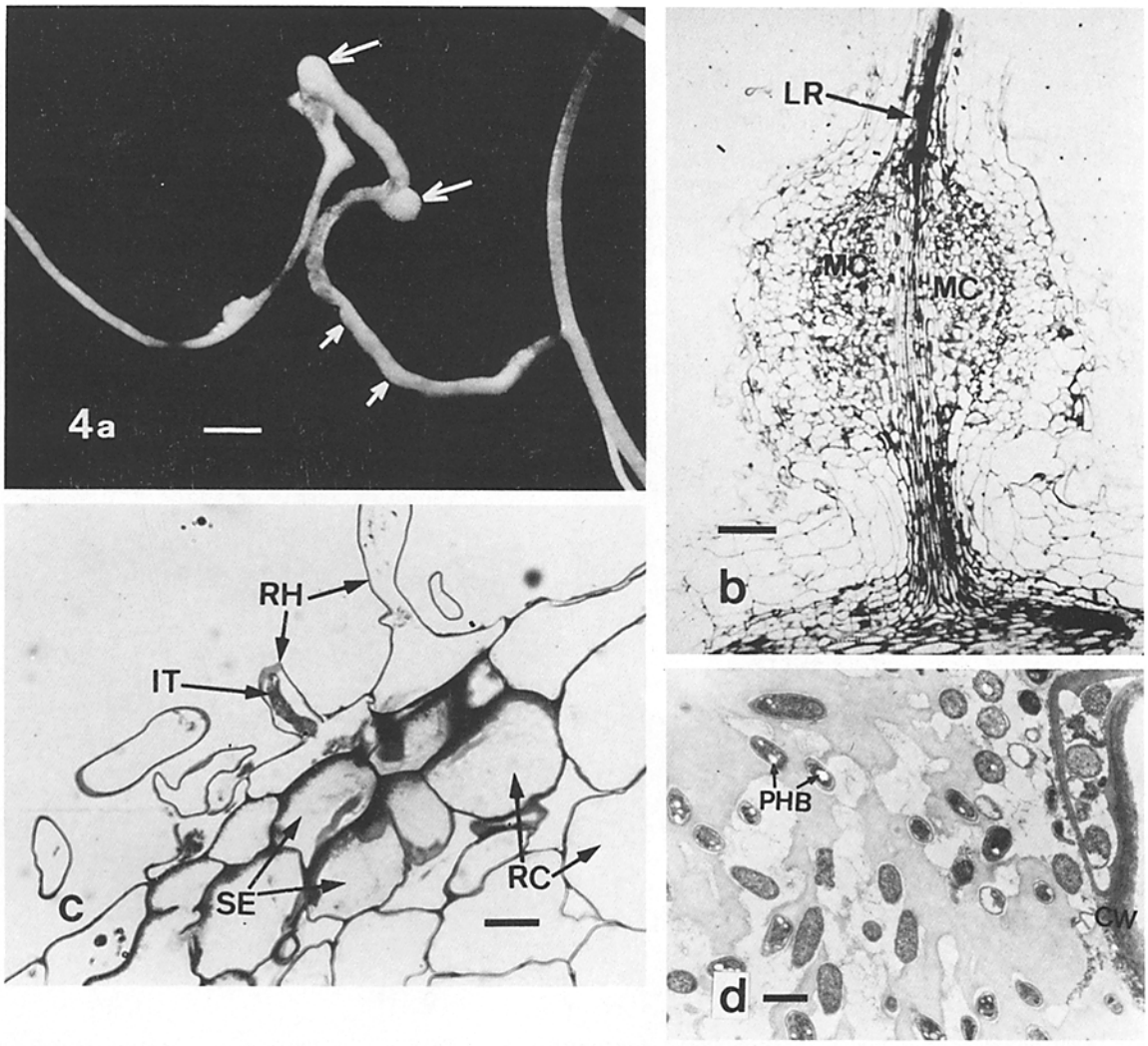


FIGURE 4 Light and electron micrographs of the nodule-like structures formed by transconjugants GV3101 of AK5026 (a) A lateral root of a *M. sativa* seedling 4 wk after inoculation with a AK5026. The root was grossly misshapen and showed regions of swelling (small arrows) and development of more distinctive nodule-like structures (large arrows). Bar, 1 mm. × 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. × 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. × 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. × 5,500.

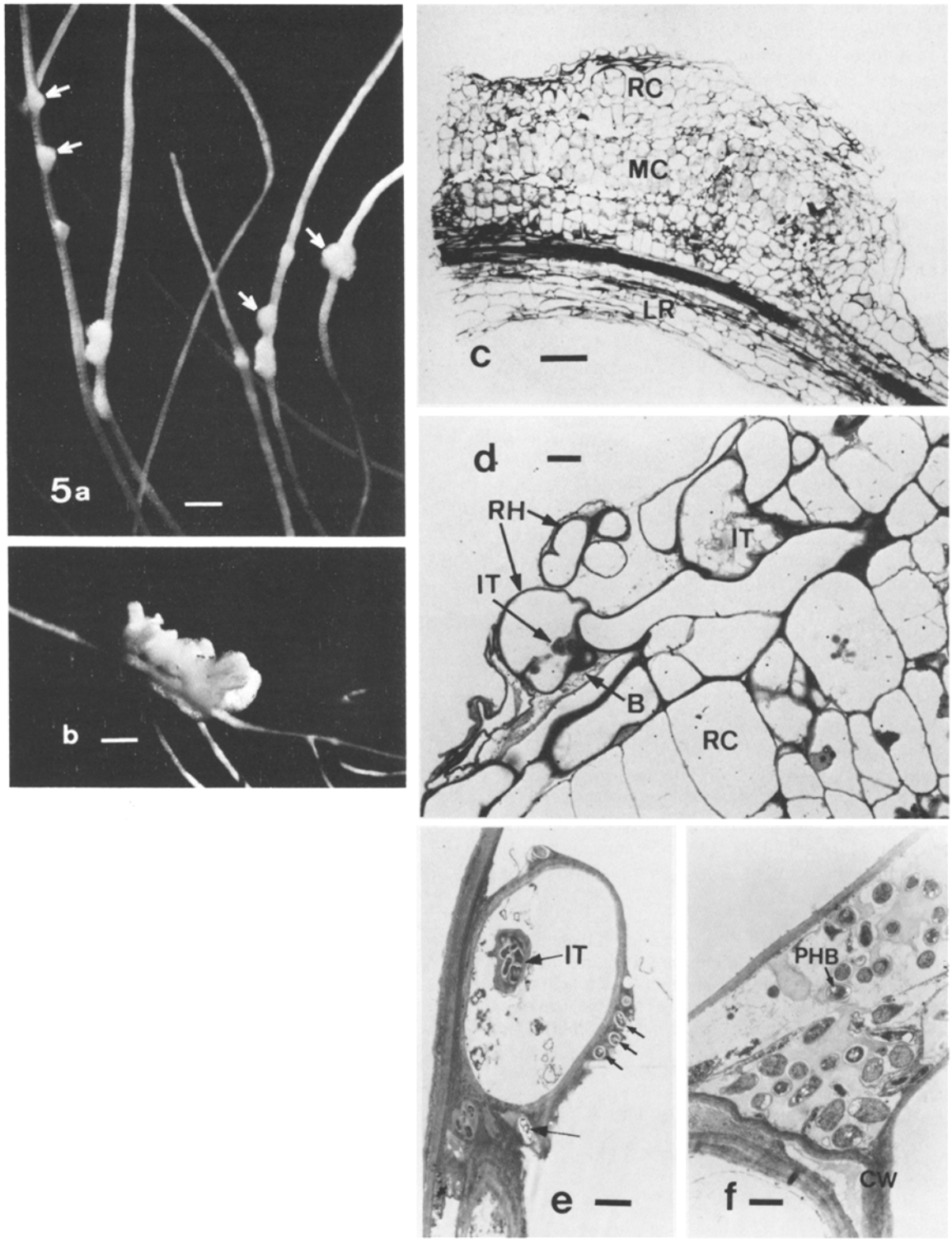


FIGURE 5 Light and electron micrographs of nodule-like structures formed by PN4003 and MPIK3030 transconjugants containing pRme41*b*. (*a*) Discrete nodule-like structures developed by the PN4003 transconjugant PN4015 (arrows). Bar, 1 mm. × 6. (*b*) An example of a large tumor-like structure formed by the MPIK3030 transconjugant CP100. Bar 1 mm. × 6. (*c*) A median longitudinal section through a nodule-like structure formed by PN4015. As in Fig. 4*b*, 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. 4 *d*), 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 pRme41*b*::pAK11 and pRme41*b*::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 pRme41*b* into *Rhizobium* strains PN4003, MPIK3030, 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 meristematic 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 meristematic 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 chromsomes of the other bacteria studied. Alternatively, it is possible that some genes on the transferred pRme41*b* megaplasmid are not expressed in the PN4003, MPIK3030, 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.

We are very grateful to J. Schell for his generous hospitality and support, to R.-D. Hirtz for his able help with the electron microscope, to E. Kondorosi for providing the strains, and to G. M. Schneider and N. Heycke for their assistance. We would also like to thank M. Kalda and D. Bock for preparing the figures and E. Schölzel for typing the manuscript.

Three of us (C. H. Wong, C. E. Pankhurst, and A. Kondorosi) were on sabbatical leave at the Max-Planck-Institut für Züchtungsforschung, Abteilung Schell. C. H. Wong wishes to thank the Universiti Sains Malaysia for financial support, C. E. Pankhurst the Alexander-von-Humboldt-Stiftung, Bonn, and A. Kondorosi the EMBO, Heidelberg, for fellowships.

*Received for publication 11 January 1983, and in revised form 23 May 1983.*

## REFERENCES

1. Banfalvi, Z., V. Sakanyan, C. Koncz, A. Kiss, I. Dusha, and A. Kondorosi. 1981. Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *R. meliloti*. *Mol. Gen. Genet.* 184:334–339.
2. Bergersen, F. J. 1957. The structure of ineffective root nodules of legumes: an unusual new type of ineffectiveness, and an appraisal of present knowledge. *Aust. J. Biol. Sci.* 10:233–242.
3. Beringer, J. E. 1974. R factor transfer in *Rhizobium leguminosarum*. *J. Gen. Microbiol.* 84:188–198.
4. Corbin, D., G. Ditta, and D. R. Helinski. 1982. Clustering of nitrogen fixation (*nif*) genes in *Rhizobium meliloti*. *J. Bacteriol.* 149:221–228.
5. Forrai, T., E. Vincze, Z. Banfalvi, G. B. Kiss, G. S. Randhawa, and A. Kondorosi. 1982. Location of symbiotic mutations in *Rhizobium meliloti*. *J. Bacteriol.* 153:635–643.
6. Hirsch, A. M., S. R. Long, M. Bang, N. Haskins, and F. M. Ausubel. 1982. Structural studies of alfalfa roots infected with nodulation mutants of *Rhizobium meliloti*. *J. Bacteriol.* 151:411–419.
7. Hooykaas, P. J. J., A. A. N. van Brussel, H. den Dulk-Ras, G. M. S. van Slogteren, and R. A. Schilperoort. 1981. Sym plasmid of *Rhizobium trifolii* expressed in different rhizobial species and *Agrobacterium tumefaciens*. *Nature (Lond.)*. 291:351–353.
8. Hooykaas, P. J. J., F. G. M. Snijdewint, and R. A. Schilperoort. 1982. Identification of the Sym plasmid of *Rhizobium leguminosarum* strain 1001 and its transfer to and expression in other rhizobia and *Agrobacterium tumefaciens*. *Plasmid.* 8:73–82.
9. Kondorosi, A., E. Kondorosi, C. E. Pankhurst, W. J. Broughton, and Z. Banfalvi. 1982. Mobilization of a *Rhizobium meliloti* megaplasmid carrying nodulation and nitrogen fixation genes into other rhizobia and *Agrobacterium*. *Mol. Gen. Genet.* 188:433–439.
10. MacGregor, A. N., and M. Alexander. 1971. Formation of tumour-like structures on legume roots by *Rhizobium*. *J. Bacteriol.* 105:728–732.
11. Maier, R. J., and W. J. Brill. 1976. Ineffective and non-nodulating mutant strains of *Rhizobium japonicum*. *J. Bacteriol.* 127:763–769.
12. Pankhurst, C. E. 1974. Ineffective *Rhizobium* mutants examined by immune-diffusion, gel electrophoresis and electron microscopy. *J. Gen. Microbiol.* 82:405–413.
13. Pankhurst, C. E. 1977. Symbiotic effectiveness of antibiotic-resistant mutants of fast- and slow-growing strains of *Rhizobium* nodulating *Lotus* species. *Can. J. Microbiol.* 23:1026–1033.
14. Pankhurst, C. E., A. S. Craig, and W. T. Jones. 1979. Effectiveness of *Lotus* root nodules. I. Morphology and flavolan content of nodules formed on *Lotus* pedunculatus by fast-growing *Lotus* rhizobia. *J. Exp. Bot.* 30:1085–1093.
15. Patel, J. J., and A. F. Yang. 1980. Light and electron microscope studies of nodule structure of alfalfa. *Can. J. Microbiol.* 27:36–43.
16. Rosenberg, C., P. Boistard, J. Dénarié, and F. L. Casse-Delbart. 1981. Genes controlling early and late functions in symbiosis are located on a megaplasmid in *Rhizobium meliloti*. *Mol. Gen. Genet.* 184:326–333.
17. Ruvkun, G., V. Sundaresan, and F. M. Ausubel. 1982. Directed transposon Tn5 mutagenesis and complementation analysis of *Rhizobium meliloti* symbiotic nitrogen fixation genes. *Cell.* 29:551–559.
18. Trinick, M. J. 1980. Relationships amongst the fast-growing rhizobia of *Lablab purpureus*, *Leucaena leucocephala*, *Mimosa* spp., *Acacia farnesiana* and *Sesbania grandiflora* and their affinities with other rhizobial groups. *J. Appl. Bacteriol.* 49:39–53.
19. Truchet, G., and J. Dénarié. 1973. Structure et activité réductrice d'acetylene des nodules de Luzerne (*Medicago sativa* L.) induits par des mutants de *Rhizobium meliloti* auxotrophes pour l'adenine et pour l'uracile. *C. R. Hébd. Séances Acad. Sci. Ser. D Sci. Nat.* 277:841–844.
20. Truchet, G., and J. Dénarié. 1973. Ultrastructure et activité réductrice d'acetylene des nodosités de Luzerne (*Medicago sativa* L.) induites par des souches de *Rhizobium meliloti* auxotrophes pour la leucine. *C. R. Hébd. Séances Acad. Sci. Ser. D Sci. Nat.* 277:925–928.
21. Truchet, M., M. Michel, and J. Dénarié. 1980. Sequential analysis of the organogenesis of lucerne (*Medicago sativa*) root nodules using symbiotically-defective mutants of *Rhizobium meliloti*. *Differentiation.* 16:163–172.
22. Tu, J. C. 1977. Structural organization of the rhizobial root nodules of alfalfa. *Can. J. Bot.* 55:35–42.
23. Van Brussel, A. A. N., T. Tak, A. Wetselaar, E. Pees, and C. A. Wijffelman. 1982. Small Leguminosae as test plants for nodulation of *Rhizobium leguminosarium* and other Rhizobia and Agrobacteria harbouring a leguminosarum Sym-plasmid. *Plant Sci. Lett.* 27:317–325.
24. Vance, C. P., L. E. B. Johnson, and G. Hardarson. 1980. Histological comparisons of plant and *Rhizobium* induced ineffective nodules in alfalfa. *Physiol. Plant Pathol.* 17:167–173.
25. Van Larebeke, N., G. Engler, M. Holsters, S. van den Elsecker, I. Zaenen, R. A. Schilperoort, and J. Schell. 1974. Large plasmid in *Agrobacterium tumefaciens* essential for crown gall-inducing activity. *Nature (Lond.)*. 252:169–170.