

Rhizobium fix Genes Mediate at Least Two Communication Steps in Symbiotic Nodule Development

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Abstract. To identify bacterial genes involved in symbiotic nodule development, ineffective nodules of alfalfa (*Medicago sativa*) induced by 64 different Fix⁻ mutants of *Rhizobium meliloti* were characterized by assaying for symbiotic gene expression and by morphological studies. The expression of leghemoglobin and nodulin-25 genes from alfalfa and of the *nifHD* genes from *R. meliloti* were monitored by hybridizing the appropriate DNA probes to RNA samples prepared from nodules. The mutants were accordingly divided into three groups. In group I none of the genes were expressed, in group II only the plant genes were expressed and in group III all three genes were transcribed. Light and electron microscopical analysis of nodules revealed that nodule development was halted at different stages in nodules induced by different

group I mutants. In most cases nodules were empty lacking infection threads and bacteroids or nodules contained infection threads and a few released bacteroids. In nodules induced by a third mutant class bacteria were released into the host cells, however the formation of the peribacteroid membrane was not normal. On this basis we suggest that peribacteroid membrane formation precedes leghemoglobin and nodulin-25 induction, moreover, after induction of nodulation by the *nod* genes at least two communication steps between the bacteria and the host plants are necessary for the development of the mature nodule. By complementing each mutant of group I with a genomic *R. meliloti* library made in pLAFR1, four new *fix* loci were identified, indicating that several bacterial genes are involved in late nodule development.

RHIZOBIA are able to cooperate with leguminous plants to fix atmospheric nitrogen. Nitrogen fixation takes place in symbiotic nodules, new plant organs developed for this purpose. Nodule cells harbor unique cell organelles, bacteroids, derived from *Rhizobium* bacteria which reduce dinitrogen to ammonia. The differentiation and function of nodules involves symbiosis-specific expression of both bacterial and plant genes (nodulin genes) in a highly coordinated manner, as well as communication between the two partners. This communication process seems to consist of signal exchange during nodule development. The molecular basis of these events are, at present, mostly unknown.

Recently, plant flavones were reported to act as signal molecules inducing the expression of bacterial nodulation (*nod*) genes (Firmin et al., 1986; Peters et al., 1986; Redmond et al., 1986). The *nod* genes are involved in a second communication step resulting in a meristematic cell division of roots culminating in nodule formation (for review see Kondorosi and Kondorosi, 1986). Govers et al. (1986) reported the induction of an early nodulin gene at this stage. They concluded that at least two signals from *Rhizobium* seem to be involved in the expression of symbiosis specific plant genes. However until now nothing is known about the second, or any further signals.

In *Rhizobium meliloti* exopolysaccharide-(EPS)¹ deficient mutants were described which induce only empty nodules on alfalfa, lacking infection threads and bacteroids (Finan et al., 1985; Leigh et al., 1985; Hynes et al., 1986). Lang-Unnasch et al. (1985) showed that these empty nodules do not contain late nodulins. Therefore it was concluded that EPS production might be involved as a signal for the expression of nodulins. With the aid of *Agrobacterium tumefaciens* chromosomal virulence genes, a second type of genes involved in nodule development (*ndv*) was identified (Dylan et al., 1986; Geremia et al., 1987). These mutants also form empty nodules, but their nodulin content was not reported.

In the above studies mutants altered in some non-symbiotic properties were isolated, then their symbiotic (Fix⁻) phenotype affecting nodule development was detected. In this paper we present the identification of several new *fix* loci which control nodule development by using a more general screening approach. In nodules induced by 64 Fix⁻ mutants of *R. meliloti*, obtained previously after random mutagenesis and assayed directly for Fix⁻ phenotypes (Forrai et al., 1983; Kondorosi et al., 1984), the lack of expression of late symbi-

1. Abbreviations used in this paper: EPS, exopolysaccharide; PBM, peribacteroid membrane.

otic genes was screened for. Using light and electron microscopy we demonstrate here that nodules failing to express late nodulin genes were halted in at least two different stages of nodule development, indicating that *Rhizobium fix* genes are involved in at least two communication steps during symbiotic nodule development. Moreover, by complementing these nodule development mutants with a genomic library, we have identified at least four different *fix* gene regions in *R. meliloti* which act before late nodulin expression.

Materials and Methods

Strains, Plasmids, and Media

Bacterial strains and plasmids are shown in Table I. Media and culture conditions have been described previously (Putnoky and Kondorosi, 1986). *Fix*⁻ mutants of *R. meliloti* 41 (AK631) were isolated in the following studies. The first sets of mutants were obtained using both chemical mutagenesis (NTG) and transposon Tn5 mutagenesis using pJB4II (Forrai et al., 1983). After Tn5 mutagenesis using plasmid pSUP101, another series of mutants were isolated (Kondorosi et al., 1984).

Table I. Bacterial Strains and Plasmids

Strains	Characteristics	Source or reference*
<i>R. meliloti</i> 41		
AK631	Wild type with compact colony morphology	This lab
AK684	Sm ^R derivative of AK631	This lab
GY896	<i>fix-535</i>	} <i>Fix</i> ⁻ derivatives of AK684 obtained by NTG mutagenesis
GY897	<i>fix-540</i>	
GY898	<i>fix-564</i>	
GY899	<i>fix-582</i>	
GY906	<i>fix-910</i>	} <i>Fix</i> ⁻ derivatives of AK631 obtained by Tn5 mutagenesis
GY911	<i>fix-1648</i>	
GY913	<i>fix-2061</i>	
GY914	<i>fix-2153</i>	
AK1282	<i>nifH::Tn5</i> of AK631	
AK1490	<i>fix-25</i>	} (AK684::Tn5)
AK1492	<i>fix-23</i>	
AK1540	<i>fix-525</i>	} Forrai et al., 1983
AK1541	<i>fix-613</i>	
AK1545	<i>fix-942</i>	
TF178	<i>fix-2</i>	
<i>R. trifolii</i>		
Resh 403		W. J. Broughton (University of Geneva, Geneva, Switzerland)
<i>A. tumefaciens</i>		
C58	wild type	C. Koncz
Plasmids		
pLAFR1	Tc ^R , cosmid derivative of pRK290	Friedman et al., 1982
Cosmid clones from the <i>R. meliloti</i> library		
PPP346	pLAFR1::common <i>nod</i> and <i>hsn</i> region	Putnoky and Kondorosi, 1986
PPP428	pLAFR1:: <i>fix23</i> region	This work
PPP662	pLAFR1:: <i>fix2</i> region	This work
PPP684	pLAFR1:: <i>fix2</i> region	This work
PPP720	pLAFR1:: <i>fix525</i> region	This work
PPP722	pLAFR1:: <i>fix2153</i> region	This work
PPP723	pLAFR1:: <i>fix2153</i> region	This work

DNA Isolation and Manipulation

Plasmid DNA was isolated according to Ish-Horowicz and Burke (1981). Isolation of DNA-fragments and use of restriction enzymes were as described in Maniatis et al. (1982).

RNA Isolation

Total RNA from *R. meliloti* cells or from alfalfa roots was isolated using the method of McGookin (1984a). Bacterial cells were opened by sonication. Total RNA from nodules was isolated in small scale preparations essentially as described by Cathala et al. (1983). 3–4 w after infection of the alfalfa seedlings, nodules were harvested into liquid nitrogen. For one preparation usually 40–50-mg nodules (~30 wild-type nodules) were used. Frozen nodules were ground to fine powder and immediately 650 μl of 5 M guanidine monothiocyanat, 10 mM EDTA, 50 mM TrisCl, pH 7.5, and 8% (vol/vol) mercaptoethanol was added. After centrifugation, 4 M LiCl was added to the supernatant to a final concentration of 3 M. RNA was precipitated overnight at 4°C and collected by centrifugation. The supernatant was carefully removed and the pellet was resuspended in 400 μl solubilization buffer containing 0.1% (wt/vol) SDS, 1 mM EDTA and 10 mM TrisCl pH 7.5. RNA was phenol extracted, ethanol precipitated, treated with DNase (Worthington Biochemical, Freehold, NJ), and reprecipitated (Maniatis et al., 1982) to yield 20–30 μg RNA from 40 to 50 mg nodules.

Quality and concentration of the RNA was analyzed in agarose gels according to the method of McGookin (1984b) and by spectrophotometric measurement.

Dot Blotting, Northern Blotting, and Hybridizations

RNA (5 µg/sample) dissolved in 10× SSC (1.5 M NaCl, 0.15 M sodium acetate, pH 7.0) was dotted on nitrocellulose filters using a Schleicher and Schuell Minifold I system. Northern blots were prepared after separating 10 µg of the RNA samples under denaturing conditions on 1% agarose gels containing 6% formaldehyde in 50 mM morpholino propane sulfonic (MOPS) acid, 1 mM EDTA, pH 7.0, as electrophoresis buffer. After electrophoresis the RNA was transferred onto Biodyne A membrane (Pall Corp., Glen Cove, NY) using 2 × SET (0.3 M NaCl, 4 mM EDTA, 60 mM TrisCl, pH 8.0) as transfer buffer according to Fuller and Verma (1984). DNA fragments used as hybridization probes were isolated from agarose gels and labeled with [³²P]ATP using the method of Feinberg and Vogelstein (1983). Prehybridization and hybridization conditions for the dot blot filters were as described earlier (Forrai et al., 1983). The Northern blots were hybridized with the denatured DNA probes at 42°C in a hybridization mixture containing 0.25 M Na₂HPO₄, 0.25 M NaCl, 1 mM EDTA, 7% (wt/vol) SDS, 50% (vol/vol) formamide and 10% (wt/vol) polyethylene glycol (PEG) according to Amasino (1986). After hybridization the Northern blots were washed twice for 30 min at 65°C in 2× SSC, 0.5% (wt/vol) SDS, and for 30 min at 65°C in 0.1× SSC, 0.5% (wt/vol) SDS, and exposed to Kodak XAR-5 film. Before rehybridization filters were washed in 20 mM Tris/HCl, pH 8.0, for 5 min at 100°C. Dot blot and Northern blot hybridization experiments were repeated at least three times with RNA samples from three different isolations.

Complementation Experiments and Plant Tests

pLAFR1 cosmid clones were transferred into Fix⁻ strains of *R. meliloti* by the method of Friedman et al. (1982). Transconjugants were selected on GTS medium containing 15 µg/ml tetracycline. When gene bank groups served as donor populations, hundreds of transconjugant colonies were suspended in 0.9% (wt/vol) NaCl and three to five plants were infected with 0.1–0.2 ml of these suspensions. Plant tests were carried out as described previously (Putnoky and Kondorosi, 1986). Experiments were evaluated 6–8 w after infection when effective symbiosis could be distinguished by the vitality and lack of chlorosis evident in N₂-fixing plants.

Phage and Calcofluor Tests

Phage 16-3 (Orosz et al., 1973) was used to test *Rhizobium* mutants for phage sensitivity. From a phage stock 10⁷ pfu were dropped onto the surface of top agar containing the *R. meliloti* strain to be tested. The calcofluor tests were carried out according to Leigh et al. (1985) using calcofluor brightener 28 (Sigma Chemical Co., St. Louis, MO).

Microscopic Studies

Nodules were harvested 3–4 w after inoculation. For each strain at least 15–30 nodules from 10–30 different plants were collected. No fewer than 10 nodules per strain were examined by light microscope and 5–10 of these nodules were further analyzed by EM. Nodule samples were fixed overnight at 0°C in 4% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer pH 6.8. After rinsing them twice in this buffer the nodules were postfixed in 2% (wt/vol) aqueous OsO₄ at 0°C for 5–6 h (Hirsch et al., 1982). After two to three rinses in cold distilled water, the samples were dehydrated in a graded acetone series at 0°C and embedded in Spurr standard low-viscosity medium (Spurr, 1969). For light microscopy thick sections (0.5 µm) were cut and stained with 0.1% (wt/vol) methylene blue. For EM ultrathin sections were collected on 300-mesh copper grids and stained with uranyl acetate-lead citrate (Reynolds, 1963). Grids were examined in a JEM 100 B electron microscope operated at 80 KV.

Results

Identification of Fix⁻ Mutants Which Fail to Induce Leghemoglobin and Nodulin-25 Expression

To study the role of *R. meliloti* genes in the development of

symbiotic nodules, 64 Fix⁻ mutants were characterized by monitoring the expression of symbiotic plant and bacterial genes in nodules induced by these mutants. Fix⁻ mutants were obtained from independent mutagenesis experiments (see Materials and Methods) and had previously been tested for their growth properties on glucose-ammonium and fumarate-nitrate media. To exclude auxotrophs only mutants unimpaired in growth on both media were used in further experiments.

Total RNA was isolated from the different nodule types using a small scale method developed for this purpose. These RNA samples were screened for the presence of mRNAs from leghemoglobin (Lb), nodulin-25 (*nod25*), and *nifHD* genes, by hybridizing with ³²P-labeled DNA fragments. The two plant DNA probes originated from an alfalfa cDNA clone bank representing a Lb gene and another nodulin gene (*nod25*). *nod25* encodes a 25-kD symbiosis-specific protein with unknown function (Kiss et al., 1987a, b). The third probe was a 2.5 kb Sal I fragment of the plasmid pID 1 (Banfalvi et al., 1981) carrying two structural genes (*nifHD*) of *R. meliloti* nitrogenase (Torok and Kondorosi, 1981). As controls, total RNA samples from uninfected alfalfa roots, from vegetatively growing wild-type *R. meliloti* AK631, and from nodules induced by AK631 were used.

First, dot blot experiments were performed in which RNA samples were fixed onto nitrocellulose filters and subsequently hybridized with the probes mentioned above. Most RNA samples hybridized with all of the three probes. Six samples hybridized only with the *nod25* and Lb probes while in ten samples no mRNA's from the examined genes were detected (data not shown).

Since we were preferentially interested in mutations blocking early nodule development, mutants which failed to induce nodulin expression were chosen for further studies. By Northern blot experiments we reexamined the results achieved in dot blot experiments. When nodules were harvested for RNA isolation, representative nodules were also collected for microscopic studies (see below). Besides analyzing RNA from group I mutants, representatives of the other two mutant groups were also chosen as controls (Fig. 1). When a dilution series of total RNA isolated from wild-

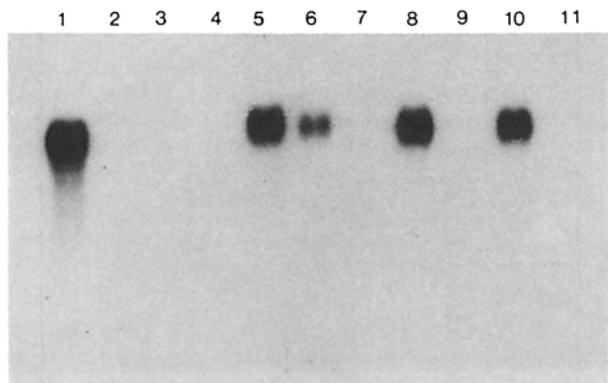


Figure 1. Expression of nodulin-25 gene in ineffective nodules. Autoradiography of Northern blot with RNA isolated from wild-type nodules (lane 1), from alfalfa root (lane 2) and from ineffective nodules induced by strains GY897 (lane 3), GY906 (lane 4), GY913 (lane 5), GY911 (lane 6), GY914 (lane 7), AK1490 (lane 8), AK1540 (lane 9), AK1282 (lane 10), and TF178 (lane 11).

type nodules was transferred onto hybridization filters, the mRNA of *nod25* gene was still visible in the sample containing 0.5 µg RNA (data not shown), indicating that in these assays at least 5% of the original messenger RNA level was detectable.

On the basis of the dot blotting and Northern blotting experiments the mutants were divided into three groups. Representatives of groups I, II, and III used for further analysis are listed in Table II. In group I neither nodulin (Lb and *nod25*) nor *nif* gene mRNAs were detectable, while in group II we could not detect any *nifHD* gene expression but both Lb and *nod25* were expressed. In group III all the three types of mRNAs were detectable. No mutant strain was found showing expression of only one of the nodulins.

Symbiotic gene expression in nodules induced by *Agrobacterium tumefaciens* and *R. trifolii* transconjugants harboring all essential *nod* genes of *R. meliloti* on cosmid clone pPP346 was also analyzed. RNA from either nodules did not show hybridization with any of the three probes used (data not shown).

Microscopic Analysis of *M. sativa* Nodules Induced by Wild-type *R. meliloti* and Its *Fix*⁻ Derivatives

The stages where nodule development was halted by mutations of group I were characterized in microscopic studies. As controls wild-type nodules formed by AK631 as well as ineffective nodules induced by mutant strains belonging to group II and group III were analyzed.

Nodules induced by wild-type strain AK631 were pink, cylindrical, or spherical, and 0.5–1.5-mm long. They had a distinct apical meristem located at the distal end followed by three other histological characteristic zones designated early, late and senescent zone (Fig. 2 a). Bacteroids in the late zone increased significantly in size (from 1.5:3.0 µm in the early zone to 3.0:12.0 µm in the late zone) and became long rod and y-shaped (Fig. 2 b). The host cytoplasm was homogeneous, electron dense, and contained numerous polysomes

and a few starch grains. These observations are consistent with previous reports (Vance et al., 1980; Patel and Young, 1981; Hirsch et al., 1983).

Although none of the nodules induced by the *Fix*⁻ mutants used in this study fixed N₂, they showed a variety of nodule shapes, except those induced by AK1282 and AK1490. Interestingly most of the examined mutants induced two types of nodules. In some nodules we could not detect infection threads and bacteroids while others contained infection threads and a variable number of invaded host cells. All ineffective nodules contained more starch grains than wild-type nodules. The ratio of empty nodules and nodules containing bacteroids was different and characteristic for each mutant strain (Table II).

Group I Mutants

In most of the examined nodules induced by GY897 or AK1545 multiple meristems were observed. The vascular bundles were found along the periphery or in the middle of these nodules. Infection threads and bacteria were observed in the subepidermal and epidermal layers, but never within the body of the nodule. All of these nodules were empty, lacking infection threads and bacteroids in the host cytoplasm (Fig. 2 c). Empty nodules induced by all other *Fix*⁻ mutants had similar structures.

In a few nodules of AK1492 and TF178 infection threads packed with deteriorated bacteria and some senescent bacteroids in the host cytoplasm were observed (cf. Fig. 2 b and Fig. 3, a and b). One nodule induced by AK1492 contained a few host cells occupied by many elongate bacteroids smaller than wild type and among these senescent bacteroids were also observed.

In contrast to the above morphology nodules of GY898, GY899, GY906, GY914, and AK1541 contained infection threads with healthy bacteria and some host cells were filled with senescent bacteroids (Fig. 3 c).

Nodules infected by AK1540 showed a very exciting

Table II. Representatives of *Fix*⁻ Mutants Characterized by RNA Hybridization

Strain	Mutation	Mutagenesis	Lb	nod 25	nif H,D	Empty nodules*	Phage test	Complementing cosmid
						%		
Group I								
GY897	<i>fix-540</i>	(1)	–	–	–	100 (15)	s	NT
AK1545	<i>fix-942</i>	(3)	–	–	–	100 (10)	r	pPP428
AK1492	<i>fix-23</i>	(3)	–	–	–	85 (20)	r	pPP428
TF178	<i>fix-2</i>	(2)	–	–	–	86 (15)	s	pPP662, pPP684
GY906	<i>fix-910</i>	(1)	–	–	–	80 (10)	s	pPP723
GY914	<i>fix-2153</i>	(1)	–	–	–	66 (12)	s	pPP722, pPP723
GY898	<i>fix-564</i>	(1)	–	–	–	85 (10)	r	pPP428
GY899	<i>fix-582</i>	(1)	–	–	–	80 (10)	r	pPP428
AK1541	<i>fix-613</i>	(3)	–	–	–	60 (10)	r	pPP428
AK1540	<i>fix-525</i>	(3)	–	–	–	25 (24)	s	pPP720
Group II								
GY911	<i>fix-1648</i>	(1)	+	+	–	70 (20)	s	NT
GY896	<i>fix-535</i>	(1)	+	+	–	30 (10)	s	NT
AK1490	<i>fix-25</i>	(3)	+	+	–	0 (12)	s	NT
Group III								
AK1282	<i>nifH</i>	(4)	+	+	NT	0 (12)	s	NT
GY913	<i>fix-2061</i>	(1)	+	+	+	0 (10)	s	NT

(1) NTG mutagenesis (Forrai et al., 1983); (2) Tn5 mutagenesis by pJB4JI (Forrai et al., 1983); (3) Tn5 mutagenesis by pSUP1011 (Kondorosi et al., 1984); (4) site-directed Tn5 mutagenesis (Forrai et al., 1983).

* The number of examined nodules is given in (). NT, not tested.

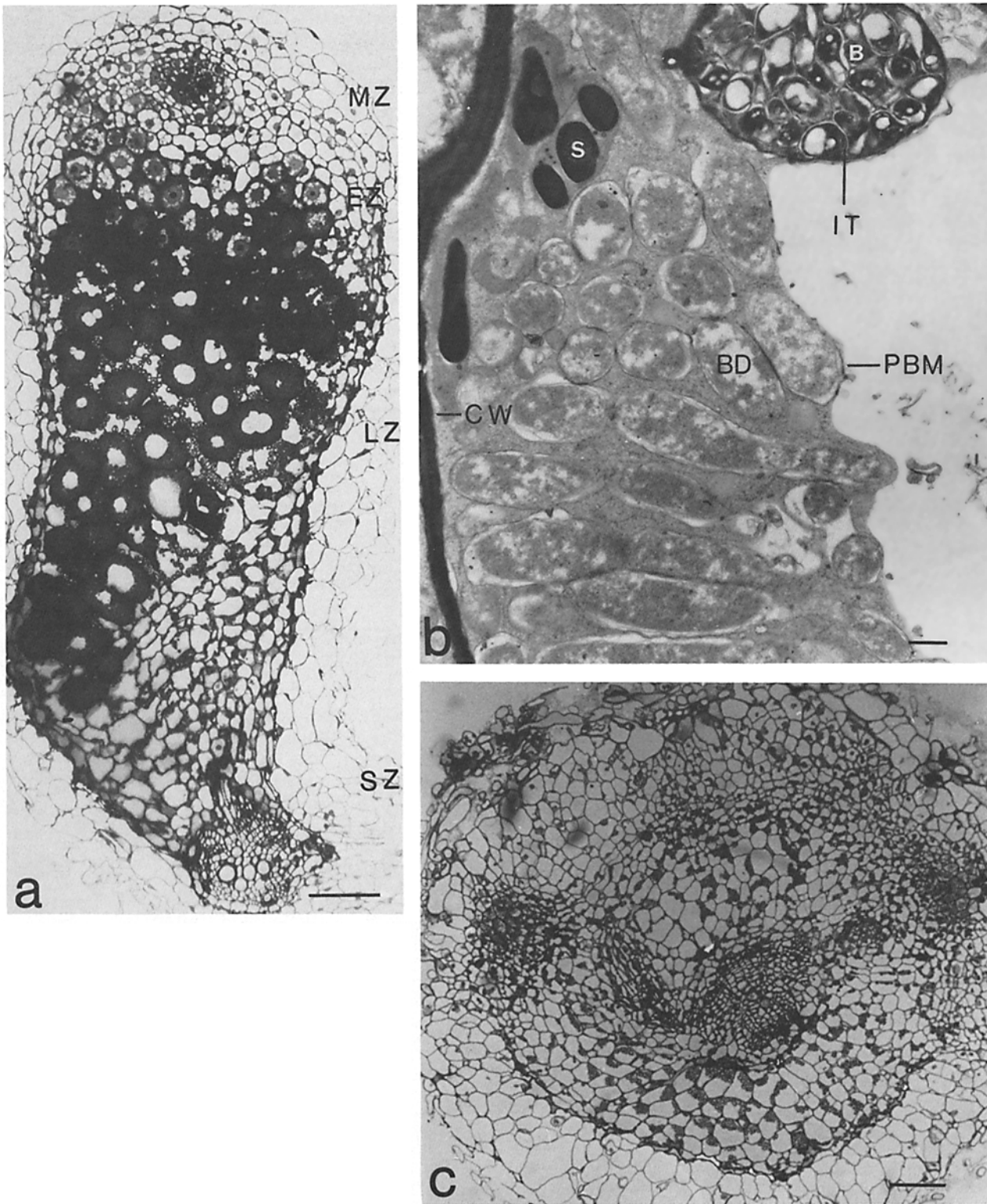


Figure 2. Structure of nodules induced by wild type (AK631) and an ineffective strain of *R. meliloti*. (a) Longitudinal section of wild-type nodule. (b) Ultrastructure of wild-type symbiotic cell. (c) Section of an ineffective nodule induced by AK1545. *MZ* meristematic zone, *EZ*, *LZ*, and *SZ*, early, late, and senescent symbiotic zones, respectively; *VB*, vascular bundles; *S*, starch granules; *B*, bacteria; *BD*, bacteroids; *IT*, infection thread; *PBM*, peribacteroid membrane; *CW*, cell wall. Bars (*A* and *C*) 100 μm ; (*B*) 1 μm .

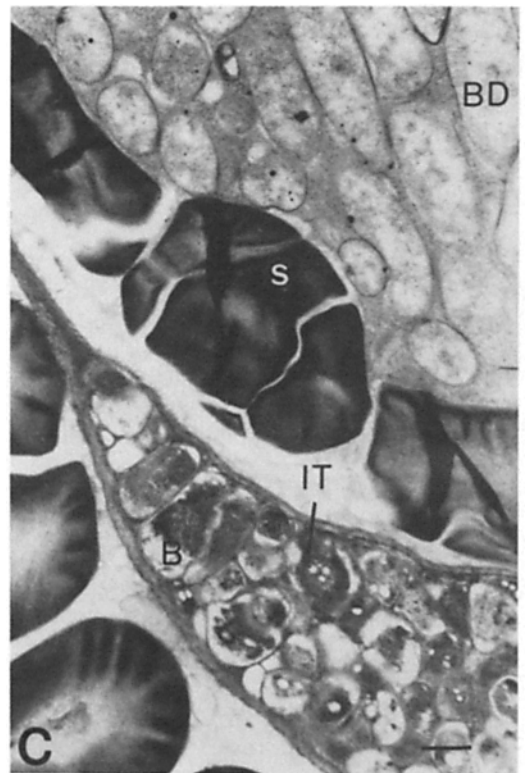
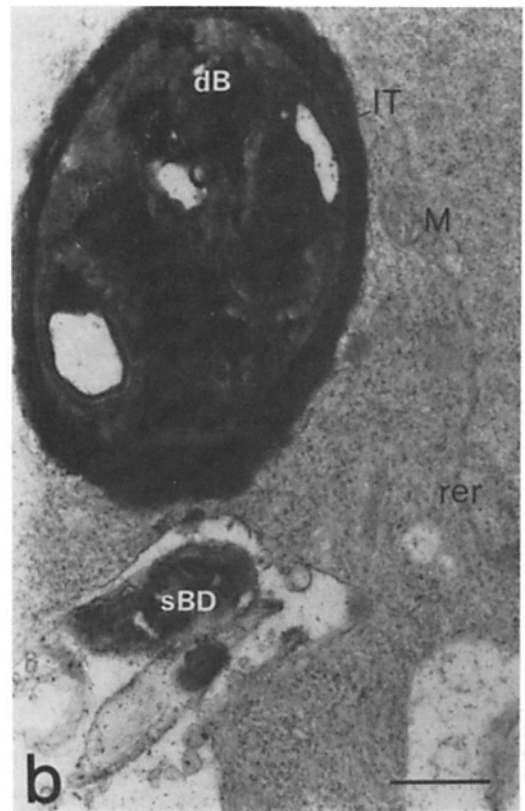
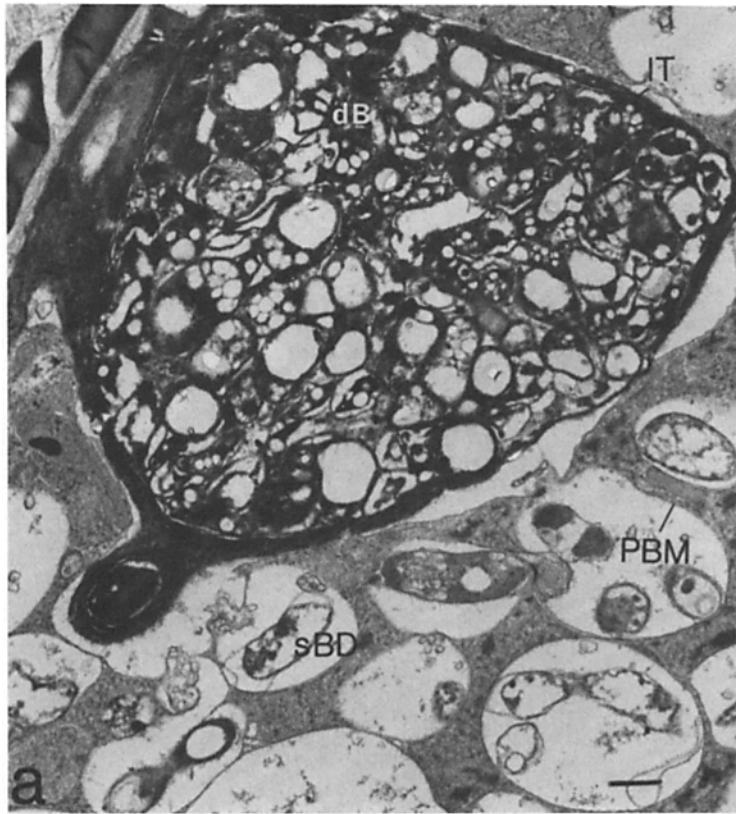


Figure 3. Ultrastructure of nodules invaded by *Fix*⁻ mutants of *R. meliloti*. (a) AK1492 (b) TF178 (c) AK1541. *IT*, infection thread; *BD*, bacteroids; *PBM*, peribacteroid membrane; *dB*, deteriorating bacteria; *S*, starch granules; *M*, mitochondrion; *rer*, rough endoplasmic reticulum; *sBD*, senescent bacteroid. Bar, 1 μ m.

phenotype. Only ~25% of the nodules were empty. The other nodules were cylindrical or spherical and contained infection threads similar to the wild type. Some of the host cells were invaded by bacteroids smaller than the wild type,

some of the bacteroids were senescent. In contrast to all other mutants the peribacteroid membranes were never clearly visible. Early, late, and senescent zones were not well distinguishable (Fig. 4, *a* and *c*).

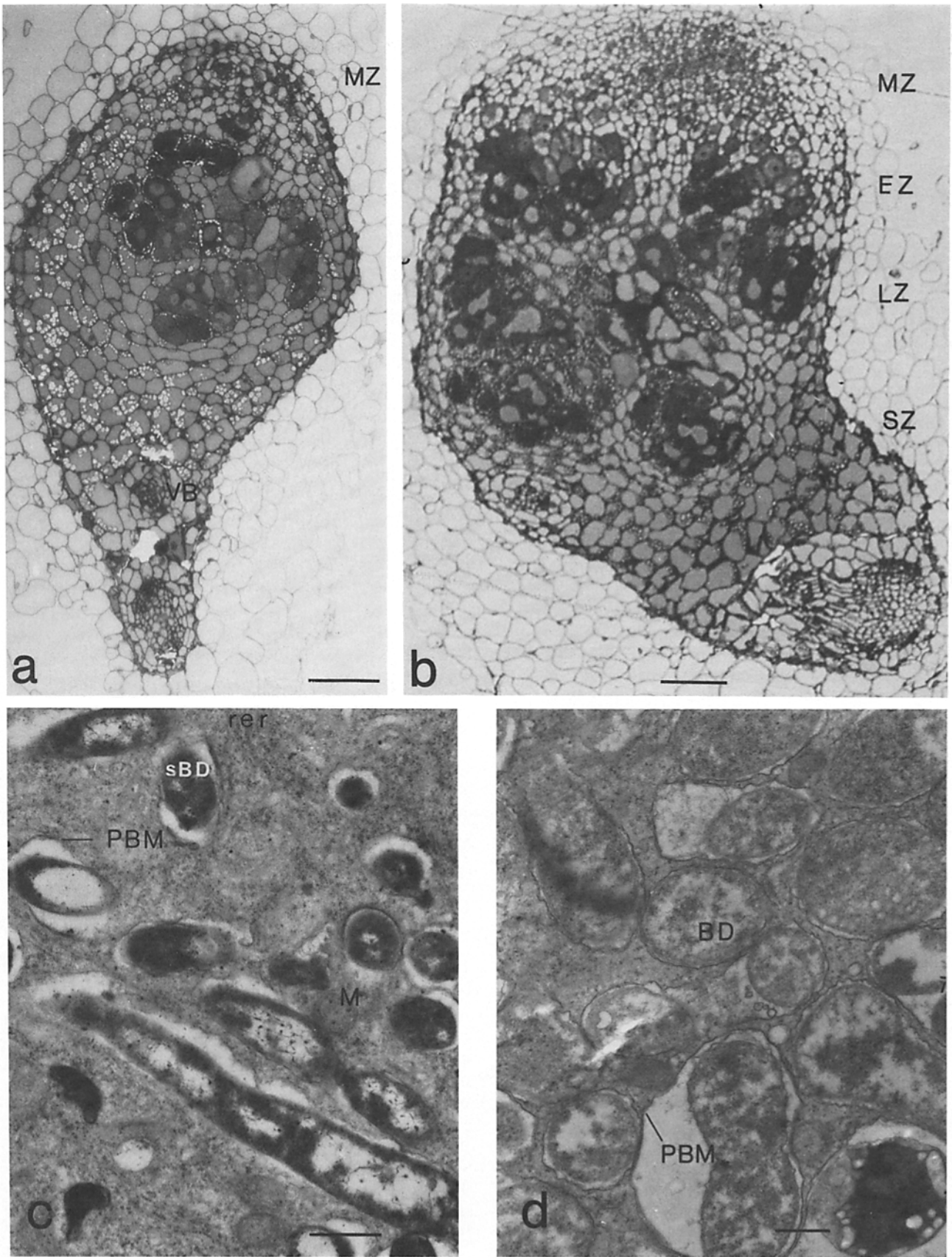


Figure 4. Structure of nodules induced by AK1540 and GY911. (a) and (b) Longitudinal sections of nodules induced by AK1540 and GY911, respectively. *MZ*, Meristematic zone; *EZ*, *LZ*, early and late symbiotic zone; *SZ*, senescent zone; *VB*, vascular bundles. (c and d) Ultrastructure of cells invaded by AK1540 and GY911, respectively. *sBD*, Senescent bacteroid; *rer*, rough endoplasmatic reticulum; *M*, mitochondrion; *PBM*, peribacteroid membrane; *BD*, bacteroid. Bars (a and b) 100 μm ; (c and d) 1 μm .

Group II Mutants

GY911. About 70% of the nodules were empty. The invaded nodules contained normal appearing infection threads, however, bacteroids were not elongate like the wild types (Fig. 4 b). In contrast to the AK1540-nodules, senescent bacteroids were never observed in the early symbiotic zone (Fig. 4 d). From light microscopic pictures of infected nodules formed by either AK1540 or GY911 the number of invaded host cells was calculated. About twice as many invaded cells were found in the case of GY911.

GY896. About 70% of the nodules contained normal looking infection threads, unusually small bacteroids, and a senescent zone which was more extensive than in wild-type nodules (Fig. 5, a and c).

AK1490. Nodules were white, in most cases spherical, sometimes cylindrical. The four histological zones were clearly distinguishable. All nodules contained infection threads and bacteroids, but the bacteroids were smaller and rapidly deteriorated. Empty granules were observed in some bacteroids.

Group III Mutants

AK1282. The histological characteristic zones were not clearly apparent. The early symbiotic zone was markedly larger, while the senescent zone seemed to be absent. The early symbiotic zone consisting of cells with a large number of small vacuoles and rough endoplasmic reticulum, was markedly increased. Host cells contained elongated, but unusually small bacteroids.

GY913. Bacteroids were senescent and the host cells were drastically abnormal, even in young cells close to the meristem. Often several bacteroids were found to be enclosed by a single bacteroid membrane, which is never observed with the wild-type bacteroids (Fig. 5, b and d).

***R. trifolii* (pPP346) and *A. tumefaciens* (pPP346).** Nodules induced by these strains harboring the *nod* and *hns* genes of *R. meliloti* were spherical, nodule-like, tumor-like, or misshapen. Their morphological structures were similar to those induced by strains GY897 and AK1545 or those induced by *A. tumefaciens* carrying the *R. meliloti* Sym-plasmid (Wong et al., 1983).

Complementation of *Fix*⁻ Mutants by a *R. meliloti* Genomic Library

As shown above, all of the group I mutants were unable to induce at least two nodulin genes and formed ineffective nodules in which the development of symbiosis was blocked at an early stage. To isolate the wild-type alleles of the mutated genes, direct complementation experiments were performed using a *R. meliloti* genomic library made in the cosmid vector pLAFRI. Earlier we isolated pLAFRI clones carrying *nod* genes by direct complementation of Nod⁻ *R. meliloti* mutants (Putnoky and Kondorosi, 1986). Plant tests provide a simple method to select for Nod⁺ bacteria carrying the complementing clones from a Nod⁻ population which carries the whole genomic library. Selection for clones complementing *Fix*⁻ mutants seemed to be more difficult since all bacteria, albeit harboring different clones of the library, are able to form nodules and therefore have the same chance to establish symbiosis.

In preliminary experiments *Fix*⁻ and *Fix*⁺ bacteria were mixed in different ratios and these populations were used to

inoculate plants. All plants established an effective symbiosis when the *Fix*⁻/*Fix*⁺ ratio was 10:1, whereas most of the nodules were ineffective when the ratio was increased to 100:1. Based on this observation a method to isolate *fix* regions was developed as follows. (a) The genomic library was divided into batches containing 10 independent clones. These gene bank groups were mated with the appropriate *Fix*⁻ strain and transconjugant populations were tested for nitrogen fixation ability on alfalfa plants. (b) Independent clones of batches which proved to harbor a wild-type allele were tested in a second experiment to identify the individual complementing clones.

(c) The identified clones were then introduced into all *Fix*⁻ mutants to test whether these DNA regions can also complement other mutations. (d) *Fix*⁻ mutants not complementable upon introduction of the identified cosmid clones were then used in further experiments to isolate other symbiotic regions.

In the first series of experiments a clone complementing the *fix-23* mutation in AK1492 was identified (pPP428). The isolated DNA region (*fix23* region) was also able to complement *Fix*⁻ mutations of other mutants (GY898, GY899, AK1541, AK1545) suggesting that the *fix-582*, *fix-564*, *fix-613* and *fix-942* mutations are located on the *R. meliloti* DNA region which is carried in pPP428 (Table II).

A further DNA region involved in nodule development was isolated by complementing the *fix-2* mutation of strain TF178. The *fix-2* mutation was previously mapped between the *gly-1* and *met-2* markers of the *R. meliloti* chromosome (Forrai et al., 1983). The identified overlapping cosmid clones pPP662 and pPP684 complemented the *fix-2* mutation but not others in group I (Table II).

In similar experiments two additional *fix* regions were identified. Cosmid clone pPP720 carried the wild-type allele of the *fix-525* mutation (AK1540) while two overlapping cosmids, pPP722 and pPP723, were able to complement the *fix-2153* mutation of GY914. pPP723 could also restore the *Fix*⁺ phenotype of GY906 (Table II).

Exopolysaccharide Deficiency and Phage Resistance of *Fix*⁻ Mutants

Surprisingly, the wild-type strain AK631 and all of its mutant derivatives with the exception of TF178, proved to be calcofluor "dark" (Leigh et al., 1985) suggesting deficiency in EPS production. AK631, having a compact colony morphology is used as the wild-type strain in our laboratory. AK631 was isolated from strain *R. meliloti* 41 which forms mucoid colonies due to the production of EPSs, and consequently is calcofluor "bright." AK631 and *R. meliloti* 41, however, did not show differences in their symbiotic properties on alfalfa plants.

To monitor phage sensitivity, the temperate rhizobial phage 16-3 was used. Both *R. meliloti* 41 and AK631 are sensitive to 16-3. Five strains, all belonging to group I, were found to be phage resistant. When cosmid clone pPP428, isolated using AK1492, was introduced into these five strains, both phage sensitivity and *Fix*⁺ phenotype were restored.

Discussion

We have described here several new *fix* loci of *R. meliloti* (*fix2*, *fix23*, *fix525*, and *fix2153*) where mutations result in

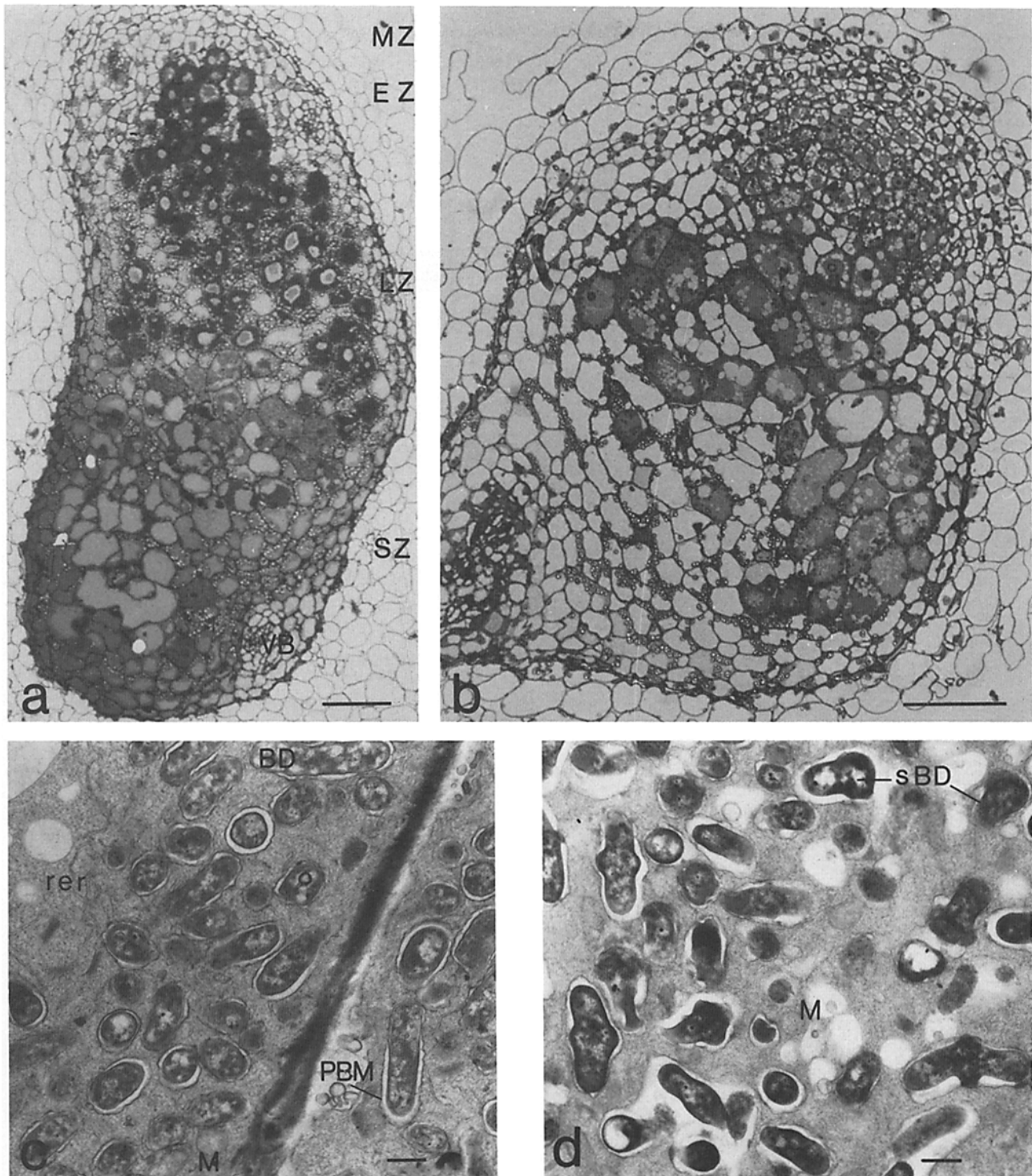


Figure 5. Structure of nodules induced by GY896 and GY913. (a and b) Longitudinal sections of nodules induced by GY896 and GY913, respectively. *MZ*, meristematic zone; *EZ*, *LZ*, early and late symbiotic zone; *SZ*, senescent zone; *VB*, vascular bundles. (c and d) Ultrastructure of cells invaded by GY896 and GY913, respectively. *sBD*, Senescent bacteroid; *rer*, rough endoplasmic reticulum; *M*, mitochondrion; *PBM*, peribacteroid membrane; *BD*, bacteroid. Bars (a and b) 100 μ m; (c and d) 1 μ m.

a similar nodule defect, that is the lack of late nodulin expression. Light and electron microscopical analysis of nodules induced by these mutants (group I) allowed to dissect the pathway of nodule development leading to late nodulin expression into several stages. Our results show that several different *fix* genes are involved in infection thread formation, release of bacteria from infection threads and normal peri-

bacteroid membrane formation, all preceding the expression of the late nodulins leghemoglobin and nodulin-25. It seems, therefore, that after induction of nodulation by the *nod* genes at least two (or more) communication steps between *Rhizobium* and the host plant are required for the development of the mature nodule.

The characterization of mutations in the different *fix* loci

suggest a rather complex interaction of the two partners during nodule development. Two mutations of group I, *fix-540* and *fix-942*, resulted in empty nodules lacking infection threads and bacteroids. The *fix-942* mutation occurs in the *fix23* region, while a complementing region for *fix-540* has not been identified yet. Changes in nodule morphology caused by these mutations are similar to those of *Exo*⁻ mutants isolated from *R. meliloti* SU47 (Leigh et al., 1985; Finan et al., 1985; Hynes et al., 1986) on the basis of their ability to interact with calcofluor. The calcofluor dye binds to the mature acidic EPS of *R. meliloti*. Under UV illumination wild-type colonies (*Exo*⁺) exhibit a green-blue fluorescence while *Exo*⁻ colonies are dark due to the lack of EPS. All *Exo*⁻ mutants of *R. meliloti* SU47 formed ineffective nodules with the exception of two mutants from group D which were incompletely dark. *R. meliloti* 41 produces a large amount of EPS and was found to be calcofluor "bright." Interestingly, its compact colony derivative AK631 used as wild-type strain in our laboratory, and the *Fix*⁻ mutants originating from AK631, proved to be calcofluor "dark." One reason for this phenotype may be that AK631 carries a mutation similar to that described in the *exo D* locus of *R. meliloti* SU47. Nevertheless, AK631 was found not to produce EPS (Petrovics, G., unpublished results) suggesting that other cell surface components may also be responsible for the same function in infection and that the *Fix*⁻ phenotype of an *exo* mutation may depend on the genetic background.

The *fix-2* and *fix-23* mutations are located at opposite sides of the *R. meliloti* chromosome (Forrai et al., 1983; Banfalvi, Z., unpublished results). Most of the induced nodules were empty, while the invaded nodules contained infection threads packed with deteriorating bacteria and bacteroids formed in host cells seemed to be immediately senescent, suggesting the activation of a plant defense mechanism (Vance, 1983).

In contrast to the above two mutations other mutations did not abolish normal infection thread formation within the invaded nodules and fell into two DNA regions (*fix23*, *fix2153*). The wild-type allele of *fix-910* mutation is present only on pPP723 while *fix-2153* mutation can be complemented by both pPP722 and pPP723 suggesting that these two mutations represent two different *fix* genes within the same region (designated *fix2153*). All *Fix*⁻ mutations localized to the *fix23* region conferred phage resistance on these strains, suggesting an alteration in the cell surface.

AK1540 carries an interesting mutation, *fix-525*, which allows the formation of infection threads and host cell invasion in most of the nodules (75%), but the expression of the two examined nodulin genes was not detectable (less than 5% of wild type). In contrast, strain GY911 formed mainly empty nodules (70%), but Lb and *nod25* expression was detectable. Since AK1540 induces about twice as many invaded nodules as GY911, the amount of RNA extracted from invaded host cells should be comparable. The signal strength obtained with GY911 RNA corresponded to ~40% of the wild-type signal. From these data we conclude that either there is no expression of the *nod25* and Lb genes in AK1540 nodules or the expression is reduced to less than ~10% of the level found in GY911 nodules.

Fortin et al. (1987) described in the soybean-*Bradyrhizobium japonicum* symbiosis that bacterial release from the infection thread and peribacteroid membrane (PBM) formation is essential for the expression of nodulin-24, whereas

nodulin-26 is expressed independent from PBM formation. Bacterial release from the infection thread was suggested as possible trigger for Lb induction in soybean (Sengupta-Gopalan et al., 1986). Recently, Gloudemans et al. (1987) described nodules induced by a *Bradyrhizobium japonicum* mutant lacking Lb and other late nodulins. The bacteria in those nodules were rarely released and infected cells appeared to be collapsed. As reported earlier (Noel et al., 1982), PBM formation seemed to be blocked in the host cells. The mutation in AK1540 results in a similar phenotype and blocks nodule development at the same stage. Thus, bacterial mutations can cause at least two types of nodule morphology before Lb expression: (a) empty nodules lacking infection threads and (b) nodules with infection threads and occasionally invaded host cells. These findings suggest the existence of at least two independent communication steps during nodule differentiation. Different bacterial gene products are necessary (a) to induce infection thread formation and (b) at a later stage for the induction of Lb and *nod25* expression. The *fix525* region of *R. meliloti* isolated by complementing *fix-525* mutation may code for genes involved directly or indirectly in a communication step between host plant and bacteria.

In nodules induced by *A. tumefaciens* and *R. trifolii* both carrying the common *nod* (Kondorosi et al., 1984) and *hsn* (Horvath et al., 1986) genes of *R. meliloti* on pPP346, no expression of Lb and *nod25* could be detected as well as the nodules were empty. Since the *R. trifolii* strain still harbored all its *fix* genes, these findings suggest the existence of host-specific recognition steps after the early events of nodulation directed by the *nod* genes. Govers et al. (1986) reported that Lb expression was detectable, when *R. leguminosarum nod* genes were introduced into a Sym-plasmid cured *R. phaseoli* strain. This finding is not surprising because of the close relatedness of *R. leguminosarum* and *R. phaseoli* which have recently been proposed to belong to the same species but represent different biovars (van Veen et al., 1986).

Our screening approach allowed to identify not only *Fix*⁻ mutations abolishing late nodulin expression, but also *Fix*⁻ mutants unable to express late symbiotic genes (*nif* genes) in *Rhizobium*. We found that bacterial mutants of group II were able to induce the expression of Lb and *nod25* but *nif* mRNA was not detected. When the structure of a group II nodule (GY896) was compared to the morphology of a group III nodule (GY913), no significant differences in the number of invaded cells or in the number of bacteroids were found, implying that the lack of *nif* mRNA is probably due to the lack of *nif* gene expression and not due to a decreased bacteroid content of a given ineffective nodule. Earlier reports (Zimmermann et al., 1983; Fuller and Verma, 1984) found late nodulin expression uncoupled from *nif* gene expression. In *R. meliloti* the *nifA* gene product was shown to regulate the transcription of *nif* genes (Szeto et al., 1984). However *ntrC*, a regulatory gene of *nifA* in *Klebsiella pneumoniae*, has no influence on the expression of *nif* genes in the symbiotic state (Szeto et al., 1987). Thus, the regulation of *nifA* in *R. meliloti* is still not understood. Preliminary results indicate that some mutations of group II are not located in the *nif* region where the *nifA* gene can be found (e.g., *fix-25* mutation of AK1490; Dusha et al., 1987). Therefore these mutants may be useful to identify further genes which may be involved in controlling the expression of *nif* genes.

In most of our mutants the nodulin genes as well as the *nif* genes were expressed (group III). Since the mutants were obtained through three independent random mutagenesis experiments (Forrai et al., 1983; Kondorosi et al., 1984), these findings suggest that far more genes exist which are directly needed for nitrogen fixation than genes which code for nodule formation and differentiation.

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