The Plant Cell, Vol. 2, 139–151, February 1990

Cooperative Action of *Rhizobium meliloti* Nodulation and Infection Mutants during the Process of Forming Mixed Infected Alfalfa Nodules

Dieter Kapp, Karsten Niehaus, Jürgen Quandt, Peter Müller,[1] and Alfred Pühler[2]

University of Bielefeld, Faculty of Biology, Department of Genetics, Postbox 8640, D-4800 Bielefeld 1, Federal Republic of Germany

Alfalfa plants co-inoculated with *Rhizobium meliloti* nodulation (Nod$^-$) and infection mutants deficient in exopolysaccharide production (Inf$^-$EPS$^-$) formed mixed infected nodules that were capable of fixing atmospheric nitrogen. The formation of infected nodules was dependent on close contact between the inoculation partners. When the partners were separated by a filter, empty Fix$^-$ nodules were formed, suggesting that infection thread formation in alfalfa is dependent on signals from the nodulation and infection genes. In mixed infected nodules, both nodulation and infection mutants colonized the plant cells and differentiated into bacteroids. The formation of bacteroids was not dependent on cell-to-cell contact between the mutants. Immunogold/silver staining revealed that the ratio of the two mutants varied considerably in colonized plant cells following mixed inoculation. The introduction of an additional *nif/fix* mutation into one of the inoculation partners did not abolish nitrogen fixation in mixed infected nodules. The expression of *nif*D::*lac*Z fusions additionally demonstrated that mutations in the nodulation and infection genes did not prevent the *nif* genes from being expressed in the mutant bacteroids.

INTRODUCTION

Bacterial mutants that block nodule formation at different stages have been used to analyze the development of leguminous root nodules (Vincent, 1980; Newcomb, 1981; Long, 1984; Rolfe and Shine, 1984). For the *Rhizobium meliloti*/alfalfa symbiosis, we have isolated rhizobial mutants and categorized them into three classes according to the point at which they impede the formation of effective nodules (Müller, 1987; Müller et al., 1988). Into the first class fall nodulation mutants (Nod$^-$) that cannot proceed beyond the early steps of nodulation (Long, Buikema, and Ausubel, 1982; Meade et al., 1982; Debelle et al., 1986; Horvath et al., 1986; Müller, 1987). The nodulation and host specificity genes have been identified and located on megaplasmid 1, the symbiotic plasmid of *R. meliloti* (Banfalvi et al., 1981; Rosenberg et al., 1981). In the second class are infection mutants (Inf$^-$) that are capable of nodule formation, although the typical infection threads do not form (Finan et al., 1985; Leigh et al., 1987; Müller et al., 1988; Niehaus and Pühler, 1988; Putnoky et al., 1988). These nodules are termed empty nodules because the central nodule tissue is devoid of bacteroids. *R. meliloti* infection mutants showed altered exopolysaccharide (EPS) production (Müller et al., 1988). Other researchers, therefore, name such mutants Exo mutants (Finan et al., 1985; Leigh et al., 1985; Long, McCune, and Walker, 1988). In infection mutants, the inability to synthesize EPS results from mutations on megaplasmid 2 (Finan et al., 1986; Hynes et al., 1986) or on the chromosome (Leigh, Signer, and Walker, 1985; Finan et al., 1986). The third class of *R. meliloti* mutants, termed nitrogen fixation mutants (Nif$^-$ or Fix$^-$), induce and colonize the root nodule as does the wild-type strain, but fail to fix atmospheric nitrogen and usually degenerate prematurely (Hirsch, Bang, and Ausubel, 1983; Aguilar, Kapp, and Pühler, 1985). These mutants have mutations in genes located on megaplasmid 1 (Banfalvi et al., 1981; Rosenberg et al., 1981).

Co-inoculation experiments have been employed to overcome the symbiotic defects of mutant rhizobia (Rolfe and Gresshof, 1980; Rolfe et al., 1980; Chen and Rolfe, 1987). Alfalfa plantlets, co-inoculated with a mixture of Nod$^-$ and Inf$^-$EPS$^-$ mutants, developed nodules of wild-type appearance that fixed atmospheric nitrogen (Klein et al., 1988; Müller et al., 1988). These nodules contained both inoculation partners in a ratio of approximately 1:1 (Müller et al., 1988) and are designated "mixed infected nodules." The formation of functional nodules implies the ability of the two inoculation partners to complement each

[1] Current address: Philipps Universität Marburg, Department of Biology/Botany, Karl-von-Frisch Strasse, D-3550 Marburg, Federal Republic of Germany.

[2] To whom correspondence should be addressed.

other on a cellular level. The Inf⁻EPS⁻ strain provides the nodulation functions, whereas the Nod⁻ strain mediates infection thread formation (Müller et al., 1988).

In this paper, the development of mixed infected alfalfa nodules is analyzed in more detail. The aim was to determine whether close contact between the nodulation and infection mutant is a prerequisite for the formation of infection threads. Immunogold/silver staining was used to distinguish the bacteroids of the co-inoculation partners from each other and to monitor their distribution in colonized plant cells. The determination of which partner (the Nod⁻ or Inf⁻EPS⁻) was capable of nitrogen fixation in mixed infected nodules was made by introducing additional *nif/fix* mutations into one of the co-inoculation partners.

RESULTS

The Formation of Alfalfa Nodules Infected by Inf⁻EPS⁻ and Nod⁻ Mutants Requires Close Contact between the Mutants

As previously shown (Klein et al., 1988; Müller et al., 1988), alfalfa plants co-inoculated with nodulation (Nod⁻) and infection (Inf⁻EPS⁻) mutants resulted in the formation of functional nodules. Both inoculation partners could be reisolated from these mixed infected nodules. We investigated whether close contact between the two inoculation partners was a prerequisite for nodule infection to occur. We designed experiments in which the inoculation partners were physically separated from each other by a filter and then tested their nodule-forming capability, as shown in Figure 1. The filter separation experiments were evaluated on three levels as follows: (1) visual scoring for nodulation and plant growth, (2) acetylene reduction assays, and (3) morphological analysis of root segments and nodules, either by Feulgen staining and clearing with phenol or by embedding, thin sectioning, and subsequent light microscopy. The results are summarized in Table 1.

In a control experiment (Table 1, experiment A) alfalfa seedlings were co-inoculated with the infection mutant 0540 and the nodulation mutant 2526. As expected, wild type-like nodules that fixed nitrogen for the plant appeared.

In experiment C, the inoculation partners were separated from each other by a membrane filter. Infection mutant 0540 was streaked on the agar surface, the alfalfa seedling was added, and a filter carrying the nodulation mutant 2526 was placed on top of the root system (experiment C, Table 1). Thirty plants were inoculated by this method. All plantlets formed exclusively ineffective nodules, which showed the typical empty nodule morphology (Finan et al., 1985; Leigh et al., 1987; Niehaus and Pühler, 1988; Müller et al., 1988). These results indicated that the nodulation mutant above the filter was unable to assist the infection mutant to invade alfalfa nodules.

The same nodule morphology was obtained when the position of the inoculation mutants was reversed. When the nodulation mutant 2526 was streaked on the agar surface and the filter carrying the infection mutant 0540 was placed on top of the root system, empty alfalfa nodules only were found (Table 1, experiment D). Again, 30 plants were tested in this experiment. Of these 30 plants, 22 formed nodules, and nodulation occurred exclusively on those roots that were located directly below the filter. The part of the roots that was not in direct contact with the filter, i.e., that grew beyond the area covered by the filter, did not form nodules (Figure 1). The same result was observed in a control experiment in which the root system was uninoculated and the filter carrying mutant 0540 was placed above (Table 1, experiment B). Contamination of the roots by Inf⁻EPS⁻ mutant 0540 cells from above the filter could be excluded because this would have resulted in the formation of effective nodules, as demonstrated by the first control experiment (see Table 1, experiment A). When bacteria adhering to the nodule surface were plated out, only the inoculant Nod⁻ strain 2526 was found to be present, further indicating that no contamination by strain 0540 had occurred. Two conclusions were drawn from these results. First, because the infection mutant was able to induce alfalfa nodule formation despite being separated from the root by a filter (Table 1, experiments B and D), we propose that a molecular signal passed

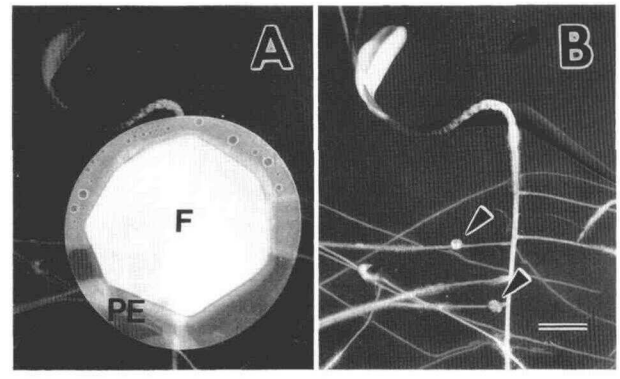

Figure 1. Filter Separation Technique.

The Nod⁻ strain 2526 that is in contact with the alfalfa root system was separated by a sterile filter (F) from the Inf⁻ EPS⁻ inoculation partner 0540. (Compare also Table 1, experiment C.) Both photographs show the same root system 3 weeks after inoculation.
(A) The root system covered by the filter (F) that bears Inf⁻EPS⁻ mutant 0540. PE, edge of the filter where paraffin was applied.
(B) Same root system with the filter removed after 3 weeks of incubation. Empty nodules (arrows) with typical spherical morphology have developed. Scale bar = 5 mm.

Table 1. Co-inoculation Experiments in which One Partner Was Separated from the Other and from the Root System by a Filter

Experiment	*R. meliloti* Co-inoculation Partner: In Contact with the Alfalfa Root System	*R. meliloti* Co-inoculation Partner: Excluded from the Alfalfa Root System by Filter	Type of Nodule Induced[a]	Fixation[b]
A	2526 and 0540	None	Wild type	+
B	None	0540 (Inf^-EPS^-)	Empty	−
C	0540 (Inf^-EPS^-)	2526 (Nod^-)	Empty	−
D	2526 (Nod^-)	0540 (Inf^-EPS^-)	Empty	−

[a] Analysis done by light microscopy of Feulgen-stained, phenol-cleared whole nodules and of thin-sectioned, plastic-embedded samples.
[b] N_2 fixation properties of whole plants were determined by the acetylene reduction assay and by evaluating plant growth 3 weeks after inoculation. +, the plants reduced acetylene and appeared healthy and green; −, acetylene was not reduced, the plants were yellow, and their growth was stunted.

through the filter to trigger the formation of nodules. Second, the nodulation mutant was unable to invade the developing empty alfalfa nodules in the absence of close contact with the Inf^-EPS^- mutant.

The general conclusion from the filter separation experiments is that a mixed inoculum of Nod^- and Inf^-EPS^- mutants can form infected nodules only if close, presumably cell-to-cell, contact exists between the two inoculation partners and the host.

The Plant Cells of Mixed Infected Alfalfa Nodules Are Colonized by Bacteroids of Both Inoculation Partners

Reisolation experiments have demonstrated that both inoculation partners, the nodulation and infection mutants, are present in nodules of co-inoculated plants (Müller et al., 1988). Because reisolation experiments reflect the colony-forming ability of strains (McRae, Miller, and Berndt, 1989), it is not possible to ascertain which partner finally colonizes the plant cells to form bacteroids. To elucidate this, immunocytochemical techniques were used to distinguish the co-inoculation partners from each other and to localize their respective bacteroids inside the infected plant cells.

The nodulation mutant strain *R. meliloti* 36070 was used in combination with the infection mutant 0540 in the co-inoculation. Mutant 36070 has a large deletion in megaplasmid 1 that includes the *nod* and *nif* HDK regions. Alfalfa plantlets co-inoculated with these mutants developed functional nodules, demonstrating that active nitrogenase was expressed in the Inf^-EPS^- mutant 0540. Thus, the presence or absence of the enzyme nitrogenase inside bacteroids could be used to distinguish the bacteroids of the co-inoculation partners from each other. This enzyme was detected in bacteroids by indirect immunogold labeling of thin sections of embedded nodule tissue. The gold label was amplified by the silver enhancement procedure to enable visualization under the light microscope.

Thus, the bacteroids of the two mutants could be distinguished from each other at both the electron and light microscopic levels, as shown in Figure 2. Nitrogenase-positive bacteroids, i.e., bacteroids of Inf^-EPS^- mutant 0540, showed dense labeling of nitrogenase following indirect immunogold staining, whereas nitrogenase-negative bacteroids, i.e., bacteroids of the nodulation/fixation mutant 36070, only had a few gold particles due to non-specific binding (Figure 2C). When 5-nm colloidal gold was used as secondary label and subsequent silver enhancement was applied, the differences in labeling were even more pronounced (Figures 2A and 2B).

In mixed infected nodules, most of the bacteroids observed were well-developed, wild type-like bacteroids (Figures 2B to 2D). Interestingly, of the labeled 0540 bacteroids, a small portion (less than 5% of the bacteroids analyzed) showed an abnormal morphology (Figure 2D). The dense gold labeling of these bacteroids showed, however, that the expression of nitrogenase did not seem to be affected by their abnormal morphology.

Most of the infected cells contained bacteroids of both strains 36070 and 0540. The ratio between the two strains varied greatly in infected cells, as can be seen in the group of cells in Figure 2B. In a number of host cells, one co-inoculation partner predominated. This was most visible in cells that harbored mainly the nitrogenase-negative bacteroids of the nodulation/fixation mutant 36070 (Figure 2B). The reduced or absent nitrogen fixation activity of the bacteroid population was also reflected by the accumulation of starch in the periphery of these plant cells. More large starch grains were observed in cells containing a low proportion than in cells containing a high proportion of nitrogenase-positive bacteroids of mutant 0540 (Figure 2B). Accumulation of starch is a feature of ineffective root nodules in alfalfa (Truchet, Michel, and Dénarié, 1980;

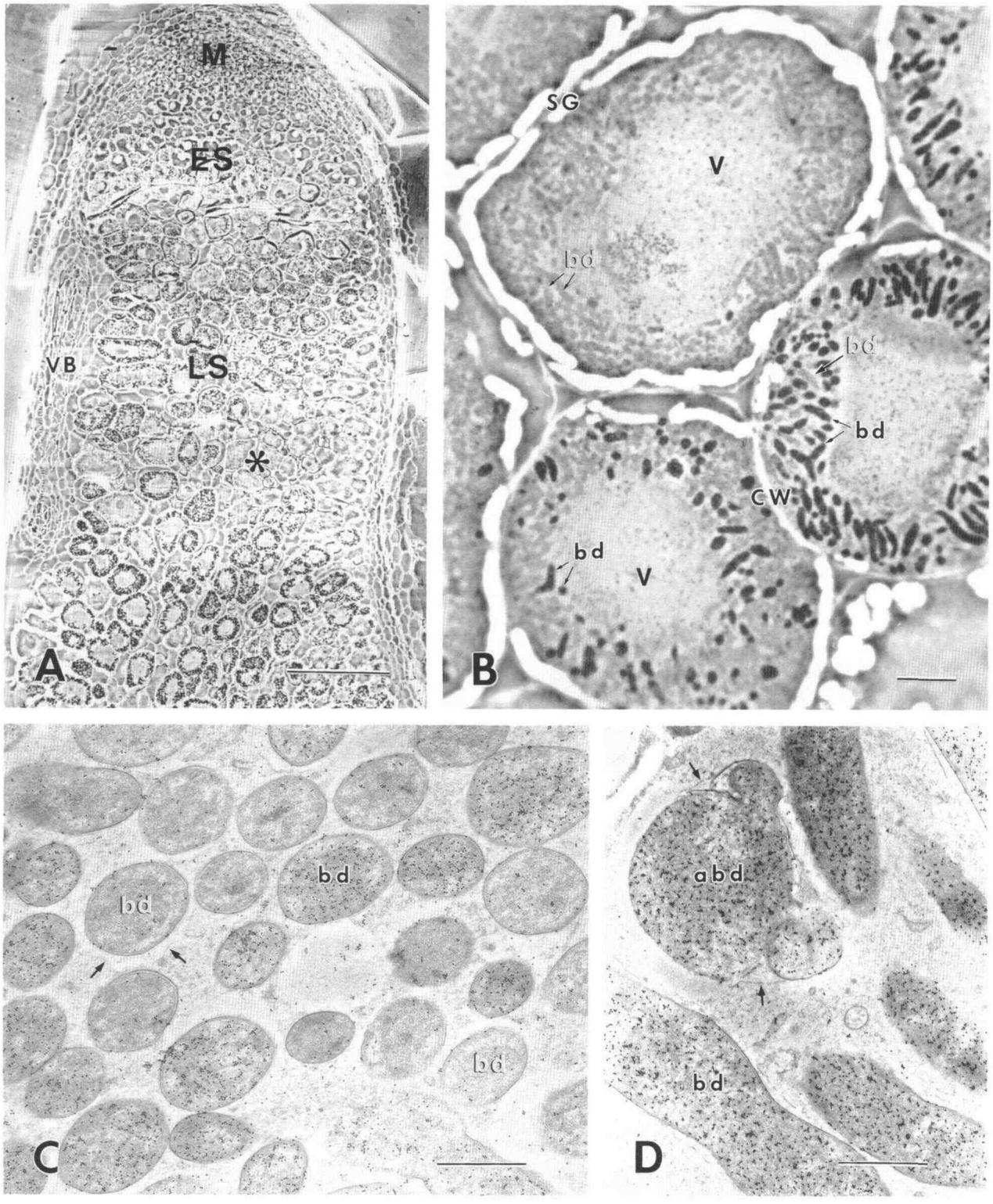

Figure 2. Immunolocalization of the *R. meliloti* Co-inoculation Partners 0540 (Inf$^-$EPS$^-$) and 36070 (Nod$^-$Fix$^-$) in Mixed Infected Alfalfa Nodules 3 Weeks after Inoculation.

Table 2. Fixation Properties of *R. meliloti* Nodulation and Infection Mutants in Alfalfa Co-inoculation Experiments

	Partner I				Partner II				
		Genotype[a]				Genotype[a]			Co-inoculated Plants,
Experiment	Designation	*nod*	*inf*	*nif/fix*	Designation	*nod*	*inf*	*nif/fix*	Fixation[b]
A	2526	−	+	+	RmJQ13	+	−	+	+
B	36070	−	+	−	RmJQ13	+	−	+	+
C	2526	−	+	+	RmJQ18	+	−	−	+
D	2526	−	+	+	RmJQ19	+	−	−	+
E	2526	−	+	+	RmJQ20	+	−	−	+

[a] +, gene region indicated is wild type; −, gene region indicated is mutated. For details, see Table 4.
[b] N_2 fixation properties of whole plants were determined by the acetylene reduction assay and by evaluating plant growth 3 weeks after inoculation. For each combination, 30 plants were tested. +, the plants reduced acetylene and appeared healthy and green.

Hirsch et al., 1983; Aguilar et al., 1985; Engelke et al., 1989). Our experiments have shown that the amount of starch in an infected host cell does reflect the extent of the nitrogen fixation activity of its bacteroid population.

A cluster of neighboring, infected host cells can be seen in Figure 2A (asterisk), all of which harbor a high proportion of, possibly exclusively, unlabeled mutant 36070 bacteroids. The accumulation of large starch granules inside these cells further indicates the absence of nitrogen-fixing 0540 bacteroids.

In Mixed Infected Alfalfa Nodules, Infection as well as Nodulation Mutants Differentiate into N_2-Fixing Bacteroids

The co-inoculation experiment in the previous section showed that, in mixed infected nodules, the infection mutant 0540 was able to synthesize the nitrogenase complex and also fix nitrogen. The nodulation and fixation mutant 36070 differentiated into bacteroids, despite the large deletion in its symbiotic plasmid. It remains to be determined whether the infection mutant only or whether the nodulation mutant carrying an intact set of *nif/fix* genes is also capable of nitrogen fixation. In a way similar to that of Klein et al. (1988), this was tested in co-inoculation experiments using infection and nodulation mutants that contained a second mutation in the fixation genes. The combinations of strains used in the co-inoculation experiments are listed in Table 2.

Combination A was a control experiment. The infection mutant RmJQ13 was derived from mutant 0540. In mutant RmJQ13, the Tn*5* was replaced by Tn*5*-Gm, which introduced another selectable marker to enable the subsequent construction of double mutants. Inf^-EPS^- Fix^- mutants were isolated after the Tn*5*-Gm marker was transduced by phage M12 from the mutant RmJQ13 to different Tn*5*-induced Fix^- mutants. Mutant RmJQ13, in combination with nodulation mutant 2526, formed fully effective, mixed infected nodules. The results of combination B confirmed the observations of the previous section: co-inoculation with the infection mutant RmJQ13 and the nodulation/fixation mutant 36070 resulted in mixed infected, effective nodules, demonstrating that the infection mutant RmJQ13,

Figure 2. (continued).

Mature bacteroids of 0540 (black bd) show intense labeling after immunogold staining [**(C)** and **(D)**] and immunogold-silver staining [**(A)** and **(B)**] of nitrogenase component II. 36070 bacteroids (white bd) show only background labeling.

(A) Light micrograph of a median longitudinal thin section, showing the overall distribution of both mutants in the infected cells of the symbiotic zone. Labeling intensity increases from early symbiotic (ES) to late symbiotic (LS) zone, reflecting the induction of *nif* genes during bacteroid development. Note the cluster of host cells (asterisk) harboring predominantly unlabeled 36070 bacteroids. M, meristem; VB, vascular bundle. Scale bar = 200 μm.

(B) Several infected cells from the same nodule as in **(A)**, showing considerable differences in the relative bacteroid number of the two strains, as well as the number of starch grains (SG) present. CW, cell wall; V, central vacuole. Scale bar = 10 μm.

(C) Electron micrograph of mutant bacteroids in a portion of a mixed infected alfalfa nodule cell from the late symbiotic zone. Unlike bacteroids of mutant 36070, 0540 bacteroids show dense labeling with 15-nm colloidal gold particles. In most cases, both mutant bacteroids were well developed and individually enclosed by peribacteroid membranes (arrows). Scale bar = 1 μm.

(D) Electron micrograph of aberrant (abd) and wild type-like (bd) bacteroids of mutant 0540 from the late symbiotic zone. The aberrant bacteroids are more spherical rather than elongated and appear to be enclosed by a common peribacteroid membrane (arrows). Scale bar = 1 μm.

like mutant 0540 from which it was derived, was able to form nitrogen-fixing bacteroids.

In combinations C, D, and E, infection mutants were used that contained a second mutation in the *nif/fix* genes. The mutants RmJQ18, RmJQ19, and RmJQ20 all contain the *inf* (0540)::Tn*5*-Gm mutation of strain RmJQ13 and, in addition to this, the *nif/fix* mutations *nif*H::Tn*5*, *fix*A::Tn*5*, and *nif*N::Tn*5*, respectively. Co-inoculation of alfalfa with these strains and the nodulation mutant 2526 resulted in mixed infected, Fix^+ nodules in all cases. This proved that the nodulation mutant 2526 is able to differentiate into nitrogen-fixing bacteroids.

To investigate which partner was able to fix nitrogen in mixed infected nodules, the activity of the *nif*H promoter in infection and nodulation mutants was tested. To enable this test, the *nif*HD-*lac*Z fusion present on plasmid pJQ71 was introduced into the *R. meliloti* strains 2011 (wild type), Rm *nif*A-d8 (Fix^-), 2526 (Nod^-), 0540 (Inf^-EPS^-), and 0544 (Nod^-). The plasmid was integrated into the genome by homologous recombination, which did not destroy the *nif*HDK operon. The nodules induced by *R. meliloti* wild-type 2011, by 2011-pJQ71, and by the *nif*-regulatory mutant Rm *nif*A-d8-pJQ71 were removed from the plant roots and their contents were assayed and compared. A high level of β-galactosidase activity was found only in those nodules induced by 2011-pJQ71. Because both vegetative cells of 2011-pJQ71 and nodules induced by Rm *nif*A-d8-pJQ71 did not exhibit increased levels of β-galactosidase activity, the assay could be used to monitor the transcriptional activity of the symbiotic *nif*H promoter. Combinations of nodulation and infection mutants with plasmid pJQ71 present in one of the two co-inoculation partners were tested. In each case, mixed infected nodules showed a high β-galactosidase activity (Table 3). These results were obtained not only in combinations in which the nodulation mutant was 2526, but also for 0544, which carried a mutation in the *hsn* region, presumably *nod*H (Müller, 1987). From these observations, it can be concluded that, in mixed infected nodules, both the Nod^- and the Inf^-EPS^- co-inoculation partners are able to form nitrogen-fixing bacteroids.

DISCUSSION

Infection Thread Formation of Alfalfa Nodules Is Dependent on Signals Generated by the Nodulation and Infection Genes of *R. meliloti*

In this paper, we analyzed the cooperative action of *R. meliloti* Nod^- and Inf^-EPS^- mutants during the formation of mixed infected alfalfa nodules. In a previous paper (Müller et al., 1988), the following model was suggested: The Nod^+ Inf^-EPS^- partner is contributing the nodulation functions, whereas the Nod^- Inf^+EPS^+ partner is responsible for normal infection thread formation. This model does not, however, indicate whether the infection step can be carried out by the Nod^- mutant alone or whether both inoculation partners are required. To elucidate this, the mutants were separated from each other by a filter such that one partner had access to the root and substances could diffuse freely between the symbiotic partners.

In an experiment in which the Nod^- mutant was in contact with the alfalfa root system and the Inf^-EPS^- mutant was separated from both the *nod* mutant and the root by the filter, only empty nodules were observed. Empty nodules also formed in a corresponding control when the root system below the filter remained uninoculated. This implies, first, that the Inf^-EPS^- strain is able to induce alfalfa root nodules from a distance without cell-to-cell contact between the micro- and macrosymbiont. A diffusible substance produced by the microsymbiont probably passes through the filter and induces meristematic activity in susceptible cortical plant cells. The absence of nodulation on some plants below the filter could have been

Table 3. *nif* Gene Activity of Nodulation and Infection Mutants in Mixed Infected Nodules

Partner I				Partner II				Co-inoculated Plants, Phenotype	
	Genotype[a]				Genotype[a]				
Designation	*nod*	*inf*	*nif*	Designation	*nod*	*inf*	*nif*	Fix[b]	β-Gal[c]
2526	−	+	+	0540	+	−	+	+	−
2526	−	+	+	0540-pJQ71[d]	+	−	+	+	+
2526-pJQ71[d]	−	+	+	0540	+	−	+	+	+
0544	−	+	+	0540	+	−	+	+	−
0544	−	+	+	0540-pJQ71[d]	+	−	+	+	+
0544-pJQ71[d]	−	+	+	0540	+	−	+	+	+

[a] +, gene region indicated is wild type; −, gene region indicated is mutated. For details, see Table 4.

[b] N_2 fixation properties of whole plants were determined by the acetylene reduction assay and by evaluating plant growth 3 weeks after inoculation. For each combination, 30 plants were tested. +, the plants reduced acetylene and appeared healthy and green.

[c] For each combination, 10 nodules were removed from different plants, broken open, and tested for β-galactosidase activity. β-Galactosidase activity is due to transcription from a translation fusion of the *nif*D gene to the *Escherichia coli lac*Z gene, and, thus, monitors transcription from the *nif*H promoter.

[d] Plasmid pJQ71 (carrying a *nif*D::*lac*Z fusion that is transcribed from the p_{nifH} promoter) was integrated into the *R. meliloti* genome by homologous recombination, without destroying the *nif*HDK operon. The strains were therefore designated 0540-pJQ71, etc.

due to air bubbles trapped between the filter and the root that impeded the diffusion.

That cortical cell divisions could be induced from a distance was concluded earlier from microscopy studies of the first observable plant responses following inoculation (Calvert et al., 1984; Dudley, Jacobs, and Long, 1987) as well as from studies of nodules induced by noninvasive mutants or heterologous, Sym-plasmid carrying strains (Truchet et al., 1980; Hirsch et al., 1984; Truchet et al., 1984; Finan et al., 1985). Bauer et al. (1985) showed, by serial sectioning, that cell divisions could be induced in the host root cortex by the homologous *Rhizobium* even though physical contact was prevented by an interposed Millipore membrane, but they did not mention whether empty nodules were formed. The authors postulated that a diffusible substance induced cell divisions in the host root cortex. Attempts have been made to isolate and characterize the factors produced by *Rhizobium* in response to its specific host plant (Yao and Vincent, 1969, 1976; Solheim and Raa, 1973; Bhuvaneswari and Solheim, 1985; Banfalvi and Kondorosi, 1989). None of the factors tested was able to induce cortical cell divisions in its specific host (Bauer et al., 1985; Banfalvi and Kondorosi, 1989). Schmidt et al. (1988) demonstrated that the *nod*AB gene products of *R. meliloti* are involved in generating low molecular weight compounds that stimulate mitosis in various plant protoplasts.

Structures that fulfill most of the histological and molecular criteria of empty nodules can also be induced in the absence of *R. meliloti* by treatment of axenic alfalfa seedlings with auxin transport inhibitors (Hirsch et al., 1989). Empty nodules can even occur spontaneously at a low rate (Truchet et al., 1989). Alfalfa apparently contains an intrinsic program for the development of empty nodules. The triggering of this program does not depend on cell-to-cell contact between the microsymbiont and the host but can occur either spontaneously, by treatment with auxin transport inhibitors, or by a still uncharacterized host-specific, diffusible substance generated by the *Rhizobium*.

The Nod^- mutant in contact with the root system was unable to infect the empty nodule in the filter separation experiment. *Rhizobia* carrying a mutation in one of the common *nod* genes are defective in both nodule meristem induction and in root hair curling (Kondorosi, Banfalvi, and Kondorosi, 1984; Rossen, Johnston, and Downie, 1984; Djordjevic, Schofield, and Rolfe, 1985; Downie et al., 1985; Jacobs, Egelhoff, and Long, 1985; Debelle et al., 1986). The latter is considered to be a prerequisite for successful infection of leguminous root hairs (Yao and Vincent, 1969, 1976). Marked root hair curling cannot be induced if a dialysis membrane prevents *Rhizobium* from being in contact with the root (Yao and Vincent, 1976), and *R. meliloti nod* mutants did not have their symbiotic functions restored by the addition of isolated root hair curling factors (Banfalvi and Kondorosi, 1989). Interestingly, in the *Bradyrhizobium japonicum*/soybean system, effective nodules that contained almost exclusively the Nod^- mutant have been observed when soybean was co-inoculated with a Nod^- mutant and a noninvasive, auxotrophic mutant (Rossbach et al., 1989). This apparently indicates that the *nod*-dependent signal(s) provided by the noninvasive mutant is only required in a very early stage of infection. When taken together, it can be concluded that these signal(s)—although diffusible components are involved—can only assist the infection of the Nod^- strain when provided at the cellular level.

In the reciprocal filter separation experiment, when the Inf^-EPS^- mutant was in contact with the roots and the Nod^- mutant separated by the filter, only empty nodules were formed. This implies that the Nod^- mutant, which is able to complement the Inf^- phenotype of the Inf^-EPS^- mutant 0540 in the filterless co-inoculation, is unable to do so when close contact is prevented. Although the factor(s) provided by the Nod^- strain remain uncharacterized, we postulate it to be the acidic EPS or a related compound, which is not produced by the Inf^-EPS^- partner. That *R. meliloti* EPS compounds are significant in nodule invasion was confirmed by Glazebrook and Walker (1989) and Zhan et al. (1989). These authors showed that *R. meliloti* has the ability to synthesize an alternative EPS that is able to function in the place of the normal EPS in root nodule invasion. Because EPS were originally isolated from *R. meliloti* culture supernatants by a procedure involving filtration (data not shown), it can be assumed that the filters employed in our experiments did not impede the EPS (or related compounds) from moving from the Nod^- to the Inf^-EPS^- strain.

The failure of the EPS producing Nod^- mutant to functionally complement the Inf^-EPS^- mutant below the filter suggests the requirement of either high localized concentrations of EPS compounds, not able to be supplied from a distance, or the permanent presence of EPS compounds during the infection process. Possible roles for the rhizobial exopolysaccharides in the symbiosis have been suggested by a number of authors, e.g., Bauer (1981), Halverson and Stacey (1986), Chen and Rolfe (1987), Djordjevic et al. (1987), Leigh et al. (1987), Long (1989), and Glazebrook and Walker (1989). These roles include recognition of susceptible root hairs, protection of rhizobia from plant defense, functioning as a carrier of extracellular enzymes, forming the infection thread matrix, and functioning as a signal to the plant. Many of these postulated functions would require the presence of the EPS-producing microsymbiont throughout the entire infection process.

The results reported show that the formation of infected nodules by Nod^- and Inf^-EPS^- mutants requires close contact and cooperation between the symbiotic partners. However, from our experiments it is not possible to discern how close the contact between the symbiotic partners must be. We assume that cell-to-cell contact is required.

On the genetic level, it can be concluded that infection thread formation is dependent on intact nodulation as well

as intact infection gene regions. The intact nodulation gene region is present in the Inf⁻EPS⁻ mutant that induces the formation of nodules, and the intact infection gene region is harbored in the Nod⁻ mutant. The experiments suggest that, during infection thread formation, both inoculation partners produce exportable substances which interact with the host tissue. The Nod⁻ partner most probably supplies EPS or a related compound. The Inf⁻EPS⁻ mutant, in contrast to this, produces still uncharacterized compound(s).

R. meliloti Nod⁻ as well as Inf⁻EPS⁻ Mutants Can Colonize Mixed Infected Alfalfa Nodules and, Independently of Each Other, Differentiate into Bacteroids

In mixed infected nodules, we were able to distinguish the two strains from each other and localize their positions by the immunogold staining of nitrogenase. These mixed infected nodules were induced by co-inoculation of alfalfa with an *R. meliloti* Inf⁻EPS⁻ mutant (0540) and a specific Nod⁻ mutant (36070) that had both *nod* and *nif/fix* genes deleted. Only 0540 bacteroids that expressed an active nitrogenase were labeled (Figure 2). In general, individual, infected plant cells varied widely with respect to the ratio of bacteroid types that they contained. Plant cells containing predominantly the bacteroids of one co-inoculation partner could be identified. These findings suggest that the close contact between Nod⁻ and Inf⁻EPS⁻ mutants necessary for infection thread formation is not necessary during the subsequent steps of nodule development. These steps include bacterial release from the infection thread, individual enclosure into peribacteroid membranes, multiplication, and differentiation into (nitrogen-fixing) bacteroids. The occurrence of clusters of host cells containing predominantly—possibly exclusively—one inoculation partner could be due to the localized separation of both strains from each other in the growing infection thread (Rolfe and Gresshof, 1980). All cells infected by a particular part of the infection thread harbor, as a consequence, a corresponding population of bacteroids. Absolute separation of the mutants from each other during infection thread growth seems to be rare because most of the infected cells harbored both mutants. Nevertheless, this result indicates that the presence of both mutants is not continually required in the growing infection thread once nodule invasion has commenced. Furthermore, microscopy indicated that the differentiation into bacteroids is largely independent of a functional *nod* gene region. It can also be concluded that, in this combination of mutants, the ability of mutant 0540 to differentiate into nitrogen-fixing bacteroids is not affected by the absence of the acidic, Cellufluor-binding EPS. However, a small portion of abnormal bacteroids, identified by their dense staining for nitrogenase, was observed among the 0540 bacteroid population. This means that, although bacteroid development is not impaired in general by the Nod⁻ and Inf⁻EPS⁻ mutations, late bacteroid development can be affected, especially in the Inf⁻EPS⁻ mutant. The abnormal bacteroids observed in our investigation were very similar to those shown by Klein et al. (1988). These authors co-inoculated alfalfa with an *R. meliloti* EPS⁻ mutant and, in contrast to our experiment, an *R. meliloti* Nod⁺Fix⁻ strain. Keller et al. (1988) demonstrated that active transcription of genes involved in EPS synthesis occurs in the bacteroid state. Therefore, some functional requirement of EPS compounds in the late stages of symbiosis seems plausible.

In host cells infected predominantly by nitrogenase-negative bacteroids (unlabeled strain 36070), starch accumulation was significantly higher than in those containing predominantly nitrogenase-positive bacteroids (labeled strain 0540). This suggests that the Fix⁻ bacteroids are unable to use the abundance of stored carbon supplied by the plant and, consequently, cannot undergo the energy-demanding nitrogenase reaction. The difference in starch accumulation was also observed when the two kinds of mixed infected host cells were located alongside each other, indicating that the cells' metabolisms are largely independent of each other. Similar observations were reported by Hahn and Studer (1986) for the determinate nodules of soybean that were mixed infected with effective and ineffective *Bradyrhizobium japonicum* strains.

R. meliloti Nod⁻ as well as Inf⁻EPS⁻ Mutants Differentiate into N_2-Fixing Bacteroids

The introduction of additional *nif* and/or *fix* mutations in the Inf⁻EPS⁻ mutant enabled further testing of combinations of mutant strains. In these co-inoculation experiments, the one partner contained functional *nif/fix* genes. Interestingly, each of these combinations resulted in effective nodulation of alfalfa. This was confirmed by nodule morphology, plant growth, and acetylene reduction assays. We concluded, therefore, that both Nod⁻ and Inf⁻EPS⁻ partners were able to differentiate into nitrogen fixing bacteroids. This concurs with the results of Klein et al. (1988) from an experiment in which a similar set of mutants was used.

Moreover, an *nif* D::*lac*Z fusion was used to investigate whether both co-inoculation partners were able to infect the nodule cells and differentiate into bacteroids that expressed *nif* genes. The *nif* D::*lac*Z fusion was introduced by homologous recombination into the symbiotic plasmids of the following strains: the Inf⁻EPS⁻ mutant 0540, the Nod⁻ mutant 2526, and another Nod⁻ mutant 0544, which carries a mutation in the *hsn* region (presumably *nod*H; Müller, 1987). Co-inoculation with the corresponding fusionless partners resulted in mixed infected, effective nodules of wild-type appearance, all of which exhibited enhanced β-galactosidase activity. This demonstrated that

symbiotic transcription of the *nif*H promoter could occur in each of the mutants tested. Thus, without altering the genetic background of the co-inoculation partners, the presence and symbiotic performance of the mutants could be demonstrated in mixed infected nodules.

METHODS

Strains, Phages, and Plasmids

The strains used or constructed are listed in Table 4 and phages and plasmids are listed in Table 5. Microbiological techniques, including culture and growth conditions, were previously described by Müller et al. (1988).

Strain Construction

The DNA manipulations employed are as described by Aguilar et al. (1985).

Construction of the *R. meliloti* Nod⁻ Deletion Mutant 36070, Which Contains a Large Deletion in the *nod-nif/fix* Region of Megaplasmid 1

R. meliloti strain 2011 was homogenotized by an EcoRI DNA fragment cloned from plasmid pRmR2 (Ruvkun, Sundaresan, and Ausubel, 1982), which carries transposon Tn*10* within the *nif*HDK operon (M. Labes, unpublished results). The homogenotized strain was subsequently subjected to heat curing at 37°C, which resulted in the frequent loss of Tn*10* (Tc^r). Excision of Tn*10* was often accompanied by the formation of deletions near the insertion site. Isolation of Tc^s colonies was facilitated by an ampicillin enrichment procedure (Miller, 1972). Tc^s colonies were analyzed by modified Eckhardt gels, which allowed the physical detection of deletions (Hynes, Simon, and Pühler, 1985). Strains that contained a reduced megaplasmid 1 were used for further experiments. Strain 36070 was Tc^s, and contained a Sym-plasmid that was reduced in size. In plant tests, this strain proved to be Nod⁻ and was, therefore, chosen for further experiments.

Construction of *R. meliloti* RmJQ10 Carrying a *fix*A::Tn*5* Mutation

Strain RmJQ10 was constructed by homogenotizing a *fix*A::Tn*5* mutation into *R. meliloti* strain 2011. This was achieved by using plasmid pRm3::Tn*5*#6 carrying the indicated mutation. The replacement of the wild-type fragment by the mutated fragment was selected for on medium supplemented by neomycin. The double recombination event was verified by DNA gel blot hybridization using a ^{32}P-labeled IS*50* *Xho*I-*Hin*dIII fragment cloned in M13mp8 as a probe (Simon et al., 1986), as well as by plant tests (data not shown).

Construction of *R. meliloti* Double Mutants Carrying *inf* (0540)::Tn*5*-Gm

Plasmid pWKR90AI was used to replace the original Tn*5* insertion in strain 0540 by Tn*5*-Gm. The resulting derivative, RmJQ13, was introduced into several Tn*5*-carrying strains by bacteriophage M12 transduction as described by Finan et al. (1984). The mutations of the transduced strains were verified by growth on selective media, by testing on Cellufluor white (Polysciences, Warrington, PA)-containing plates (Müller et al., 1988), and by plant tests. After the introduction of the *inf* complementing cosmid pRmPM551 (Müller et al., 1988), the Fix⁻ phenotype of the double mutants was tested in additional plant tests. The genetic composition of the transduced strains was checked by DNA gel blot hybridization using a biotinylated Tn*5* probe as described by Keller et al. (1988) (data not shown).

Table 4. *R. meliloti* Strains

Strain	Relevant Genotype[a]			Source
	nod/hsn	*inf*[b]	*nif/fix*	
2011	+	+	+	Casse et al., 1979
2526	*nod*(2526)::Tn*5*	+	+	Müller et al., 1988
0544	*hsn*(0544)	+	+	Müller, 1987
0540	+	*inf*(0540)::Tn*5*	+	Müller et al., 1988
RmJQ13	+	*inf*(0540)::Tn*5*-Gm	+	This work
RmRS81	+	+	*nif*H::Tn*5*	Simon, Priefer, and Pühler, 1983[c]
36070	Δ*nod/hsn*	+	Δ*nif/fix*	This work
Rm *nif*A-d8	+	+	Δ*nif*A	Klipp et al., 1989
RmJQ10	+	+	*fix*A::Tn*5*	This work
MA3	+	+	*nif*N::Tn*5*	Aguilar et al., 1987
RmJQ18	+	*inf*(0540)::Tn*5*-Gm	*nif*H:Tn*5*	This work
RmJQ19	+	*inf*(0540)::Tn*5*-Gm	*fix*A::Tn*5*	This work
RmJQ20	+	*inf*(0540)::Tn*5*-Gm	*nif*N::Tn*5*	This work

[a] +, gene region indicated is wild type.
[b] Inf⁻EPS⁻ mutant 0540 is unable to infect alfalfa nodules and forms no acidic exopolysaccharide (Müller et al., 1988).
[c] Strain construction. Strain designation was introduced in this work.

Table 5. Plasmids and Phages

Plasmid or Phage	Relevant Characteristics	Source
pWKR561	Tc^r, Km^r, Mob$^+$	Klipp, Masepohl, and Pühler, 1988
pWKR90AI	Internal XhoI-fragment of Tn*5*-Gm (Hirsch, Wang, and Woodward, 1986) cloned into XhoI-site of pWKR561	W. Klipp, unpublished results
pGW7	p_{nifH}, *nif*HD-*lac*Z, Ap^r	Weber, Reiländer, and Pühler, 1985
pJQ71	EcoRI-cassette from pUS31A cloned into EcoRI site of pGW7	This work
pUS31A	pACYC184 derivative carrying an EcoRI cassette containing the HindIII-BamHI (Gm^r) fragment from Tn*1696* (Hirsch, Wang, and Woodward, 1986) and the Mob site from pSUP201-1 (Simon, Priefer, and Paühler, 1983)	U. Schramm, unpublished results
pRmR3	pACYC184 carrying *fix* genes, Tc^r	Ruvkun, Sundaresan, and Ausubel, 1982
pRmR3::Tn*5*#6	pACYC184*fix*A::Tn*5*	G. Weber, unpublished results
pJQ135	EcoRI fragment from pRmR3::Tn*5*#6	This work
pSUP202	pBR325, Ap^r, Cm^r, Tc^r, Mob$^+$	Simon, Priefer, and Pühler, 1983
ΦM12	*R. meliloti* transducing phage	Finan et al., 1984

Plant Test

Nodulation of *Medicago sativa* cv du Puits was tested as described by Hynes et al. (1986). Nitrogenase activity was measured by acetylene reduction assay 3 to 4 weeks after inoculation. For co-inoculation experiments, strains were grown overnight in TY medium, diluted to the same optical density (OD_{580} = 0.8), and mixed in equal volumes. In each assay, 100 μL of the cell suspension was streaked on the plant agar below the roots.

Filter Separation Technique

The filter separation technique was used to separate the two inoculation partners from each other in co-inoculation experiments. Surface sterilized seeds of *M. sativa* were germinated for 2 days on plant agar. Two seedlings were transferred to fresh agar plates and incubated for an additional 2 days. Each seedling was then inoculated with approximately 8×10^6 cells of the first co-inoculation partner using 50 μL from an overnight culture in TY medium (adjusted to $OD_{580} = 0.8$). Sterile filters (cellulose acetate, pore size 0.2 μm; Sartorius, Göttingen, Federal Republic of Germany), coated around the rim by hot paraffin to prevent bacterial migration, were placed on top of the inoculated plant roots. Finally, 50 μL of bacterial suspension of the second co-inoculation partner (approximately 8×10^6 cells) was placed on top of the filter. The plants were incubated for 3 weeks in a growth chamber, as reported previously (Müller et al., 1988). Nodulation was analyzed by Feulgen staining and phenol clearing, as described by Niehaus and Pühler (1988). A more detailed examination was performed by thin sectioning of embedded root tissue, followed by light microscopy.

Microscopy

Specimen Preparation

Nodules were harvested 3 weeks after inoculation, placed in fixative, cut longitudinally with a razor blade, and fixed for 2 hr at room temperature in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). They were then dehydrated in a graded ethanol series, infiltrated, and embedded in Lowicryl K4M resin (Chemische Werke Lowi, Waldkraiburg, Federal Republic of Germany) at low temperatures as described by Acker et al. (1986).

Light and Electron Microscopy

With the use of glass knives, semithin sections of 0.5 μm to 1.0 μm were cut on a Reichert Ultracut microtome. Each section was transferred to a drop of distilled water on a gelatin-coated slide (Berlyn and Miksche, 1976) and dried overnight at 40°C. Sections were examined and photographed in phase-contrast mode with a Zeiss Universal R scientific microscope. Ultrathin sections of about 70 nm were cut using a diamond knife and collected on Parlodion-carbon-coated 200-mesh nickel grids. Sections were stained for 5 min in 2% aqueous uranyl acetate, followed by 1 min in Reynolds lead citrate (Reynolds, 1963), and examined in a Hitachi H500 electron microscope at 75 kV or a Zeiss EM109 at 50 kV.

Immunolabeling

To saturate the nonspecific binding sites, semithin sections were covered by a drop of PBS (10 mM sodium phosphate buffer, pH 7.2, 500 mM NaCl) containing 2% BSA and incubated for 30 min on a shaker. The drops were then pipetted off and replaced by 50 μL of diluted *Rhizobium leguminosarum* PRE (Schetgens et al., 1984) nitrogenase antiserum (component I or component II, 1:30 in 0.2% BSA in PBS; kind gift of R. van den Bos, Wageningen, The Netherlands). Sections were placed on a shaker and incubated for 6 hr to 12 hr at 4°C. The slides were rinsed three times in PBS-Tween (0.5% w/v Tween 20 in PBS) and three times in PBS in a coplin jar for 10 min each. Excess liquid around the sections was drained away. The sections were then covered with 100 μL of diluted, gold-labeled secondary antibodies (5 nm, goat anti-rabbit; light microscopy grade, Janssen Pharmaceutica, Beerse, Belgium; 1:40 in 0.2% BSA in PBS), incubated, and washed as described above. To detect bound colloidal gold

particles by light microscopy, the slides were silver-enhanced using a commercial silver enhancement kit (Janssen Pharmaceutica), according to the manufacturer's instructions. For electron microscopy, the grids were placed face-down on drops of the solutions mentioned above. The drops were arranged on a sheet of Parafilm placed on a laboratory shaker in a humid chamber. Colloidal gold (15 nm, goat anti-rabbit, EM-grade, Janssen Pharmaceutica, diluted 1:20 in 0.2% BSA in PBS) was used as the secondary labeled antibody. The grids were washed for about 30 sec under a mild jet of the washing solution created by a plastic spray bottle. The grids were blotted with a piece of filter paper and rapidly transferred to the next drop. Incubation times were the same as above. After a final rinse in distilled water, the grids were blotted and air-dried. Eight mixed infected nodules from different plants were examined, as well as several *R. meliloti* wild-type and Rm *nif* A-d8 induced nodules as controls.

ACKNOWLEDGMENTS

We gratefully acknowledge John G. Robertson, Brian Wells, and Georg Acker for their kind help and advice during initial embedding of root nodules and immunocytochemistry. We are endebted to Rommert van den Bos for the generous gift of anti-nitrogenase sera and Monika Labes, Werner Klipp, and Uwe Schramm, Federal Republic of Germany, for providing strains and plasmids. We thank Inge Pretorius-Güth and Bob Kosier for editing the manuscript. This work was financially supported by a grant from Deutsche Forschungsgemeinschaft (Pu 28/13). K.N. gratefully acknowledges his fellowship from the Studienstiftung des deutschen Volkes. Part of this work was presented at the 4th International Symposium on Molecular Genetics of Plant-Microbe Interactions, Acapulco, Mexico, 1988.

Received July 18, 1989; revised December 8, 1989.

REFERENCES

Acker, G., Bitter-Suermann, D., Meier-Dieter, U., Peters, H., and Mayer, H. (1986). Immunocytochemical localization of enterobacterial common antigen in *Escherichia coli* and *Yersinia enterocolitica* cells. J. Bacteriol. **168,** 348–356.

Aguilar, O.M., Kapp, D., and Pühler, A. (1985). Characterization of the *Rhizobium meliloti* fixation gene (*fix*F) located near the common nodulation region. J. Bacteriol. **164,** 245–254.

Aguilar, O.M., Reiländer, H., Arnold, W., and Pühler, A. (1987). *Rhizobium meliloti nif* N (*fix*F) gene is part of an operon regulated by a *nif* A-dependent promoter and codes for a polypeptide homologous to the *nif* K gene product. J. Bacteriol. **169,** 5393–5400.

Banfalvi, Z., and Kondorosi, A. (1989). Production of root hair deformation factors by *Rhizobium meliloti* nodulation genes in *Escherichia coli*: HsnD (NodH) is involved in the plant host-specific modification of the NodABC factor. Plant Mol. Biol. **13,** 1–12.

Banfalvi, Z., Sakanyan, V., Koncz, C., Kiss, A., Dusha, I., and Kondorosi, A. (1981). Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid in *R. meliloti*. Mol. Gen. Genet. **184,** 318–325.

Bauer, W.D. (1981). Infection of legumes by rhizobia. Annu. Rev. Plant Physiol. **32,** 407–449.

Bauer, W.D., Bhuvaneswari, T.V., Calvert, H.E., Law, I.J., Malik, N.S.A., and Vesper, S.J. (1985). Recognition and infection by slow-growing rhizobia. In Nitrogen Fixation Research Progress, H.J. Evans, P.J. Bottomley, and W.E. Newton, eds (Dordrecht, The Netherlands: Martinus Nijhoff Publishers), pp. 247–253.

Berlyn, G.P., and Miksche, J.P. (1976). Botanical Microtechnique and Cytochemistry. (Ames, IA: Iowa State University Press), pp. 65–67.

Bhuvaneswari, T.V., and Solheim, B. (1985). Root hair deformation in the white clover/*Rhizobium trifolii* symbiosis. Physiol. Plant. **63,** 25–34.

Calvert, H.E., Pence, M.K., Pierce, M., Malik, N.S.A., and Bauer, D. (1984). Anatomical analysis of the development and distribution of *Rhizobium* infections in the soybean roots. Can. J. Bot. **62,** 2375–2384.

Casse, F., Boucher, C., Julliot, J.S., Michel, M., and Denarie, J. (1979). Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. J. Gen. Microbiol. **113,** 229–242.

Chen, H., and Rolfe, B.G. (1987). Cooperativity between *Rhizobium* mutant strains: Induction of nitrogen-fixing nodules on the tropical legume *Leucaena leucocephala*. J. Plant Physiol. **127,** 307–322.

Debelle, F., Rosenberg, C., Vasse, J., Maillet, F., Martinez, E., Denarie, J., and Truchet, G. (1986). Assignment of symbiotic developmental phenotypes to common and specific nodulation (*nod*) genetic loci of *Rhizobium meliloti*. J. Bacteriol. **168,** 1075–1086.

Djordjevic, M.A., Schofield, P.R., and Rolfe, B.G. (1985). Tn5 mutagenesis of *Rhizobium trifolii* host-specific nodulation genes results in mutants with altered host-range ability. Mol. Gen. Genet. **200,** 463–471.

Djordjevic, S.P., Chen, H., Batley, M., Redmond, J.W., and Rolfe, B.G. (1987). Nitrogen fixing ability of exopolysaccharide synthesis mutants of *Rhizobium sp.* strain NGR234 and *Rhizobium trifolii* is restored by the addition of homologous exopolysaccharides. J. Bacteriol. **169,** 53–60.

Downie, J.A., Knight, C.D., Johnston, A.W.B., and Rossen, L. (1985). Identification of genes and gene products involved in the nodulation of peas by *Rhizobium leguminosarum*. Mol. Gen. Genet. **198,** 255–262.

Dudley, M.E., Jacobs, T.W., and Long, S.R. (1987). Microscopic studies of cell divisions induced in alfalfa roots by *Rhizobium meliloti*. Planta **171,** 289–301.

Engelke, T., Jording, D., Kapp, D., and Pühler, A. (1989). Identification and sequence analysis of the *Rhizobium meliloti dct*A gene encoding the C_4-dicarboxylate carrier. J. Bacteriol. **171,** 5551–5560.

Finan, T.M., Hartwieg, E., LeMieux, K., Bergman, K., Walker, G.C., and Signer, E.R. (1984). General transduction in *Rhizobium meliloti*. J. Bacteriol. **159,** 120–124.

Finan, T.M., Hirsch, A.M., Leigh, J.A., Johansen, E., Kuldau, G.A., Deegan, S., Walker, G.C., and Signer, E.R. (1985). Symbiotic mutants of *Rhizobium meliloti* that uncouple plant from bacterial differentiation. Cell **40,** 869–877.

Finan, T.M., Kunkel, B., De Vos, G.F., and Signer, E.R. (1986). Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol. **167,** 66–72.

Glazebrook, J., and Walker, G.C. (1989). A novel exopolysaccharide can function in place of the Calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti*. Cell **56,** 661–672.

Hahn, M., and Studer, D. (1986). Competitiveness of a *nif*⁻ *Bradyrhizobium japonicum* mutant against the wild type strain. FEMS Microbiol. Lett. **33,** 143–148.

Halverson, L., and Stacey, G. (1986). Signal exchange in plant-microbe interactions. Microbiol. Rev. **50,** 193–211.

Hirsch, A.M., Bang, M., and Ausubel, F.M. (1983). Ultrastructural analysis of ineffective alfalfa nodules formed by *nif*::Tn*5* mutants of *Rhizobium meliloti*. J. Bacteriol. **155,** 367–380.

Hirsch, A.M., Wilson, K.J., Jones, J.D.G., Bang, M., Walker, V.V., and Ausubel, F.M. (1984). *Rhizobium meliloti* nodulation genes allow *Agrobacterium tumefaciens* and *Escherichia coli* to form pseudonodules on alfalfa. J. Bacteriol. **158,** 1133–1143.

Hirsch, P.R., Wang, C.L., and Woodward, M.J. (1986). Construction of a Tn*5* derivative determining resistance to gentamycin and spectinomycin using a fragment cloned from R1033. Gene **48,** 203–209

Hirsch, A.M., Bhuvaneswari, T.V., Torrey, J.G., and Bisseling, T. (1989). Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. Proc. Natl. Acad. Sci. USA **86,** 1244–1248.

Horvath, B., Kondorosi, E., John, M., Schmidt, J., Török, I., Györgypal, Z., Barabas, I., Wieneke, U., Schell, J., and Kondorosi, A. (1986). Organization, structure and symbiotic function of *Rhizobium meliloti* nodulation genes determining host specificity for alfalfa. Cell **46,** 335–343.

Hynes, M.F., Simon, R., and Pühler, A. (1985). The development of plasmid-free strains of *Agrobacterium tumefaciens* by using incompatibility with a *Rhizobium meliloti* plasmid to eliminate pAtC58. Plasmid **13,** 99–105.

Hynes, M.F., Simon, R., Müller, P., Niehaus, K., Labes, M., and Pühler, A. (1986). The two megaplasmids of *Rhizobium meliloti* are involved in the effective nodulation of alfalfa. Mol. Gen. Genet. **202,** 356–362.

Jacobs, T.W., Egelhoff, T.T., and Long, S.R. (1985). Physical and genetic map of a *Rhizobium meliloti* nodulation gene region and nucleotide sequence of *nod*C. J. Bacteriol. **162,** 469–476.

Keller, M., Müller, P., Simon, R., and Pühler, A. (1988). *Rhizobium meliloti* genes for exopolysaccharide synthesis and nodule infection located on megaplasmid 2 are actively transcribed during symbiosis. Mol. Plant-Microbe Interact. **1,** 267–274.

Klein, S., Hirsch, A.M., Smith, C.A., and Signer, E.R. (1988). Interaction of *nod* and *exo Rhizobium meliloti* in alfalfa nodulation. Mol. Plant-Microbe Interact. **1,** 94–100.

Klipp, W., Masepohl, B., and Pühler, A. (1988). Identification and mapping of nitrogen fixation genes of *Rhodopseudomonas capsulatus*: Duplication of a *nif*A-*nif*B region. J. Bacteriol. **170,** 693–699.

Klipp, W., Reiländer, H., Schlüter, A., Krey, R., and Pühler, A. (1989). The *Rhizobium meliloti fdx*N gene encoding a ferredoxin-like protein is necessary for nitrogen fixation and is cotranscribed with *nif*A and *nif*B. Mol. Gen. Genet. **216,** 293–302.

Kondorosi, E., Banfalvi, Z., and Kondorosi, A. (1984). Physical and genetic analysis of a symbiotic region of *Rhizobium meliloti*. Identification of nodulation genes. Mol. Gen. Genet. **193,** 445–452.

Leigh, J.A., Signer, E.R., and Walker, G.C. (1985). Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. Proc. Natl. Acad. Sci. USA **82,** 6231–6235.

Leigh, J.A., Reed, J.W., Hanks, J.F., Hirsch, A.M., and Walker, G.C. (1987). *Rhizobium meliloti* mutants that fail to succinylate their Calcofluor-binding exopolysaccharide are defective in nodule invasion. Cell **51,** 579–587.

Long, S.R. (1984). Genetics of nodulation. In Plant Microbe Interactions, T. Kosuge, and E. Nester, eds (New York: Macmillan Publishing Corp.), pp. 265–306.

Long, S.R. (1989). *Rhizobium*-legume nodulation: Life together in the underground. Cell **56,** 203–214.

Long, S.R., Buikema, W.J., and Ausubel, F.M. (1982). Cloning of *Rhizobium meliloti* nodulation genes by direct complementation of Nod⁻ mutants. Nature **298,** 485–488.

Long, S., McCune, S., and Walker, G.C. (1988). Symbiotic loci of *Rhizobium meliloti* identified by random Tn*phoA* mutagenesis. J. Bacteriol. **170,** 4257–4265.

McRae, D.G., Miller, R.W., and Berndt, W.B. (1989). Viability of alfalfa nodule bacteroids isolated by density gradient centrifugation. Symbiosis **7,** 67–80.

Meade, H.M., Long, S.R., Ruvkun, G.B., Brown, S.E., and Ausubel, F.M. (1982). Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149,** 114–122.

Miller, J.H. (1972). Penicillin and ampicillin treatment for the isolation of auxotrophic mutants. In Experiments in Molecular Genetics. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory), pp. 230–234.

Müller, P. (1987). Infektionsmutanten von *Rhizobium meliloti* mit Defekten in der EPS-Synthese. PhD thesis, University of Bielefeld, Federal Republic of Germany.

Müller, P., Hynes, M., Kapp, D., Niehaus, K., and Pühler, A. (1988). Two classes of *Rhizobium meliloti* infection mutants differ in exopolysaccharide production and in coinoculation properties with nodulation mutants. Mol. Gen. Genet. **211,** 17–26.

Newcomb, W. (1981). Nodule morphogenesis and differentiation. Int. Rev. Cytol. Suppl. 13, 246–298.

Niehaus, K., and Pühler, A. (1988). Light microscopic analysis of entire alfalfa (*Medicago sativa*)) nodules by a fast staining and clearing method. Endocyt. C. Res. **5,** 59–68.

Putnoky, P., Grosskopf, E., Cam Ha, D.T., Kiss, G.B., and Kondorosi, A. (1988). *Rhizobium fix* genes mediate at least two communication steps in symbiotic nodule development. J. Cell Biol. **106,** 597–607.

Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17,** 208–213.

Rolfe, B.G., and Gresshof, P.M. (1980). *Rhizobium trifolii* mutant interaction during the establishment of nodulation in white clover. Aust. J. Biol. Sci. **33,** 491–504.

Rolfe, B.G., and Shine, J. (1984). *Rhizobium*-legume symbiosis: The bacterial point of view. In Genes Involved in Plant-Microbe Interactions, D.P.S. Verma and T. Hohn, eds (New York: Springer Verlag).

Rolfe, B.G., Gresshof, P.M., Shine, J., and Vincent, J.M. (1980). Interaction between a non-nodulating and an ineffective mutant of *Rhizobium trifolii* resulting in effective (nitrogen-fixing) nodulation. Appl. Environ. Microbiol. **39,** 449–452.

Rosenberg, C., Boistard, P., Dénarié, J., and Casse-Delbart, F.F. (1981). Genes controlling early and late functions in symbiosis are located on a megaplasmid in *R. meliloti*. Mol. Gen. Genet. **184,** 326–333.

Rossbach, S., Gloudemans, T., Bisseling, T., Studer, D., Kaluza, B., Ebeling, S., and Hennecke, H. (1989). Genetic and physiologic characterization of a *Bradyrhizobium japonicum* mutant defective in early bacteroid development. Mol. Plant-Microbe Interact. **2,** 233–240.

Rossen, L., Johnston, A.W.B., and Downie, J.A. (1984). DNA sequence of the *Rhizobium leguminosarum* nodulation genes *nod*AB and C required for root hair curling. Nucl. Acids Res. **12,** 9497–9508.

Ruvkun, G.B., Sundaresan, V., and Ausubel, F.M. (1982). Directed transposon Tn*5* mutagenesis and complementation analysis of *Rhizobium meliloti* symbiotic nitrogen fixation genes. Cell **29,** 551–559.

Schetgens, T.M.P., Bakkeren, G., Van Dun, C., Hontelez, J.G.J., van den Bos, R.C., and van Kammen, A. (1984). Molecular cloning and functional characterization of *Rhizobium leguminosarum* structural *nif* genes by site-directed transposon mutagenesis and expression in *Escherichia coli* minicells. J. Mol. Appl. Genet. **2,** 406–421.

Schmidt, J., Wingender, R., John, M., Wieneke, U., and Schell, J. (1988). *Rhizobium meliloti nod*A and *nod*B genes are involved in generating compounds that stimulate mitosis of the host plant cells. Proc. Natl. Acad. Sci. USA **85,** 1133–1143.

Simon, R., Priefer, U., and Pühler, A. (1983). A broad host range mobilization system for in vivo genetic engineering: Transposon mutagenesis in Gram-negative bacteria. Bio/Technology **2,** 269–278.

Simon, R., O'Connell, M., Labes, M., and Pühler, A. (1986). Plasmid vectors for the genetic analysis and manipulation of rhizobia and other Gram-negative bacteria. Methods Enzymol. **118,** 640–659.

Solheim, B., and Raa, J. (1973). Characterization of the substances causing deformations of the root hairs on *Trifolium repens* when inoculated with *Rhizobium trifolii*. J. Gen. Microbiol. **77,** 241–247.

Truchet, G., Michel, M., and Dénarié, J. (1980). Sequential analysis of the organogenesis of lucerne (*Medicago sativa*) root nodules using symbiotically defective mutants of *Rhizobium meliloti*. Differentiation **16,** 163–172.

Truchet, G., Rosenberg, C., Vasse, J., Julliot, J.-S., Camut, S., and Dénarié, J. (1984). Transfer of *Rhizobium meliloti* pSym genes into *Agrobacterium tumefaciens*: Host specific nodulation by atypical infection. J. Bacteriol. **157,** 134–142.

Truchet, G., Barker, D.G., Camut, S., de Billy, F., Vasse, J., and Huguet, T. (1989). Alfalfa nodulation in the absence of *Rhizobium*. Mol. Gen. Genet. **219,** 65–68.

Vincent, J.M. (1980). Factors controlling the legume-*Rhizobium* symbiosis. In Nitrogen Fixation, W.E. Newton and W.H. Orme-Johnson, eds (Baltimore: University Park Press), Vol. II, pp. 103–129.

Weber, G., Reiländer, H., and Pühler, A. (1985). Mapping and expression of a regulatory nitrogen fixation gene (*fix*D) of *Rhizobium meliloti*. EMBO J. **4,** 2751–2756.

Yao, P.Y., and Vincent, J.M. (1969). Host specificity in the root hair "curling factor" of *Rhizobium* spp. Aust. J. Biol. Sci. **22,** 413–423.

Yao, P.Y., and Vincent, J.M. (1976). Factors responsible for the curling and branching of clover root hairs by *Rhizobium*. Plant Soil **45,** 1–46.

Zhan, H., Levery, S.B., Lee, C.C., and Leigh, J.A. (1989). A second exopolysaccharide of *Rhizobium meliloti* strain SU47 that can function in root nodule invasion. Proc. Natl. Acad. Sci. USA **86,** 3055–3059.