JOURNAL OF BACTERIOLOGY, Jan. 1984, p. 134–142
0021-9193/84/010134-09$02.00/0

Vol. 157, No. 1

Transfer of *Rhizobium meliloti* pSym Genes into *Agrobacterium tumefaciens*: Host-Specific Nodulation by Atypical Infection†

GEORGES TRUCHET,[1] CHARLES ROSENBERG,[2] JACQUES VASSE,[1] JEAN-SIMON JULLIOT,[2] SYLVIE CAMUT,[2] AND JEAN DENARIE[2]*

Laboratoire de Cytologie et de Biologie Cellulaire, Faculté des Sciences Marseille-Luminy, F-13288 Marseille Cedex 2,[1] and Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, Centre National de la Recherche Scientifique-Institut National de la Recherche Agronomique, Groupement Scientifique Microbiologie Toulouse, F-31320 Castanet-Tolosan, France[2]

Received 20 June 1983/Accepted 23 October 1983

The pSym megaplasmid of *Rhizobium meliloti* 2011 mobilized by plasmid RP4, or plasmid pGMI42, an RP4-prime derivative which carries a 290-kilobase pSym fragment including nitrogenase and *nod* genes, was introduced into *Agrobacterium tumefaciens*. The resulting transconjugants induced root deformations specifically on the homologous hosts *Medicago sativa* and *Melilotus alba* and not on the heterologous hosts *Trifolium pratense* and *Trifolium repens*. The root deformations were shown to be genuine nodules by physiological and cytological studies. Thus, host specificity nodulation genes are located on the pSym megaplasmid. Host nodulation specificity did not seem to require recognition at the root hair level since no infection threads could be detected in the root hairs. Cytological observations indicated that bacteria penetrated only the superficial layers of the host root tissue by an atypical infection process. The submeristematic zone and the central tissue of the nodules were bacteria free. Thus, nodule organogenesis was probably triggered from a distance by the bacteria. *Agrobacterium* transconjugants carrying pSym induced the formation of more numerous and larger nodules than those carrying the RP4-prime plasmid pGMI42, suggesting that some genes influencing nodule organogenesis are located in a pSym region(s) outside that which has been cloned into pGMI42.

Rhizobium strains are gram-negative soil bacteria which induce the formation of nodules on the roots of leguminous hosts, in which they fix nitrogen. The induction of nodule organogenesis is specific in that a particular strain of *Rhizobium* can usually form root nodules with only a limited range of plants, e.g., *Rhizobium meliloti* strains with species of *Medicago*, *Melilotus*, and *Trigonella*, or *Rhizobium trifolii* strains with *Trifolium* species (see reference 32). In *Rhizobium leguminosarum*, *Rhizobium phaseoli*, and *R. trifolii*, genes controlling nodule formation and nitrogen fixation are located on a large pSym plasmid varying in size from 160 kilobases (kb) to more than 500 kb (12–14, 20, 26). Interspecific crosses have shown that genes controlling host-range specificity are also located on these pSym plasmids in *R. leguminosarum* (13, 16), *R. phaseoli* (20), and *R. trifolii* (14). Genetic analysis of host-range nodulation specificity was facilitated in these three species because they are closely related genetically (8, 19, 32) and their pSym plasmids can be self-transmissible (14, 16, 20). The introduction of the *R. leguminosarum* or *R. trifolii* pSym plasmid into *Agrobacterium tumefaciens* results in the transfer of the ability to nodulate pea and clover, respectively (13, 14). The resulting nodules are ineffective and do not contain bacteroids characteristic of nitrogen-fixing nodules (14). *R. meliloti* strains are distantly related physiologically and genetically to other *Rhizobium* species (8, 19, 32). They can be differentiated by their pRme plasmids content (6, 28), but all are characterized by the presence of a megaplasmid greater than 700 kb (1, 28, 29) which carries the nitrogenase genes (1, 28, 31) and genes controlling alfalfa (*Medicago sativa*) nodulation (1, 22, 28). This pSym megaplasmid is not self-transmissible at detectable frequency, but recently Kondorosi et al. (18) mobilized it into *A. tumefaciens* by cloning a *mob* region into the *R. meliloti* 41 pSym megaplasmid; the transconjugants were able to induce ineffective nodule-like deformations on *Medicago sativa* roots.

In this paper we show that in *R. meliloti* RCR2011 host specificity nodulation genes are located on the pSym megaplasmid. By using the broad host-range plasmid RP4, the pSym plasmid or a 290-kb pSym fragment carrying nitrogenase and *nod* genes was introduced into *A. tumefaciens*. Transconjugants induced root deformations specifically on the homologous hosts *Medicago sativa* and *Melilotus alba* and not on the heterologous hosts *Trifolium pratense* and *Trifolium repens*. The root deformations were shown to be genuine nodules by physiological and cytological studies. This host nodulation specificity did not seem to require recognition at the root hair level since no infection thread could be detected in the root hairs. Cytological observations indicated that bacteria superficially penetrated the host.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are shown in Table 1. *A. tumefaciens* GMI9012 is a spontaneous streptomycin-resistant (200 μg/ml) derivative of strain C58C1, and strain GMI9013 is a spontaneous rifampicin-resistant (100 μg/ml) derivative of strain GV3350. *R. meliloti* Rm1531, *A. tumefaciens* GV3350, and bacteriophages S2 and S5 were kindly provided by F. M. Ausubel (Boston, Mass.) and M. Van Montagu (Gent, Belgium).

Growth conditions. *Escherichia coli*, *Rhizobium* and *Agrobacterium* strains were grown as described previously (28). Phage sensitivity tests were performed by the double-layer technique on yeast extract-mannitol C medium (2).

* Corresponding author.

† This paper is dedicated to the memory of our much valued colleague Jean-Simon Julliot, who died on 21 February 1982.

TABLE 1. Bacteria, bacteriophages, and plasmids used

Bacterial strain, bacteriophage, or plasmid	Relevant characteristics[a]	Source or reference
E. coli		
ED8767	*supE supF hsdS met recA56*	(3)
GMI3180	Rif^r derivative of ED8767	17a
GMI3232	*thr leu rpsL hsdR rpoB* (Mu cts62)	17a
GMI3506	*thy deoC* Tn*7* (Mu cts62)	17a
R. meliloti		
RCR2011=SU47	Wild type; symbiotically effective	(28)
Rm1021	Spontaneous Sm^r derivative of RCR2011	(23)
Rm1531	Derivative of Rm1021 carrying *nif-20*::Tn*5*; Fix$^+$	(30)
GMI243	Derivative of RCR2011 carrying two insertions in the pSym plasmid: *nif-20*::Tn*5* and Tn*7* insertion no. 5002	This work
A. tumefaciens		
C58C1	Derivative of C58 cured of Ti plasmid	(28)
GMI9012	Sm^r derivative of C58C1	This work
GV3350	Ery^r Cm^r derivative of C58C1 with a Tn*1* insertion in pAt	M. Van Montagu (Gent University)
GMI9013	Rif^r derivative of GV3350	This work
Bacteriophages S_2 and S_5	Specific to *A. tumefaciens*	M. Van Montagu (Gent University)
Plasmid		
RP4	Tc^r Ap^r Km^r	(4)
pGMI106	Derivative of RP4 carrying 5 kb of the pSym2011 *nif* region	17a
pGMI42	Derivative of RP4 carrying 290 kb of pSym2011	17a
pGMI104	Derivative of pGMI42 carrying a bacteriophage Mu insertion in the *tet* gene	17a
pGMI27	Derivative of pSym2011 carrying *nif-20*::Tn*5* and Tn*7* insertion no. 5002	This work

[a] Antibiotic resistances: Ap^r, ampicillin; Cm^r, chloramphenicol; Ery^r, erythromycin; Km^r, kanamycin; Rif^r, rifampicin; Str^r, streptomycin; Tc^r, tetracycline.

Mating conditions. Donor and recipient strains were grown in TY liquid medium (28) to mid-log phase, mixed in equal volumes, collected on a filter membrane, and incubated overnight on a TY agar plate. Crosses resulting in the tagging of pSym 2011 by transposons Tn*5* and Tn*7* have been described elsewhere (17a). In crosses involving *Agrobacterium* strains, donors were counterselected with 200 μg/ml streptomycin (Sm), 100 μg/ml rifampicin (Rif), or 25 μg/ml chloramphenicol (Cm), and plasmid transfers were selected with 30 μg/ml kanamycin (Km), 2.5 μg/ml tetracycline (Tc), or 200 μg/ml spectinomycin (Sp).

Plasmid visualization. The in-gel lysis method of Eckhardt (7), as modified by Rosenberg et al. (29), was used for plasmid detection.

Plant material. Seeds of *Medicago sativa* cv. Gemini were obtained from Tourneur Frères (F77120 Coulommiers, France), seeds of *Melilotus alba* line 9158 were from Estacao Nacional de Melhoramento de Plantas (Elvas, Portugal), and seeds of *T. pratense* cv. Alpille and *T. repens* cv. Crau were from Service Production de Semences, Institut National de la Recherche Agronomique (F78280 Guyancourt, France).

Nodulation assays. Seeds were sterilized and germinated, and the seedlings were aseptically grown in test tubes on nitrogen-free agar slants as previously described (34). Two seedlings were grown per tube, and 10 tubes were used for each treatment. Bacteria were inoculated 5 days after seed sterilization. To avoid nitrogen starvation in the case of ineffective bacteria-plant associations, 0.3 mM urea was added 3 weeks after inoculation. When the effect of a high level of combined nitrogen on nodule formation was studied, 20 mM KNO_3 was added to the medium before seedling planting. The nitrogenase activity of nodules was assayed by the acetylene reduction technique as already described (34).

Light microscopy of the infection process. Seeds were sterilized by immersion first in 70% (vol/vol) ethanol for 30 s and then in 0.1% mecuric chloride for 5 min and incubated for 36 h on Fahraeus medium solidified with 1.2% agar (9). Germinating seeds with radicles of 0.5 to 1 cm were inoculated by adding Fahraeus liquid medium containing ca. 2×10^8 bacteria per ml and transferred in a slide assembly (9) or on a solidified medium in petri dishes. With seedlings grown and inoculated on agar medium, plants were then transferred between a slide and a cover slip for observation. Root hairs were observed daily from day 3 after inoculation up to day 11. Rootlets were stained for 30 s with 0.01% methylene blue in liquid Fahraeus medium as described elsewhere (J. Vasse and G. Truchet, submitted for publication); after being rinsed, roots were observed by photomicroscopy with direct illumination. Roots were also examined after "clearing" as follows: fixation by 4% glutaraldehyde, ethanol dehydration, and treatment with an undiluted commercial solution of methyl benzoate, a cytoplasm clearing agent (J. Vasse and G. Truchet, submitted for publication). After rehydration, plants were stained with methylene blue and rinsed before observation by light microscopy.

Microscopy of nodules. Nodules of *Medicago sativa* and *Melilotus alba* were fixed for 90 min with 4% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) followed by 1 h in 1% OsO_4, dehydrated in an ethanol series, and embedded in Epon. Semithin sections were laid on a slide, stained by the basic fuchsin-methylene blue method (15), and observed with the microscope with direct illumination. Ultrathin sections on Formvar membrane grids were stained according to the method of Reynolds (27) and observed by transmission electron microscopy (Hitachi EM 600).

RESULTS

Transfer of pSym2011 genes into *A. tumefaciens*. (i) Introduction of an RP4-prime episome carrying pSym genes. In *R. meliloti* RCR2011, nitrogenase and nodulation genes are located on a pSym megaplasmid (22, 28, 31). A mutant carrying a Tn*5* insertion in the *nif* region (30), but not altered

in its symbiotic properties, was used to clone into RP4 a 290-kb fragment of the pSym plasmid (17a) containing the nitrogenase genes and the *nod* region mapped by Long et al. (22). This RP4-prime episome, pGMI42, was introduced into *A. tumefaciens* GMI9013 by mating with an *E. coli* donor GMI3232(pGMI42). Strain GMI9013 is a derivative of *A. tumefaciens* C58 which is cured of the Ti plasmid and carries chromosomal antibiotic resistance markers chloramphenicol (Cm^r), erythromycin (Ery^r), and rifampicin (Rif^r), and a Tn*1* insertion (Cb^r) to tag the pAtC58 large cryptic plasmid. From the cross, *Agrobacterium* tranconjugants had retained the Ery^r unselected marker and the Cb^r marker of the pAtC58 plasmid and were sensitive to the *Agrobacterium* phages S2 and S5. Agarose gel electrophoresis showed, in addition to the indigenous pAtC58 plasmid (410 kb), the presence of a plasmid of the same electrophoretic mobility as pGMI42 (Fig. 1, lanes B and D). A procedure to cure strain C58 of its cryptic pAt plasmid has been recently found (C. Rosenberg and T. Huguet, manuscript in preparation). It was thus possible to introduce pGMI42 into *Agrobacterium* C58 derivatives with the following combinations of indigenous plasmids: pTi^- pAt^+, pTi^+ pAt^-, and pTi^- pAt^-.

(ii) Mobilization of pSym by RP4. To mobilize the pSym plasmid, we needed to tag it with a second genetic marker to (i) avoid the problem caused by the rather high Tn*5* transposition frequency during introduction into *R. meliloti* (10, 23) (ii) avoid the selection of small episomes carrying only a short fragment of pSym carrying the Tn*5* insertion, and (iii) allow the use of a pSym unselected marker. The Tn*7* transposon (Tp^r Sm^r Sp^r) was recently shown not to transpose at a high frequency in strain 2011 and therefore was used to mutagenize the pSym region cloned in the episome pGMI42 (17a). The region-specific Tn*7* mutagenesis procedure used was the following. Bacteriophage Mu was inserted into pGMI42 in order to generate a "suicide" plasmid unable to replicate stably in *R. meliloti* 2011 (2, 10, 23). The pGMI42::Mu episome (pGMI104) was next mutagenized with Tn*7* by transfer from *E. coli* GMI3506(pGMI104) to strain GMI3232 and then introduced into a *R. meliloti* 2011 recipient by mating. A Neo^r Tp^r Sm^r Sp^r *R. meliloti* transconjugant, GMI243, in which it was not possible to detect the pGMI104 suicide episome (Fig. 1., lane C) was assumed to be a strain carrying both Tn*5* and Tn*7* insertions in the pSym plasmid (plasmid pGMI27) and was chosen for the following experiments.

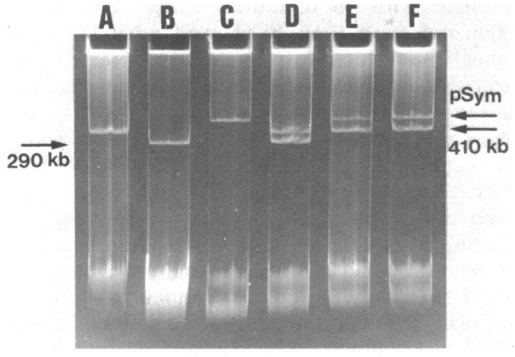

FIG. 1. Electrophoretic characterization of plasmids in *E. coli*, *R. meliloti*, *A. tumefaciens*, and their transconjugants. Lane A, the *A. tumefaciens* 9013 recipient harboring the resident pAtC58::Tn*1* plasmid (410 kb); lane B, *E. coli* donor ED8767 carrying pGMI42; lane C, *R. meliloti* GMI243 with its pSym megaplasmid; lane D, *A. tumefaciens* 9013 transconjugant with pAtC58::Tn*1* (upper band) and pGMI42 (lower band); lanes E and F, *A. tumefaciens* 9013 transconjugants with pAtC58::Tn*1* (lower band) and pGMI27 megaplasmid (upper band).

When *R. meliloti* GMI243 was mated with *A. tumefaciens* GMI9013, no transfer of Km^r Sp^r could be detected, suggesting that the pSym2011 plasmid was not self-transmissible at a detectable frequency. In an attempt to mobilize pSym, RP4, or pGMI106, an in vitro-constructed RP4 derivative carrying a 4-kb fragment of pSym2011 to create a source of DNA homology between the RP4 sex factor and pSym was introduced into strain GMI243. Both RP4 and the RP4-prime episome pGMI106 mobilized transfer of Sp^r into *A. tumefaciens* at a frequency of about 2×10^{-4} per initial donor. Agarose gel electrophoresis showed the presence of a band corresponding to the resident plasmid pAtC58::Tn*1* in all the Sp^r *Agrobacterium* transconjugants tested. All the transconjugants had the unselected markers, the phage sensitivity, and the erythromycin and carbenicillin resistance of *A. tumefaciens*. A sample of transconjugants, obtained either with GMI243(RP4) or GMI243(pGMI106) donors, was submitted to plasmid profile analysis. Five Tc^s transconjugants exhibited a band of the same electrophoretic mobility as the pGMI27 megaplasmid of *R. meliloti* (Fig. 1, lanes C, E, and F), showing that RP4 as well as pGMI106 can mobilize pSym2011 into *A. tumefaciens*. In 12 Tc^r transconjugants, a plasmid of about 150 kb could be detected, corresponding probably to an RP4-prime carrying an insertion of a pSym fragment which included Tn*5* and Tn*7*. No transconjugants were found to carry both pGMI27 and RP4.

Root deformation and nodulation by *Agrobacterium* transconjugants. *Agrobacterium* transconjugants GMI9013 (pGMI27) and GMI9013(pGMI42) were inoculated onto the root system of the four leguminous hosts *Medicago sativa* and *Melilotus alba* (which are effectively nodulated by *R. meliloti* 2011) and *T. pratense* and *T. repens* (which are not nodulated).

The *Agrobacterium* control strain GMI9013 had no detectable effect on the roots of the four legumes. *R. meliloti* GMI243 did not initiate any response on roots of the two clover species but induced the formation of pink oval nodules on the two homologous hosts. The *Agrobacterium* transconjugants had no detectable effect on the roots of *Trifolium* seedlings but induced thickening, yellowing, and swelling of the roots of *Medicago sativa* and *Melilotus alba* (Fig. 2). These root swellings culminated in the formation of nodule-like structures which appeared sooner and were more numerous with strain GMI9013(pGMI27) than with strain GMI9013(pGMI42) on *Medicago sativa* (Fig. 3A), as well as on *Melilotus alba* (Fig. 3B). These nodule-like structures were small, spherical, and white-yellow and had no detectable nitrogenase activity 4 weeks after inoculation. Their rates of appearance were much slower than in the case of seedlings inoculated with the *R. meliloti* control strain GMI243 (Fig. 3). An *Agrobacterium* strain cured of the Ti plasmid and of the pAtC58 plasmid and containing plasmid pGMI42 induced nodules on alfalfa roots showing that the *Agrobacterium* plasmids are not involved in the nodule-inducing ability of the transconjugants.

Effect of combined nitrogen on nodule formation. On *Medicago sativa*, nodule formation is inhibited by NO_3^- concentrations well below the level necessary to cause injury to the bacterial symbiont or the host plant (33). Addition of 20 mM KNO_3 to the plant growth medium repressed nodule formation by *R. meliloti* GMI243 on *Medicago sativa* and also repressed the visible reactions of plant roots to inoculation by *Agrobacterium* hybrids GMI9013(pGMI27) and

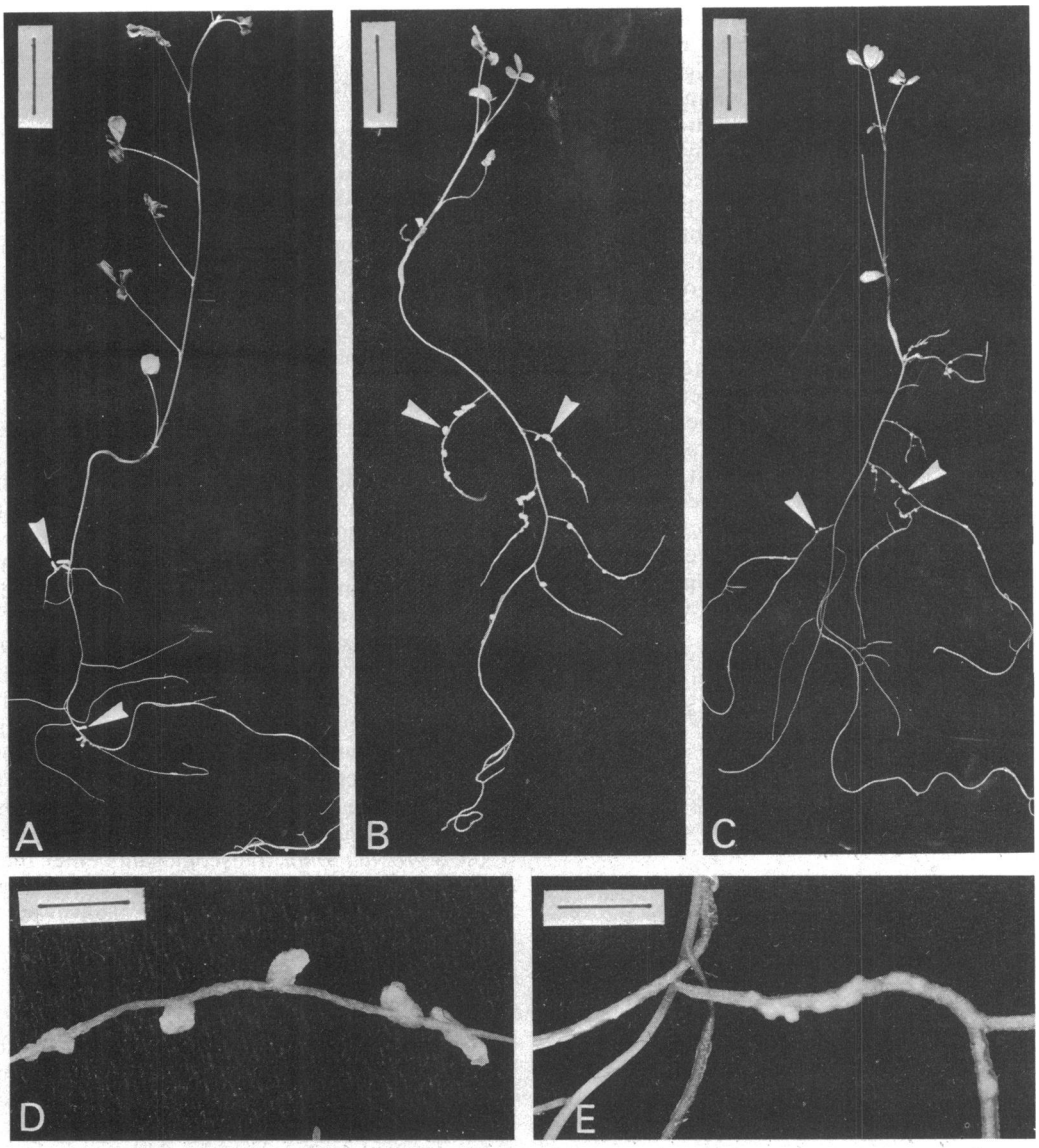

FIG. 2. Root systems of *Medicago sativa* inoculated with (A) *R. meliloti* GMI243, (B and D) *A. tumefaciens* GMI9013(pGHI27) and (C and E) strain GMI9013(pGMI42). Arrows point to differentiated nodules. Note the difference in morphology between nodules in (D) and (E). Panels A, B, and C. bar = 1 cm; panels D and E. bar = 2 mm.

GMI9013(pGMI42): roots remained white and thin, and root swelling was strongly depressed and delayed (Table 2).

Nodule organogenesis. Twenty nodule-like structures induced on the root system of *Medicago sativa* and *Melilotus alba* seedlings by strains GMI9013(pGMI42) and GMI9013 (pGMI27) were observed by light and electron microscopy. These structures originated from the inner cortex of the roots outside of the endodermis area (as illustrated in Fig. 4). Thus, they are not derived from deformations of secondary roots but have a cortical origin, as do the normal root nodules of alfalfa (11, 33, 34). They have a localized apical meristem, an endodermis separating a central zone from the outer nodular cortex, and vascular bundles (Fig. 4B) localized as in normal alfalfa root nodules. All these observations of the root deformations induced by *Agrobacterium* transconjugants show clearly that they are not tumors or deformed secondary roots but have a similar developmental origin and anatomy to the alfalfa nitrogen-fixing nodules induced by *R. meliloti*.

In root nodules induced by effective *R. meliloti* strains, the submeristematic zone, or zone II, contained numerous infection threads (Fig. 4D), and bacteria were released from the threads into the host cells; most cells of the central tissue, zone III, were filled by bacteroids (33, 34). In contrast, in the nodules induced by the *Agrobacterium* transconjugants GMI9013(pGMI27) and GMI9013(pGMI42), no infection thread could be seen in semithin sections of the submeristematic zone (Fig. 4C). In the central tissue, the plant cells had small nuclei, contained large amyloplasts, and were devoid of bacteria (Fig. 4E). Thus, the *Agrobacterium* transconjugants appear to induce specifically the formation of bacteria-free root nodules.

The infection process. In the *R. meliloti-Medicago sativa* symbiosis, the early steps of the specific infection can be visualized as a tight curling of root hairs called "shepherd's crook," followed by the formation of the infection threads in root hairs (Fig. 5A). Although the *Agrobacterium* control strain was not able to induce root hair curling on young roots

of *Medicago sativa* and *Melilotus alba*, *Agrobacterium* transconjugant GMI9013(pGMI27) clearly induced shepherd's crook formation on host root hairs (Fig. 5B). However, infection threads could not be seen within the root hairs (Fig. 5B). To ensure that this lack of detection was not due to a technical limitation, roots were stained by two different techniques. Half of the samples were stained directly with methylene blue, whereas the other half were pretreated with the clearing agent methylbezoate and then stained (J. Vasse and G. Truchet, submitted for publication). A total of 90 inoculated young roots grown in either liquid or agar medium were observed, and no infection threads were seen either in the root hairs or in the root cortex from day 3 to day 11 after inoculation. However, diffuse areas in the outer cortex of the root appeared heavily stained by methylene blue. This observation prompted us to determine whether these stained diffuse areas could correspond to local infiltrations of bacteria through the outer cortex of the plant host.

Light microscopy of semithin sections of either young or well-developed nodules triggered by *Agrobacterium* hybrids on *Medicago sativa* and *Melilotus alba* revealed the presence of thread-like structures infiltrating some intercellular spaces in the outer cortex. These structures were generally

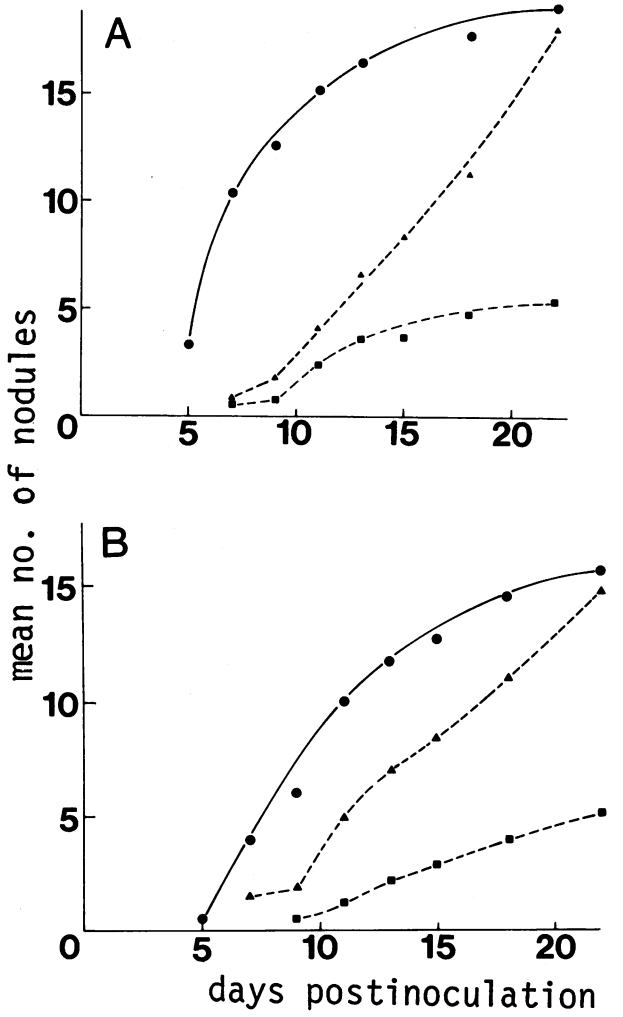

FIG. 3. Kinetics of nodule formation on (A) *Medicago sativa* and (B) *Melilotus alba*. Symbols: ○, *R. meliloti* GMI243; ▲, *A. tumefaciens* GMI9013(pGMI27); and ■, *A. tumefaciens* GMI9013(pGMI42).

TABLE 2. Inhibition of nodule formation by KNO_3

Bacterial strain	No. of nodules[a]	
	Medicago sativa	*Melilotus alba*
E. coli ED8767(pGMI42)	0	0
A. tumefaciens GMI9013	0	0
R. meliloti(pGMI27)	19	15.6
+ 20 mM KNO_3	4.1	
A. tumefaciens GMI9013(pGMI42)	5.4	5.2
+ 20 mM KNO_3	0	
A. tumefaciens GMI9013(pGMI27)	18	14.7
+ 20 mM KNO_3	0.3	

[a] Mean number of nodules per tube 22 days after inoculation.

located in an apical position and did not generate invaginations within the host cell volume (Fig. 6A and B). In contrast, in nodules induced by the *R. meliloti* control strain the infection threads were located in the submeristematic zone (Fig. 4C) and produced invaginations into the host cells. Electron microscope observation of the thread-like structures induced by *Agrobacterium* hybrids showed that they were similar to typical infection threads induced by *R. meliloti* in that bacteria were enclosed by a fibrillar matrix of mucilage surrounded by the host cell wall (Fig. 6C, D, E, and F), but they could be observed only within the intercellular space and never in the cell volume. Thus, it appears that the *Agrobacterium* transconjugants enter the host plant by an atypical infection process. Instead of entering at the root hair level by the formation of infection threads which grow toward the inner cortex of the root, the results indicate that the *Agrobacterium* transconjugants enter the host plant by infiltration of the intercellular space, forming diffuse infection traces in the outer cortex. However, this restricted infection is sufficient to trigger specifically the organogenesis of root nodules.

DISCUSSION

The induction of nodule organogenesis on the root system of an homologous host by a *Rhizobium* strain is a complex process entailing a sequence of specific interactions between the host plant and the invading bacteria (36). In *R. meliloti* are grouped bacterial strains which are reported to show a strong host specificity and nodulate only species of *Medicago, Melilotus*, and *Trigonella* (32). The *R. meliloti* strains are a homogenous group from the physiological, taxonomical, and genetic points of view (8, 19, 32). All the *R. meliloti* strains studied so far contain a pSym megaplasmid which carries nitrogenase genes (1, 28, 29, 31), as well as genes controlling nodule formation (1, 22, 28), but the genetic basis of *R. meliloti* host specificity has not been studied. Recently, however, Kondorosi et al. (18) reported that the mobilization of the pSym megaplasmid of *R. meliloti* 41 into *A. tumefaciens* was associated with the transfer of the ability to induce nodule-like structures on *Medicago sativa*, suggesting that host-specificity genes were also located on pSym.

We have shown that the pSym megaplasmid of *R. meliloti* 2011, which is not self-transmissible at detectable frequencies, can be mobilized into *A. tumefaciens* by the RP4 plasmid. That RP4 behaves as a sex factor in strain 2011 was already shown by its mobilization of the chromosome (17, 24). It was not clear from our experiments why RP4 or pGMI106 were not present in the transconjugants after mobilization of pGMI27.

The transfer of the entire pSym or of a large region of pSym into *A. tumefaciens* is associated with the transfer of the ability to induce the formation of nodules specifically on

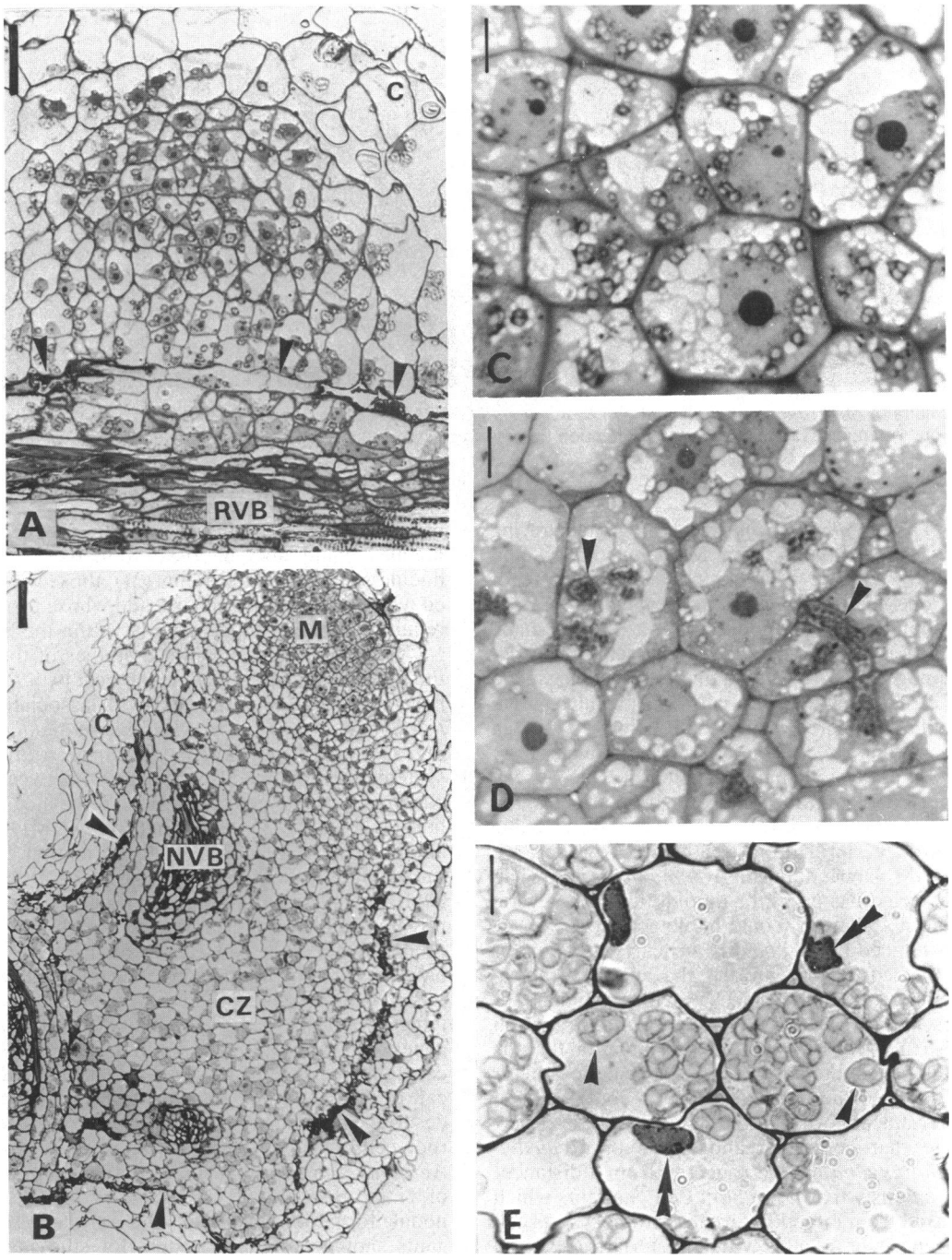

FIG. 4. Nodule organogenesis. Light microscopy of nodules induced on *Medicago sativa* by (A) *A. tumefaciens* GMI9013(pGMI42), (B, C, and E) strain GMI9013(pGMI27), and (D) *R. meliloti* GMI243. (A) Transverse section showing the cortical origin of the initial meristem of the nodule. Arrows, root endodermis; C, root cortex; RVB, root vascular bundles. Bar = 40 μm. (B) Nodule induced by strain GMI9013(pGMI27). M, apical meristem; CZ, central zone; NVB nodule vascular bundles; C, nodule cortex; arrows, nodule endodermis. Bar = 100 μm. (C and D) Semithin sections of the submeristematic zones. Infection threads are visible in the section of *R. meliloti* nodule (arrows, panel D) but not in the section of *A. tumefaciens* GMI9013(pGMI27) nodule (C). Bar = 10 μm. (E) Semithin section of the central zone of a strain 9013(pGMI27) nodule. Plant cells, devoid of bacteria, are filled with large amyloplasts (arrows). Note the small size of nuclei (double arrows). Bar = 10 μm.

Medicago sativa and *Melilotus alba; Agrobacterium* transconjugants do not provoke detectable root deformation on heterologous clover hosts. This result shows that the genetic information required for the specific induction of nodule formation is located on the pSym megaplasmid, more precisely in the 290-kb region of pSym which has been cloned into RP4 and which contains root hair curling nodulation genes (17a, 22). Thus, in *R. meliloti*, genes controlling host-range specificity, nodulation, and nitrogen fixation are located on the same replicon as has been shown for *R. leguminosarum* (12), *R. trifolii* (14, 26), and *R. phaseoli* (20). One difference is that these symbiotic genes are located on a megaplasmid of more than 700 kb in *R. meliloti* and on plasmids of varying size (160 to 500 kb) in the *R. legumino-*

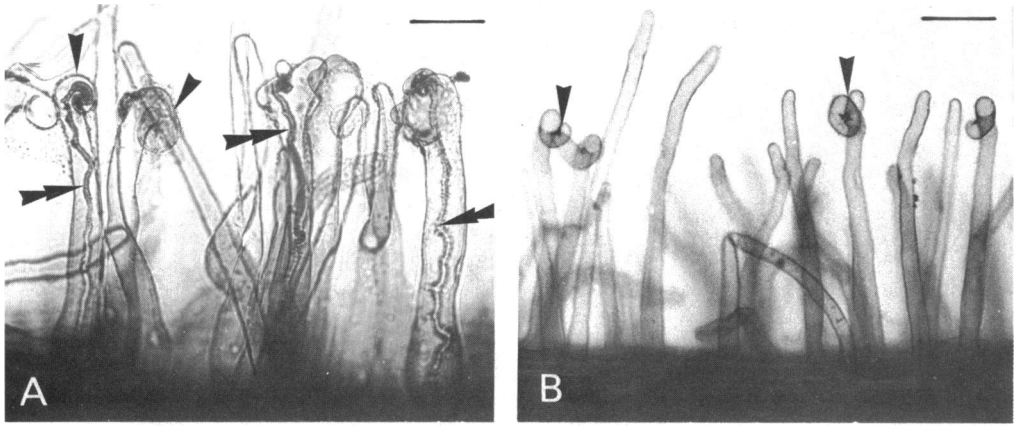

FIG. 5. Root hairs of *Medicago sativa* infected by (A) *R. meliloti* GMI243 or (B) *A. tumefaciens* GMI9013(pGMI27). Typical shepherd's crooks are seen on both micrographs (arrows), but infection threads were detected only in plants inoculated with *R. meliloti* (double arrows, panel A). (A) Bar = 35 μm; (B) bar = 23 μm.

sarum cluster. The *Agrobacterium* transconjugants carrying pSym behaved differently on both *Medicago sativa* and *Melilotus alba* from those carrying pGMI42; the rate of nodule formation was faster, and the proportion of root deformations culminating in a nodule with a definite shape was higher. These results suggest that some genes influencing nodule organogenesis are located on the pSym megaplasmid outside the region cloned into pGMI42. An alternative explanation could be an instability of the R-prime pGMI42 in *A. tumefaciens*, giving rise to deletions in the cloned pSym fragment. This hypothesis cannot be ruled out, because it was not possible to isolate transconjugants from nodules, the bacteria present in the superficial intercellular spaces being killed by the surface sterilization treatment. However, we observed that pGMI42 was stable in *A. tumefaciens* grown in the absence of selective pressure (antibiotics) for several generations, and no deletions could be detected.

The nodules induced by *A. tumefaciens* carrying pSym or pGMI42 have the cortical origin and the anatomy (apical meristem, peripherical endodermis, vascular bundles) of typical nodules induced by effective *R. meliloti* strains on *Medicago sativa* and *Melilotus alba*, but no infection threads or bacteria could be detected in the submeristematic zone or in the central tissue of the nodules. Bacteria could be seen only in the outer apical cortex of the nodules. These observations indicate that nodule organogenesis of *Medicago sativa* and *Melilotus alba* can be triggered from a distance. We reported previously that *R. meliloti* Leu$^-$ mutants which cannot be released from infection threads into host cells can induce organogenesis of nodules with a bacteria-free central tissue. We hypothesized the existence of a diffusible or transferable morphogen of bacterial origin, the nodule organogenesis-inducing principle (34). The present results are in agreement with this hypothesis and suggest that the required gene(s) is located in the pGMI42 region of the pSym megaplasmid, that is, in a region located to the left of the nitrogenase genes on the pSym map (22, 31; Julliot et al., in press). *A. tumefaciens* is a suitable host for cloning *R. meliloti* genes involved in nodule organogenesis induction. The resulting "bacteria-free" nodules could be used to investigate by nucleic acid hybridization whether triggering of nodulation is mediated by the transfer of a bacterial DNA fragment into the host genome as occurs before the tumor formation induced by of *A. tumefaciens*. If such a fragment exists, it should be located on the pGMI42 plasmid to the left of the *nif* region of the megaplasmid. These bacteria-free nodules could also be used to detect, from among the nodule-specific plant proteins (21), those which are associated with nodule organogenesis and whose formation does not require the presence of bacteria within the host cells.

Cytological and biochemical studies of the early steps in the specific infection of a legume host by a *Rhizobium* strain have mostly been performed with association between *R. trifolii* and *T. repens* (5), *Rhizobium japonicum* and *Glycine max* (35), and *R. meliloti* and *Medicago sativa* (25). In these systems the first specific recognition between the plant and bacterial partners is hypothesized to be mediated at the root hair level (5, 25, 35). *A. tumefaciens* GMI9013(pGMI27) is able to induce shepherds crook formation, which confirms the previous observation of Long et al. (22) that in *R. meliloti* 2011 gene(s) controlling root hair curling are located on the pSym megaplasmid. However, no infection thread could be seen in these root hairs. Instead, bacterial infection must have occurred by infiltration of the intercellular spaces of the outer cortical cells of the roots. Nevertheless, this atypical infection results in the specific induction of nodule organogenesis in *Medicago sativa* and *Melilotus alba*, which indicates that the specific recognition between bacteria and plant can be mediated not only at the root hair level as already described in alfalfa (25) but also at the level of other cortical root cells. This atypical infection is not controlled by an *Agrobacterium* plasmid since an *Agrobacterium* strain cured of Ti and pAtC58 and containing pGMI42 is able to induce nodule formation on alfalfa. The *Agrobacterium* transconjugants should provide a system for studying the molecular basis of specific recognition: the nature of the bacterial surface components coded for by pSym or pGMI42 and the interactions of the hybrid strains with the host roots and host lectins (25).

The transfer of pSym from *R. leguminosarum* (13) or *R. trifolii* (14) into *A. tumefaciens* rendered it able to induce nodules on pea and clover, respectively. Infection threads were present in the nodules, and bacteria were released into the host cells. The fact that the introduction of pSym strain 2011 into *A. tumefaciens* did not result in the formation of infection threads and infection of host cells suggests that either pSym strain 2011 does not carry all of the information required for this process or that the *Agrobacterium* recipient strain does not allow correct expression of these genes.

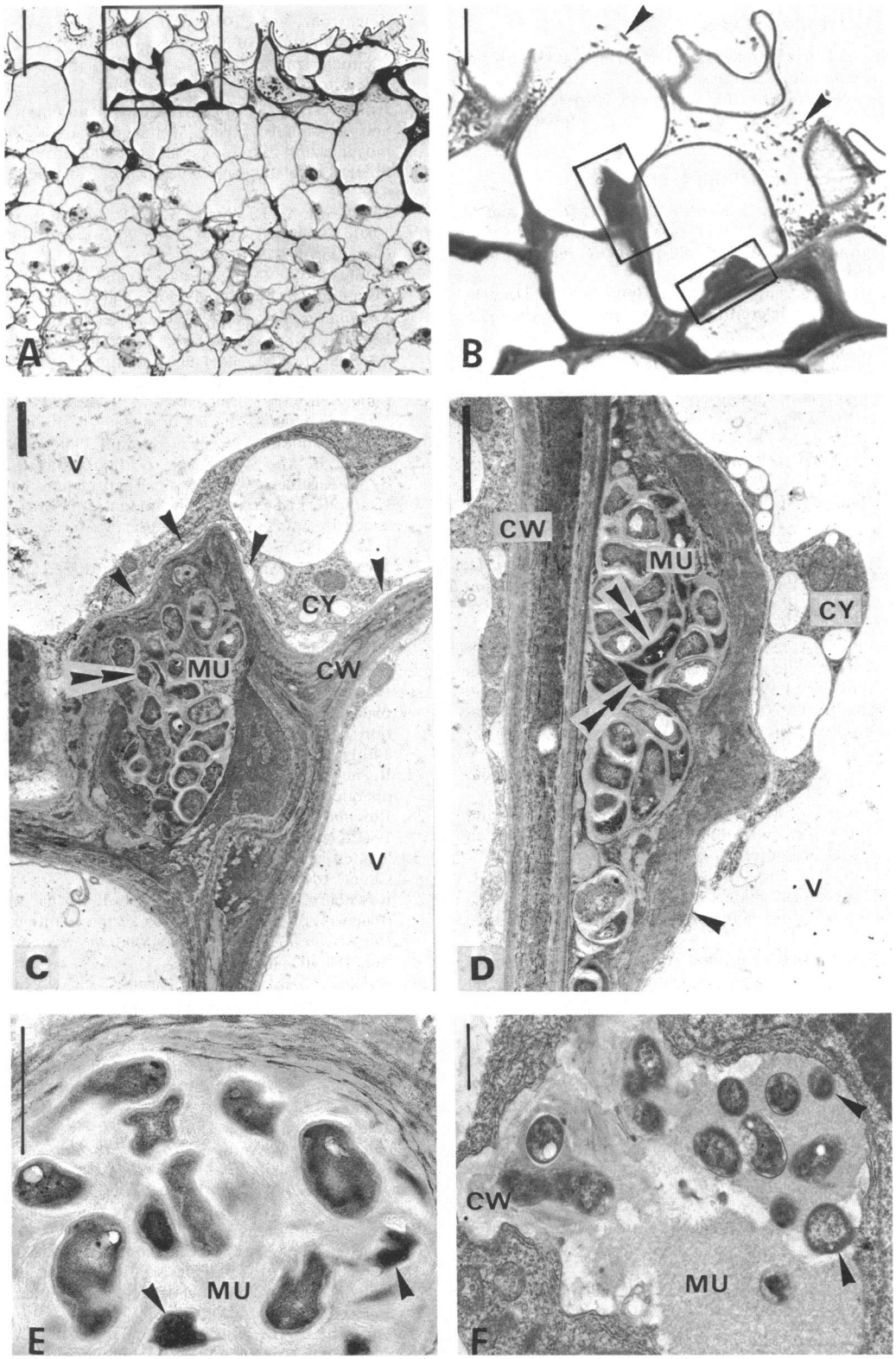

FIG. 6. The infection process. (A, B, C, D, and E) Sections of nodules of *Medicago sativa* inoculated woth *A. tumefaciens* GMI9013(pGMI42). (A) Apical part of a nodule. Bar = 40 μm; (B) Enlargement of the insert in A. Arrows, bacteria; bar = 10 μm. (C and D) Electron micrographs of inserts 1 and 2 in (B). Infection traces: intercellular structures which appear to be homologous to the infection threads in the nodules induced by *R. meliloti*. Bacteria are enclosed in mucilage (Mu) located between the two cellulose sheathes of the plant cell wall (CW). Plant plasmalemma (arrows) is clearly seen. In the mucilage some bacteria undergo precocious degeneration (double arrows); V, plant cell vacuole; CY, plant cytoplasm. (E) Magnification of an infection trace showing the fibrillar matrix of the mucilage (Mu); arrows, degenerating bacteria. (F) Typical infection thread located in the submeristematic part of a nodule induced by *R. meliloti*. Arrows, bacterial release from the infection thread; Mu, mucilage; CW, plant cell wall. Panels C, D, E, and F: bars = 1 μm.

ACKNOWLEDGMENTS

We thank Betty and Eric Terzaghi for reviewing the manuscript and T. Huguet for stimulating discussions.

This work was supported by the Biological Nitrogen Fixation grant from Société Nationale Elf Aquitaine and Entreprise Minière et Chimique.

LITERATURE CITED

1. **Banfalvi, Z., V. Sakanyan, C. Koncz, A. Kiss, I. Dusha, and A. Kondorosi.** 1981. Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *R. meliloti*. Mol. Gen. Genet. **184**:334–339.
2. **Boucher, C., B. Bergeron, M. De Bertalmio, and J. Dénarié.** 1977. Introduction of bacteriophage Mu into *Pseudomonas solanacearum* and *Rhizobium meliloti* using the R factor RP4. J. Gen. Microbiol. **98**:253–263.
3. **Cami, B., and P. Kourilsky.** 1978. Screening of cloned recombinant DNA in bacteria by in situ colony hybridization. Nucleic Acids Res. **5**:2381–2390.
4. **Datta, N., R. W. Hedges, E. J. Shaw, R. B. Sykes, and M. H. Richmond.** 1971. Properties of an R factor from *Pseudomonas aeruginosa*. J. Bacteriol. **108**:1244–1249.
5. **Dazzo, F. B., and D. H. Hubbell.** 1982. Control of root hair infection, p. 274–310. *In* W. J. Broughton (ed.), Nitrogen fixation, vol. 2, *Rhizobium*. Oxford University Press, Oxford.
6. **Dénarié, J., P. Boistard, F. Casse-Delbart, A. G. Atherly, J. O. Berry, and P. Russell.** 1981. Indigenous plasmids in *Rhizobium*, p. 225–246. *In* K. L. Giles and A. G. Atherly (ed.), Biology of the Rhizobiaceae. Academic Press, Inc., New York.
7. **Eckhardt, T.** 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid **1**:584–588.
8. **Elkan, G. H.** 1981. The taxonomy of the *Rhizobiaceae*, p. 1–14. *In* K. L. Giles and A. G. Atherly (ed.), Biology of the Rhizobiaceae. Academic Press, Inc., New York.
9. **Fahraeus, G.** 1957. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J. Gen. Microbiol. **16**:374–381.
10. **Forrai, T., E. Vincze, Z. Banfalvi, G. Kiss, G. S. Randhawa, and A. Kondorosi.** 1983. Localization of symbiotic mutations in *Rhizobium meliloti*. J. Bacteriol. **153**:635–643.
11. **Hirsch, A. M., S. R. Long, M. Bang, N. Haskins, and F. M. Ausubel.** 1982. Structural studies of alfalfa roots infected with nodulation mutants of *Rhizobium meliloti*. J. Bacteriol. **151**:411–419.
12. **Hombrecher, G., N. J. Brewin, and A. W. B. Johnston.** 1981. Linkage of genes for nitrogenase and nodulation ability on plasmids of *Rhizobium leguminosarum* and *R. phaseoli*. Mol. Gen. Genet. **182**:133–136.
13. **Hooykaas, P. J. J., F. G. M. Snijdewint, and R. A. Shilperoort.** 1982. Identification of the Sym plasmid of *Rhizobium leguminosarum* strain 1001 and its transfer to and expression in other rhizobia and *Agrobacterium tumefaciens*. Plasmid **8**:73–82.
14. **Hooykaas, P. J. J., A. A. N. Van Brussel, H. Den Dulk-Ras, G. M. S. Von Slogteren, and R. A. Schilperoort.** 1981. Sym-plasmid of *Rhizobium trifolii* expressed in different rhizobial species and in *Agrobacterium tumefaciens*. Nature (London) **291**:351–353.
15. **Huber, J. D., F. Parker, and G. F. Odland.** 1968. A basic fuchsin and alkanized methylene blue rapid stain for epoxy embedded tissue. Stain Technol. **43**:83–87.
16. **Johnston, A. W. B., J. L. Beynon, A. V. Buchanan-Wollaston, S. M. Setchell, P. R. Hirsch, and J. E. Beringer.** 1978. High frequency transfer of nodulating ability between strains and species of *Rhizobium*. Nature (London) **276**:635–636.
17. **Julliot, J. S., and P. Boistard.** 1979. Use of RP4-prime plasmids constructed in vitro to promote a polarized transfer of the chromosome in *Escherichia coli* and *Rhizobium meliloti*. Mol. Gen. Genet. **173**:289–298.

17a. **Julliot, J. S., I. Dusha, M. H. Renalier, B. Terzaghi, A. M. Garnerone, and P. Boistard.** 1984. An RP4-prime containing a 285-kb fragment of *Rhizobium meliloti* pSym plasmid: structural characterization and utilization for genetic studies of symbiotic functions controlled by pSym. Mol. Gen. Genet. **193**:17–26.

18. **Kondorosi, A., E. Kondorosi, C. E. Pankhurst, W. J. Broughton, and Z. Banfalvi.** 1982. Mobilization of a *Rhizobium meliloti* megaplasmid carrying nodulation and nitrogen fixation genes in other rhizobia and *Agrobacterium*. Mol. Gen. Genet. **188**:433–439.
19. **Kondorosi, A., E. Vincze, A. W. B. Johnston, and J. E. Beringer.** 1980. A comparison of three *Rhizobium* linkage maps. Mol. Gen. Genet. **178**:403–408.
20. **Lamb, J. W., G. Hombrecher, and A. W. B. Johnston.** 1982. Plasmid determined nodulation and nitrogen-fixation abilities in *Rhizobium phaseoli*. Mol. Gen. Genet. **186**:449–452.
21. **Legocki, R. P., and D. P. S. Verma.** 1980. Identification of "nodule-specific" host proteins (nodulins) involved in the development of *Rhizobium*-legume symbiosis. Cell **20**:153–164.
22. **Long, S. R., W. J. Buikema, and F. M. Ausubel.** 1982. Cloning of *Rhizobium meliloti* nodulation genes by direct complementation of Nod$^-$ mutants. Nature (London) **298**:485–488.
23. **Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149**:114–122.
24. **Meade, H. M., and E. R. Signer.** 1977. Genetic mapping of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. U.S.A. **74**:2076–2078.
25. **Paau, A. S., W. T. Leps, and W. J. Brill.** 1978. Agglutinin from alfalfa necessary for binding and nodulation by *Rhizobium meliloti*. Science **213**:1513–1515.
26. **Prakash, R. K., R. A. Schilperoort, and M. P. Nuti.** 1981. Large plasmids of fast-growing rhizobia: homology studies and location of structural nitrogen fixation (*nif*) genes. J. Bacteriol. **145**:1129–1136.
27. **Reynolds, E. I.** 1963. The use of lead citrate as an electron opaque stain in electron microscopy. J. Cell Biol. **17**:208–212.
28. **Rosenberg, C., P. Boistard, J. Dénarié, and F. Casse-Delbart.** 1981. Genes controlling early and late functions in symbiosis are located on a megaplasmid in *Rhizobium meliloti*. Mol. Gen. Genet. **184**:326–333.
29. **Rosenberg, C., F. Casse-Delbart, I. Dusha, M. David, and C. Boucher.** 1982. Megaplasmids in the plant-associated bacteria *Rhizobium meliloti* and *Pseudomonas solanacearum*. J. Bacteriol. **150**:402–406.
30. **Ruvkun, G. B., and F. M. Ausubel.** 1981. A general method for site-directed mutagenesis in prokaryotes. Nature (London) **289**:85–88.
31. **Ruvkun, G. B., V. Sundaresan, and F. M. Ausubel.** 1982. Directed transposon mutagenesis and complementation analysis of *Rhizobium meliloti* symbiotic nitrogen fixation genes. Cell **29**:551–559.
32. **Trinick, M. J.** 1982. Biology, p. 76–146. *In* W. J. Broughton (ed.), Nitrogen fixation, vol. 2, *Rhizobium*. Oxford University Press, Oxford.
33. **Truchet, G. L., and F. B. Dazzo.** 1982. Morphogenesis of lucerne root nodules incited by *Rhizobium meliloti* in the presence of combined nitrogen. Planta **154**:352–360.
34. **Truchet, G., M. Michel, and J. Dénarié.** 1980. Sequential analysis of the organogenesis of lucerne (*Medicago sativa*) root nodules using symbiotically-defective mutants of *Rhizobium meliloti*. Differentiation **16**:163–172.
35. **Turgeon, B. G., and W. D. Bauer.** 1982. Early events in the infection of soybean by *Rhizobium japonicum*. Time course and cytology of the initial infection process. Can. J. Bot. **60**:152–161.
36. **Vincent, J. M.** 1980. Factors controlling the legume-*Rhizobium* symbiosis, p. 103–129. *In* W. E. Newton and W. H. Orme-Johnson (ed.), Nitrogen fixation, vol. 2. University Park Press, Baltimore.