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Two Gene Clusters of *Rhizobium meliloti* Code for Early Essential Nodulation Functions and a Third Influences Nodulation Efficiency

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A pLAFR1 cosmid clone (pPP346) carrying the nodulation region of the symbiotic plasmid pRme41b was isolated from a gene library of *Rhizobium meliloti* 41 by direct complementation of a Nod⁻ deletion mutant of *R. meliloti*. *Agrobacterium tumefaciens* and *Rhizobium* species containing pPP346 were able to form ineffective nodules on alfalfa. The 24-kilobase insert in pPP346 carries both the common nodulation genes and genes involved in host specificity of nodulation. It was shown that these two regions are essential and sufficient to determine the early events in nodulation. A new DNA region influencing the kinetics and efficiency of nodulation was also localized on the symbiotic megaplasmid at the right side of the *nif* genes.

Rhizobia induce the formation of nitrogen-fixing nodules on the roots of leguminous plants. In the fast-growing species of rhizobia, genes determining the early steps of nodule development (*nod* genes) were located on large indigenous plasmids (2, 11, 22, 23, 26, 38, 47).

In *Rhizobium meliloti* a very high molecular weight plasmid (megaplasmid) confers the ability to nodulate the host plant alfalfa (*Medicago sativa*) (2, 38). When this megaplasmid was mobilized into other *Rhizobium* species or *Agrobacterium tumefaciens*, the transconjugants induced nodules on alfalfa, indicating that the early steps of nodulation and host range specificity are coded by this symbiotic plasmid (pSym) (31, 44). These nodules, however, developed only to a certain stage: there was no initiation of infection thread formation, and bacteria were not released into the inner part of the nodule (44, 46).

The *nod* genes are closely linked to the *nif* genes in *R. meliloti* (2, 38). In strain 1021, *nod* mutations were mapped 25 kilobases (kb) away from the *nifKDH* genes (33). In strain 41, R-prime plasmids carrying the essential *nod* genes and the *nif* structural genes were isolated (1), and the physical map of this region was constructed (29). The smallest *nod-nif* R-prime carried an insert of 90 kb. *nod* mutations were localized in two clusters within this region. On an 8.5-kb *Eco*RI fragment, nodulation genes conserved in a wide range of rhizobia (common *nod* genes) (29) and consisting of four genes (*nodABC* and *nodD*) were identified (41, 43; M. Gottfert, B. Horvath, E. Kondorosi, P. Putnoky, F. Rodriguez-Quiñones, and A. Kondorosi, J. Mol. Biol., in press, manuscript in preparation). A similar arrangement of the *nodABC* and *nodD* genes was found in strain 1021 (14, 25). Genes determining host range specificity (*hsn* genes) for alfalfa were localized on a 6.8-kb *Eco*RI fragment about 6 kb away from the 8.5-kb common *nod* fragment (29). The 6.8-kb fragment carries four *hsn* genes (*hsnEFGH*; B. Horvath, E. Kondorosi, M. John, T. Schmidt, I. Török, Z. Györgypal, I. Barabas, V. Wieneke, T. Schell, and A. Kondorosi, Cell, in press, manuscript in preparation).

In this paper we report the isolation of a recombinant cosmid carrying both the 8.5-kb and the 6.8-kb *nod* fragments and show that only these two fragments carry the essential early nodulation genes. Moreover, the existence of a new class of genes influencing the kinetics and efficiency of nodulation (*efn* genes) located on the symbiotic megaplasmid is demonstrated.

(Parts of this work were presented at the Second International Symposium on the Molecular Genetics of the Bacteria-Plant Interaction, Cornell University, Ithaca, N.Y., in June 1984.)

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used are listed in Table 1.

Media and growth conditions. For *R. meliloti* and *A. tumefaciens* strains, the growth conditions and complete medium (YTB) used have been described by Orosz et al. (36). Minimal medium (GTS) supplemented with 1 mg of NH_4Cl per ml was that described by Kiss et al. (28). For *R. leguminosarum*, *R. trifolii*, *R. lupini*, the broad-host-range *Rhizobium* sp. strain MPIK3030, and *R. fredii*, growth conditions and complete medium TY were those of Beringer (3). For *Escherichia coli* strains, YTB or GTS medium supplemented with appropriate amino acids, vitamins, and antibiotics was used. The concentration of antibiotics was as described by Kiss et al. (27).

Bacterial matings and transformations. Matings were carried out by the method of Kondorosi et al. (32) or Ditta et al. (9). For *E. coli* transformation, the method of Dagert and Ehrlich was used (8).

DNA isolation procedures. Plasmids were prepared from bacterial cultures grown in complete medium by the method of Ish-Horowicz and Burke (24). Total *R. meliloti* DNA was isolated as described by Meade et al. (35).

Restriction enzyme digestions and gel electrophoresis. Restriction endonucleases were purchased from REANAL (Budapest, Hungary). Digestions and gel electrophoresis were carried out as described by Maniatis et al. (34). T4 DNA ligase was provided by K. Burg.

Construction of *R. meliloti* gene bank. *R. meliloti* AK631 total DNA was partially digested with *Eco*RI and separated in a 0.5% (wt/vol) low-melting-temperature agarose gel (type VII; Sigma Chemical Co.). DNA in the size range of ca. 10 to 30 kb was recovered from the gel (45) and ligated with *Eco*RI-digested pLAFR1 DNA in 20 μg to 1 μg ratio (7, 17).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source or reference
Strains		
R. meliloti 41		
AK631	Wild-type variant with compact colony morphology	This lab
AK194	*cys-46 gly-1 ade-4 tyr-1 str-3*	30
AK245	*his-1*	L. Orosz
AK635	Nod⁻ deletion derivative of AK631 (see Fig. 1)	2
AK1212	Nod⁻ deletion derivative of AK631 (see Fig. 1)	2
ZB121	Nod⁻ deletion derivative of AK631 (see Fig. 1)	2
ZB129	Nod⁻ deletion derivative of AK631 (see Fig. 1)	Z. Banfalvi
ZB138	Nod⁻ deletion derivative of AK631 (see Fig. 1)	2
ZB157	Nod⁻ deletion derivative of AK631 (see Fig. 1)	2
AK1655	*nodA*::Tn*5*	29
AK1679	*nodB*::Tn*5*	29
AK1657	*nodC*::Tn*5*	29
R. leguminosarum 6015	*phe-1 trp-12 rif-392 str-37 nod-6007*	26
R. trifolii Resh403		W. J. Broughton
R. fredii USDA201	Wild type	M. Sadowsky
R. lupini CsfK1	Wild type	E. Bakondi
Rhizobium sp. strain MPIK3030 ZB710	Rif^r 5-Fu^r	Z. Banfalvi (37)
A. tumefaciens C58	Wild type	C. Koncz
E. coli		
HB101	*hsdS hsdM pro leu thi gal lacY recA str*	4
PP347	HB101(pPP346)	This work
PP370	HB101(pPP369)	This work
Plasmids		
pJB3JI	Km^s derivative of R68.45	5
pIJ1089	Cosmid clone carrying *nod* region of pRL1JI	12
pGR1	R′ derivative of pJB3JI	1
pGR3	R′ derivative of pJB3JI	1
pRK290	Tc^r, Inc-P1	9
pKSK5	pRK290 carrying 8.5-kb common *nod* fragment of pRme41b	29
pEK10	pRK290 carrying 6.8-kb *hsn* fragment of pRme41b	29
pLAFR1	Derivative of pRK290	17
pRK2013	Km^r helper plasmid	15
pPP346	Cosmid clone carrying 24-kb fragment of pRme41b	This work
pPP369	Cosmid clone carrying 25-kb fragment of pRme41b	This work

The in vitro packaging was carried out by the method of Scalenghe et al. (40). The components of the packaging system were prepared and provided by I. Takacs.

The transduction of *E. coli* HB101 bacteria was performed as described by Hohn (21).

Plant test. Alfalfa (*Medicago sativa* L. var. Nagyszenas) seeds were sterilized with 95% (vol/vol) ethanol for 3 min and with 0.1% (wt/vol) acidic mercuric chloride for 10 min. The seeds were then washed five times with sterile distilled water and germinated on 1% (wt/vol) distilled water agar. Seedlings were grown on slopes of Gibson medium (with 1% [wt/vol] agar) (18) in a greenhouse with a 16-h photoperiod and temperatures of 22°C.

For plant inoculation, bacteria were grown on the appropriate medium and suspended in 0.9% (wt/vol) NaCl, and 0.1 ml of the suspension was added to 2- to 4-day-old seedlings.

Bacteria were reisolated from the nodules after surface sterilization with 95% (vol/vol) ethanol for 2 min, followed by several washes with sterile distilled water.

In kinetics experiments, the number of nodulated plants and the number of nodules per plant were counted. From 15 to 20 plants were inoculated with the appropriate strain, and the experiment was repeated at least three times.

RESULTS

Construction and identification of recombinant cosmids carrying the essential early nodulation genes. To isolate the DNA region conferring the essential *nod* genes from the symbiotic megaplasmid, a gene library in *E. coli* was constructed by using total DNA from the wild-type *R. meliloti* AK631. For this purpose the cosmid vector pLAFR1, coding for tetracycline resistance (Tc^r), was used, since it is mobilizable into *Rhizobium*, it can replicate in both *E. coli* and *Rhizobium*, and inserts of 10 to 30 kb can be packaged into phage lambda heads (17). The total DNA was digested partially with *Eco*RI, and DNA fragments between 10 and 30 kb in size were ligated into the *Eco*RI site of pLAFR1 and packaged into lambda heads. After transduction into *E. coli* HB101, about 3,000 Tc^r colonies were obtained.

The recombinant cosmid DNA was analyzed in 24 randomly selected clones; 16 clones (67%) contained an insert with a mean size of 25 kb, indicating at least a 99.9% probability that the gene bank contains the complete *Rhizobium* genome.

This was also supported by the complementation of five auxotrophic mutations (*cys-46*, *gly-1*, *ade-4*, *tyr-1*, and *his-1*) located at different regions of the *R. meliloti* chromosomal linkage map (30). When the clone bank was conjugated into two mutant strains, AK194 and AK245, carrying the five auxotrophic mutations, the appearance of the wild-type phenotype was observed at a frequency of 10^{-2} to 10^{-3} per transconjugant for each allele.

To identify clones carrying the *nod* genes, the method of Long et al. (33) was used. The gene bank was mass conjugated into two different Nod⁻ deletion mutants. By infecting alfalfa seedlings with the transconjugant populations, the formation of nodules was checked for on the roots. In strain AK635, the common *nod* genes are deleted but the *hsn* genes are still present, whereas in strain ZB121 a very large region of the megaplasmid, including the two so-far identified *nod* regions and the *nif-fix* region, is deleted (Fig. 1).

Nodule induction on 50% of the plants was observed with transconjugants of both strains. Bacteria were reisolated from several independent nodules, and the restriction pattern of the recombinant cosmids in these isolates was

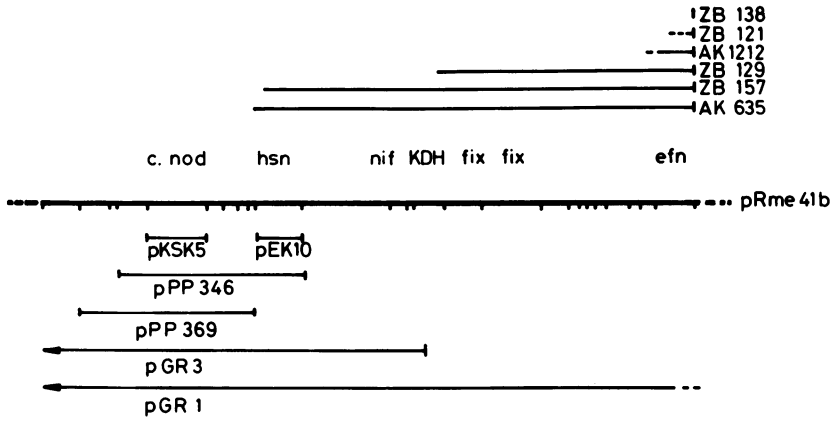

FIG. 1. Schematic drawing of the *nod-nif* region of *R. meliloti* 41. The thick line represents the *Eco*RI map. Horizontal lines indicate the regions present in the deletion mutants (upper part). Subclones and R-primes used in this work are shown below.

determined after digestion with *Eco*RI. Cosmids in the Nod$^+$ transconjugants of AK635 carried a 25-kb insert comprising the 8.5-kb common *nod* region (pPP369 on Fig. 1), while all Nod$^+$ transconjugants of ZB121 contained a 24-kb insert, including both the 8.5-kb and the 6.8-kb *Eco*RI *nod* fragments (pPP346 on Fig. 1).

Plasmid pPP346 carries the essential early nodulation genes from pRme41b. It was shown earlier that after introduction of R-prime plasmids carrying the *nod-nif* region of pRme41b into *A. tumefaciens*, the transconjugants formed nodules or nodulelike structures on alfalfa (1). To demonstrate the presence of all essential early *nod* genes on pPP346, this plasmid was transferred into *A. tumefaciens* and into different *Rhizobium* species (*R. trifolii*, *R. lupini*, and a broad-host-range strain, *Rhizobium* sp. strain MPIK3030 [37]). The transconjugants were then tested for nodulation ability on alfalfa. As a control, the *nod-nif* R-prime plasmid pGR1 was also introduced into these recipients. *R. trifolii* and *Rhizobium* sp. strain MPIK3030 carrying either pGR1 or pPP346 efficiently nodulated alfalfa (Fig. 2). *A. tumefaciens* and the other *Rhizobium* species containing either pGR1 or pPP346 were also able to form nodules or nodulelike structures on alfalfa roots, although with somewhat lower efficiency. These results indicate that pPP346 indeed carries the essential early nodulation genes from pRme41b.

All the above-mentioned transconjugants containing pGR1 or pPP346 induced such nodules, but these nodules did not fix nitrogen. The nodules and nodulelike structures were

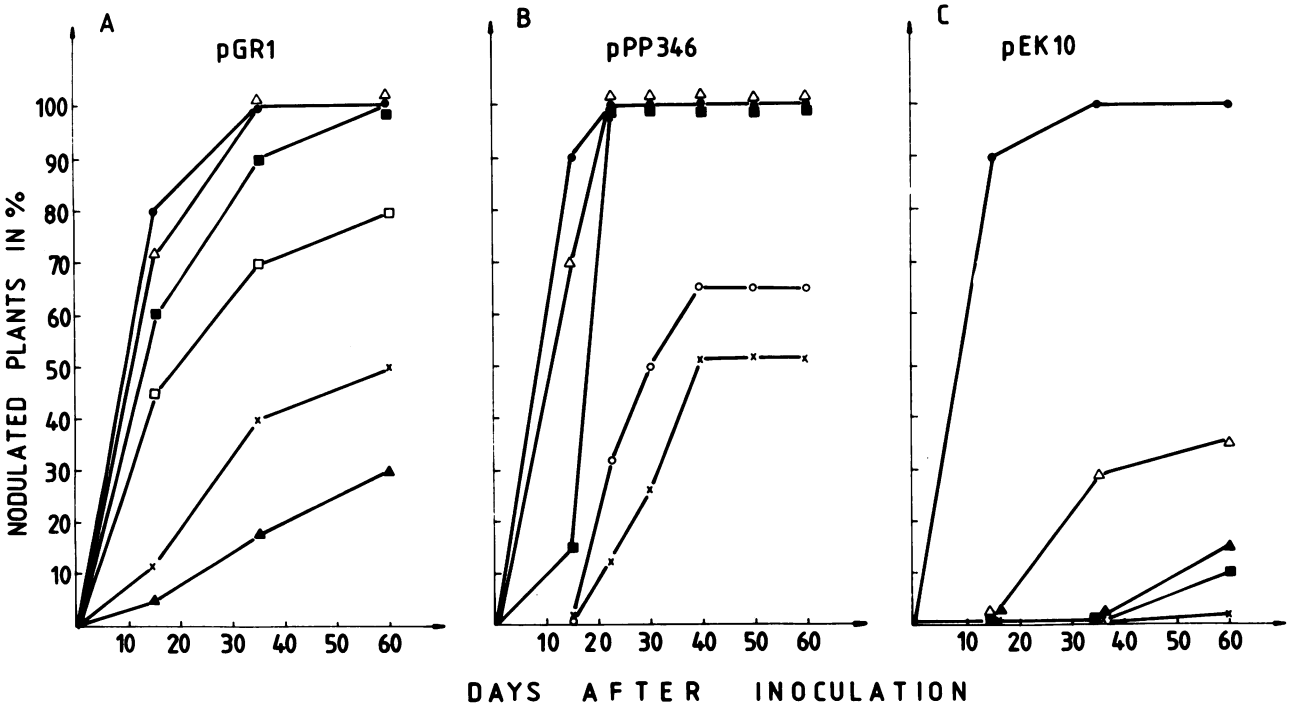

FIG. 2. Kinetics of nodule formation of wild-type *R. meliloti* and different transconjugants. Transconjugants carried the pGR1 R-prime (A), pPP346 cosmid (B), or pEK10 plasmid (C). Strains: △, *Rhizobium* sp. strain MPIK3030; ▲, *R. leguminosarum*; □, *R. fredii*; ■, *R. trifolii*; ○, *R. lupini*; ×, *A. tumefaciens*; ●, *R. meliloti* AK631.

similar to those reported for different *Rhizobium* species and *A. tumefaciens* carrying pRme41b (31, 46).

Nodulation genes on the 8.5- and 6.8-kb fragments are both essential and sufficient for nodule induction. As demonstrated above, the essential nodulation genes of pRme41b have been delimited to a 24-kb region present in pPP346 which includes the 8.5- and 6.8-kb *Eco*RI fragments coding for common and host-specific nodulation functions. The involvement of several additional *Eco*RI fragments on pPP346, however, was not known. Therefore, complementation analysis of Nod$^-$ deletion and transposon Tn*5* insertion mutants by the 8.5-kb *nod* fragment (pKSK5 on Fig. 1) was carried out. Since in pPP346 the 6.8-kb fragment represents the right-hand border of the insert, two Nod$^-$ mutants, AK635, containing the 6.8-kb fragment but lacking all the other fragments present in pPP346, and ZB157, with an even larger deletion extending to the very left-hand end of the 6.8-kb fragment, were chosen as recipients (Fig. 1). The Nod$^+$ phenotype of both AK635 and ZB157 was restored by introduction of the 8.5-kb common nodulation region (pKSK5) (Fig. 3A). Nod$^-$ mutants carrying the Tn*5* insertions in the *nodA*, *B* or *C* genes (AK1655, AK1678, and AK1657, respectively) were also complemented by pKSK5 and showed kinetics of nodulation similar to those of the two deletion mutants ZB157 and AK635. Although complementation of various Tn*5* insertion or deletion Nod$^-$ mutants was somewhat delayed compared with that with the wild-type *R. meliloti*, these results suggest that no essential early nodulation genes are located on megaplasmid regions other than the 8.5- and 6.8-kb *nod* fragments.

Another set of complementation experiments provided further support for this finding. As the 8.5-kb fragment contains common *nod* genes, mutants lacking the 8.5-kb fragment should be complementable after the introduction of symbiotic plasmids from other *Rhizobium* species, as reported earlier (2). When plasmid pIJ1089 carrying the cloned *nod* region of *R. leguminosarum* was introduced into the same Nod$^-$ mutants, the ability to nodulate alfalfa was restored (Fig. 3C). Nodulation by the complemented insertion mutants, however, was delayed by about 10 days and even slightly longer with the two deletion mutants. We must note that these experiments cannot exclude the existence of other nonessential *nod* genes between the two *nod* fragments.

When the 8.5-kb *Eco*RI fragment (pKSK5) or the 6.8-kb *Eco*RI fragment (pEK10) was transferred into *A. tumefaciens*, the transconjugants did not induce nodules on alfalfa. Similarly, mutants having large megaplasmid deletions and carrying either pKSK5 or pEK10 did not nodulate alfalfa. Occasionally, however, some nodulelike structures appeared 6 to 8 weeks after infection with both types of transconjugants on less than 5% of the plants.

A DNA region located on the right side of the *nif* genes influences the kinetics and efficiency of nodulation. It was observed that nodulation of alfalfa inoculated with Nod$^-$ mutants ZB138 or ZB121, with very large deletions in pRme41b but containing pPP346, was delayed about 1 week and was less efficient than with the wild-type strain AK631 (Fig. 4C). This delay was not observed with complemented deletion mutants carrying smaller deletions (Fig. 4C) or when the large-deletion mutants (ZB138 and ZB121) were complemented wtih pGR1 (Fig. 4A). A smaller R-prime plasmid (pGR3, Fig. 1), however, exhibited complementation properties similar to those of pPP346 (Fig. 4B). The total number of nodules on the infected plants showed similar variations that were in good correlation with the delayed-type phenotypes. When delay was observed, the number of nodules was also reduced to at least half of the normal value. These data indicate that a DNA region influencing the kinetics and efficiency of nodulation is present on pGR1 and

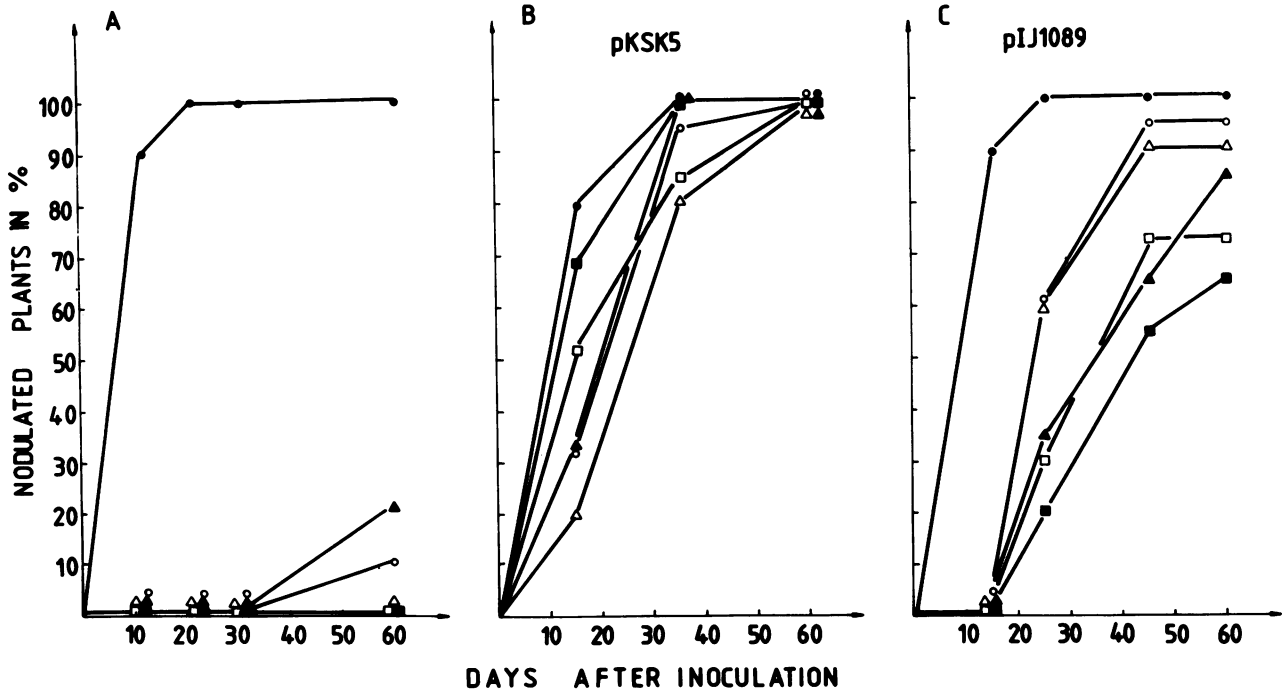

FIG. 3. Kinetics of nodule formation of wild-type *R. meliloti* and the transconjugants of its Nod$^-$ mutants. (A) Control; (B) transconjugants containing the 8.5-kb common *nod* region on pKSK5; (C) transconjugants carrying the *nod* region of *R. leguminosarum* on pIJ1089. Symbols: ●, wild type (AK631); ○, AK1679; □, ZB157; ■, AK635; △, AK1657; ▲, AK1655.

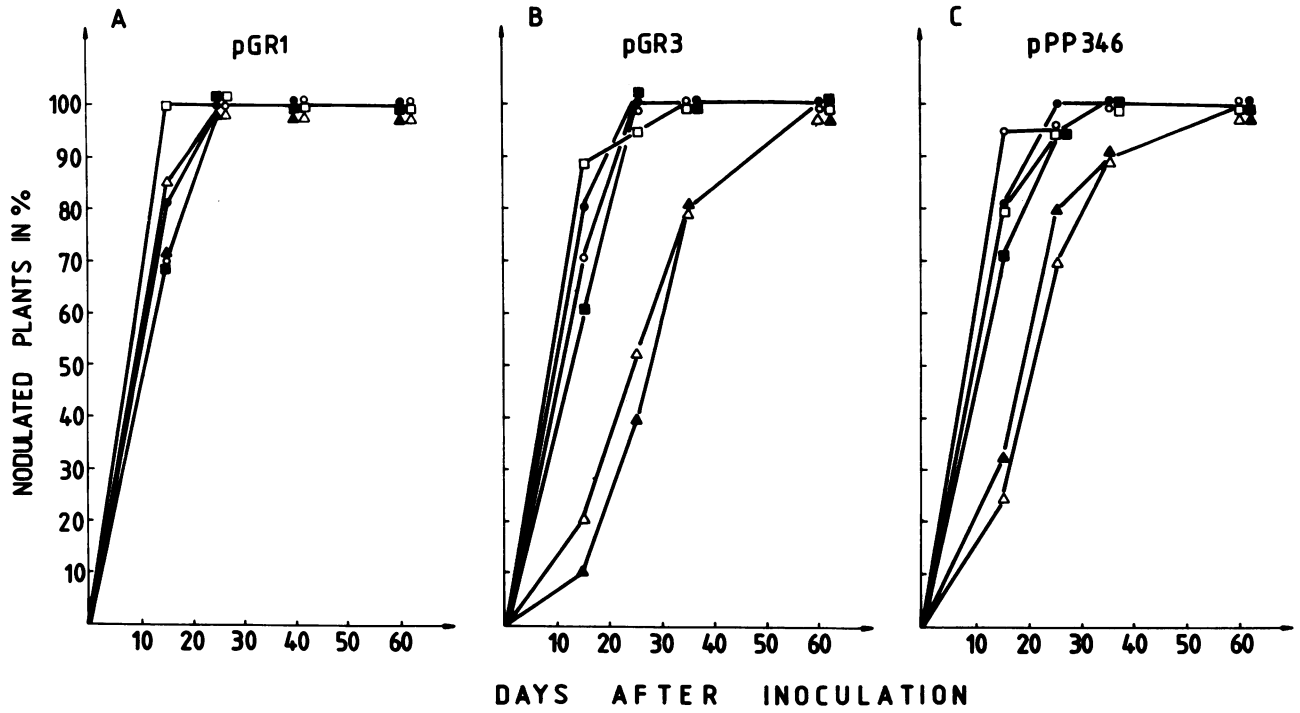

FIG. 4. Kinetics of nodule formation of wild-type *R. meliloti* and its Nod⁻ pSym deletion derivatives carrying pGR1 (A), pGR3 (B), or pPP346 (C). Symbols: ●, wild type (AK631); ○, ZB129; ▲, ZB138; △, ZB121; ■, AK1212; □, ZB157.

in Nod⁻ mutants with a short deletion (AK1212, ZB127, and ZB157), but missing from pPP346 and pGR3, as well as from the Nod⁻ large-deletion mutants ZB121 and ZB138.

These results show that a DNA region influencing nodulation kinetics and efficiency (*efn*) is located on a 10-kb *Eco*RI fragment between the right border endpoints of the deletions in AK1212 and ZB121 (Fig. 1).

DISCUSSION

We have isolated a recombinant plasmid, pPP346, with a 24-kb insert from pSym (pRme41b) of *R. meliloti* coding for the essential early nodulation genes. This plasmid was transferred into different *Rhizobium* species and into *A. tumefaciens*, and at least 50% of the *M. sativa* plantlets infected with the various transconjugants were nodulated. The structure of nodules was similar to that of those induced by *Agrobacterium* or *Rhizobium* species carrying pRme41b (31) or pGR1 (Fig. 1) (1), indicating that the essential early *nod* genes of the roughly 1,500-kb megaplasmid (6) are clustered on the 24-kb segment inserted into pPP346. Clustering of essential nodulation genes has been reported also for *R. leguminosarum* (12) and *R. trifolii* (42).

Two *nod* clusters have been described on the 24-kb region so far (29). One cluster, located on an 8.5-kb *Eco*RI fragment, contains the *nodABC* and *D* genes determining common nodulation functions (14, 16, 25, 29, 41, 43; Gottfert et al., in press). The other cluster comprises genes determining the host specificity of nodulation and consists of the *hsnEFG* and *H* genes (Horvath et al., in press) carried on a 6.8-kb *Eco*RI fragment. *R. meliloti* AK635(pKSK5) and ZB157(pKSK5), containing only these two *Eco*RI fragments of the 24-kb region, were able to induce nodules on alfalfa, indicating that these two fragments carry sufficient genetic information for nodulation of alfalfa. When only one of the two fragments was introduced into *A. tumefaciens*, no nodulation occurred, providing further support that both DNA regions are essential for nodulation of alfalfa. Hirsch et al. (19, 20) found that *A. tumefaciens* and *R. trifolii* transconjugants harboring pRmSL26, a recombinant plasmid carrying the nodulation region of *R. meliloti* 1021 (33), induced nodules on about 15% of the alfalfa plants infected. A similar observation was made with these bacteria carrying pRmJ30, with an 8.7-kb *Eco*RI fragment coding for the *nodABC* and *D* genes. In contrast to these data, Djordjevic et al. (10) did not observe nodulation of alfalfa with either the same *R. trifolii* strain carrying pRmSL26 or a *nod-nif* deletion mutant of *R. meliloti* containing pRmSL26. On the basis of these latter results, it is likely that the organization of *nod* genes in *R. meliloti* 41 and 1021 is very similar and that the second *nod* region (the *hsn* genes) is outside the megaplasmid region present in pRmSL26. The differences between the nodulation results obtained by Hirsch et al. (19, 20) and Djordjevic et al. (10) (or in our laboratory) may be explained by differences in the *M. sativa* seeds used and their different responsiveness to bacterial infection. For instance, we have noted that in some of our plant tests occasional swellings appeared on the roots about 8 weeks after infection even with other bacteria, such as *Agrobacterium* and *E. coli*.

Analysis of the nodulation ability of Tn*5*-induced mutants carrying the insertions in different *nod* genes indicated that mutations in different *nod* genes resulted in different Nod⁻ phenotypes. For instance, *nodC* and *hsnH* mutations produced a very clear Nod⁻ phenotype, since no nodules were formed even 6 weeks after infection (13, 16, 29; Horvath et al., in press), while mutation in *hsnG* only delayed the appearance of nodules by about 4 days, indicating that *hsnG* is nonessential for nodulation (Horvath et al., in press). We still cannot exclude the possibility that other genes of this

latter type may be present somewhere else on the DNA region carried by pPP346.

In this report we demonstrated the existence of a Psym megaplasmid region outside the segment carried in pPP346 which codes for ancillary nodulation functions. If this region is missing from *R. meliloti*, nodulation does occur, but with about a 10-day delay, and it is less efficient (*efn* genes). Interestingly, the *efn* region harbors the conserved *nod* promoter sequence ("*nod*-box") (38a); moreover, it contains a second copy of *nodD* gene as well (Göttfert et al., in press). To identify and locate the *efn* genes more precisely, direct Tn*5* mutagenesis of this DNA segment is being carried out in our laboratory.

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