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Identification of a Conserved, Reiterated DNA Region That Influences the Efficiency of Nodulation in Strain RS1051 of *Rhizobium leguminosarum* bv. *trifolii*

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The symbiotic plasmid of strain RS1051 of *Rhizobium leguminosarum* bv. *trifolii* has been identified by: an indirect approach through isolation of deleted and cured derivatives, mobilization of the plasmid into *Agrobacterium*, and hybridization with *nod* and *nif* gene probes. Two cosmids carrying the RS1051 *nod* region were selected from a genomic clone bank. Subcloning and deletion analysis indicate that an 11.45-kb DNA region on the symbiotic plasmid carries all the essential genes for red and white clover nodulation in *R. l.* bv. *trifolii* and for red clover nodulation in the heterologous strains *Agrobacterium* and *R. l.* bv. *viceae*, whereas an additional 2.55-kb region has been proven to be necessary for white clover nodulation by those hosts. In addition, a 1.7-kb region located adjacent to the *nodFE* genes has been found to influence the efficiency of nodulation of both red and white clover. This region is structurally conserved among the rhizobia examined and structurally as well as functionally conserved in *R. l.* bv. *viceae*. In *R. l.* bv. *trifolii* RS1051 the 1.7-kb *nod* locus is reiterated on the pSym, and our results indicate that at least two of the copies are functional and necessary for successful nodulation. Furthermore, evidence is presented that strongly indicates that the RS1051 *nodD* gene is functionally reiterated and works in a host-specific manner.

Additional keywords: host range, nodulation efficiency, nodulation genes, *Rhizobium*, Sym plasmid, Tn*5*.

The genus *Rhizobium* comprises gram-negative bacteria that interact with Leguminosae plants resulting in the formation of nitrogen-fixing root nodules. This is a complex process that involves the differentiation of the cortical root cells into meristematic cells that further develop into an organized nodule structure.

Detailed analysis of the genes involved in the early steps of nodule development (nodulation genes) have been done on a limited number of rhizobial strains from the species *Rhizobium meliloti* (Török *et al.* 1984; Egelhoff *et al.* 1985; Horvath *et al.* 1986), *Rhizobium leguminosarum* bv. *viceae* (Shearman *et al.* 1986; Surin and Downie 1988), *Rhizobium leguminosarum* bv. *trifolii* (Redmond *et al.* 1986; Djordjevic *et al.* 1986), *Rhizobium* sp. NGR234 (Bachem *et al.* 1986; Bassam *et al.* 1986; Nayudu and Rolfe 1987), and more recently, *Rhizobium fredii* (Sadowsky *et al.* 1988; Appelbaum *et al.* 1988).

The nodulation genes have been proposed to be clustered in two groups. The "common" nod genes are conserved among a wide variety of rhizobia at the nucleotide level as well as functionally (Kondorosi *et al.* 1984; Török *et al.* 1984; Egelhoff *et al.* 1985; Jacobs *et al.* 1985). In *R. meliloti* there are four genes organized in two transcriptional units (*nodABC* and *nodD*). Additionally, in *R. l.* bv. *viceae* another operon, *nodIJ* (Evans and Downie 1986), which functionally corresponds with region II of *R. l.* bv. *trifolii* (Djordjevic *et al.* 1985) has been described. Mutations in the *nodABC* genes exhibit a Nod$^-$ phenotype, whereas those in region II (*nodIJ* genes) cause a reduced nodulation ability. A regulatory role has been attributed to *nodD* gene as it controls the expression of the other genes in conjunction with a plant exudate factor (Mulligan and Long 1985; Rossen *et al.* 1985; Firmin *et al.* 1986; Horvath *et al.* 1987). The interaction between *nodD* and plant factors has been reported to be plant-specific (Horvath *et al.* 1987; Spaink *et al.* 1987; Györgypal *et al.* 1988). On the other hand, the *nodD* gene appears to be reiterated in several rhizobia (Rodríguez-Quiñones *et al.* 1987). In *R. meliloti* the three *nodD* copies are involved in nodulation to an extent depending on the actual host (Honma and Ausubel 1987; Györgypal *et al.* 1988). Similarly, two *nodD* gene copies of different function have been found in *R. fredii* (Appelbaum *et al.* 1988).

The second *nod* gene cluster has been designated *hsn* as it is involved in host-specific nodulation functions (Kondorosi *et al.* 1984). At least four genes, *hsnABC* and *D* (otherwise termed *nodFEG* and *H*, respectively), form part of this region in *R. meliloti* (Horvath *et al.* 1986; Debelle and Sharma 1986). Mutations in *hsnABC* delay nodulation, whereas mutations in *hsnD* lead to loss of nodulation ability. The *hsnAB* (*nodFE*) genes are structurally, but not functionally, conserved in *R. l.* bv. *trifolii* and *viceae* (Shearman *et al.* 1986; Schofield and Watson 1986). In addition to the *nodFE* genes (region III), other regions (IV and V) have been reported to alter the nodulation as well as the host range properties of *R. l.* bv. *trifolii* (Djordjevic *et al.* 1986). More recently, the genes *nodLMN* have been identified in *R. l.* bv. *viceae*. These genes are adjacent to the *nodFE* operon and are structurally conserved in *R. l.* bv. *trifolii* (Surin and Downie 1988).

In the present paper we report on the characterization of

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the symbiotic region of strain RS1051 of *R. l.* bv. *trifolii*. The Sym plasmid has been identified along with several loci involved in clover nodulation, including a conserved reiterated region that influences the efficiency of nodulation. Results that strongly indicate that RS1051 contains two functional *nodD* copies are also provided.

MATERIALS AND METHODS

Media and growth conditions. Strains, phages, and plasmids used are listed in Table 1. *R. meliloti* was grown at 34° C in YTB medium (Orosz *et al.* 1973). All other *Rhizobium* were grown at 30° C in tryptone-yeast extract (TY) medium (Beringer 1974) or minimal medium MM (Hooykaas *et al.* 1981) supplemented with biotin (0.250 mg/L) and thiamine (0.1 mg/L). *Agrobacterium tumefaciens* was grown at 30° C in rich medium Luria-Bertani (LB; Maniatis *et al.* 1982) or minimal GTS (Kiss *et al.* 1979). Strains of *Escherichia coli* were grown at 37° C in LB medium (strains C2110 and HB101) or TYE medium (strain JM101; Messing 1983).

Matings and plasmid mobilization. Bacterial crosses and triparental matings were done as described earlier (Kondorosi *et al.* 1977, 1982). Mobilization of the *Rhizobium* plasmids was done by labeling the plasmids with the chimeric transposon Tn*5*-Mob and later mobilization with or without the help of plasmid pJB3JI, which provides Tra functions *in trans* (Simon *et al.* 1983). Selection was with tetracycline and/or kanamycin at final concentrations of 10 and 200 μg/ml (15 and 50 μg/ml for *E. coli*), respectively.

Heat-curing experiments. TY liquid media containing 10^8 cells per milliliter were exposed to high temperature for 7 days (Zurkowski 1982). Temperatures ranged from 37° C to 47° C. Samples of the cultures were plated onto rich medium to obtain single colonies after heat treatments.

Analysis of plasmid profile. Plasmids were detected by a modified technique of Eckhardt (1978) adapted to a horizontal gel system, as described by Hynes *et al.* (1985), with 0.5% agarose gels. *Rhizobium* and *Agrobacterium* strains were grown overnight in rich medium, and 100–300 μl of the culture was centrifuged and the supernatant removed. The pellet was loosened by brief shaking, thoroughly resuspended in 35 μl of lysis mixture (10% Ficoll 400, 0.01% lysozyme, 0.002% RNase I, 0.025% bromophenol blue in Tris-borate buffer), and immediately loaded into the gel. Electrophoresis was carried out at 20 V for 1 hr and then at 100 V for 3–4 hr. The gel was stained, visualized, and photographed as previously described (Maniatis *et al.* 1982).

Enzymes and isotopes. Restriction endonucleases were purchased from either REANAL (Hungary) or Boehringer (West Germany). T4 DNA ligase was from Bethesda Research Laboratories (U.S.A.), calf intestinal alkaline phosphatase was from Worthington (U.S.A.), and Klenow

Table 1. Bacterial strains, plasmid,[a] and phages

Strains, plasmids, and phages	Characteristic	Reference or source
Strains		
Rhizobium leguminosarum bv. *trifolii*		
RS1051	Wild type	University of Seville, Spain
RS1000 to RS1042, RS1045 to RS1061	RS1051ΔpRtr1051*a*, Nod$^-$	This work
RS1043, RS1044	RS1051 pRtr1051*a*$^-$, Nod$^-$	This work
R. l. bv. *viceae*		
300	Wild type	J. E. Beringer
R. meliloti		
AK631	Wild type, compact colony morphology variant of *R. meliloti* 41	Banfalvi *et al.* 1981
Rhizobium sp.		
MPIK3030	An Sm^r derivative of strain NGR234	Trinick 1980
Agrobacterium tumefaciens		
GMI9017	Plasmid-cured derivative (pAt$^-$, Ti$^-$) of strain C58	C. Rosenberg
Escherichia coli		
HB101	Sm^r *recA pro leu*	Boyer and Roulland-Dussoix 1969
JM101	*sup*E *thi* Sm^{r}Δ(*lac pro*) F′ (*tra*D36 *pro*AB *lac*I^{q}ZΔM15	Messing 1983
UNF510	HB101Ω::Tn*5*	Merrick *et al.* 1978
C2110nal	A Nal^r derivative of C2110	Ditta 1986
Plasmids		
pVK102	Cosmid derivative of pRK290	Knauf and Nester 1982
pGS72	Cosmid derivative of pRK290	Selvaraj and Iyer 1985
pRK2013	Km^r helper plasmid	Ditta 1986
pRK2073	Spc^r helper plasmid	Ditta 1986
pSUP5011	pBR322::Tn*5*-Mob	Simon *et al.* 1983
pJB3JI	A Km^s derivative of R68.45	Brewin *et al.* 1980
pID1	pBR322::4.8-kb *Pst*I fragment of *R. meliloti* 41 carrying *nifHDK*	Banfalvi *et al.* 1981
pFR14, pFR15	pVK102 derivatives carrying RS1051 *nod* region	This work
pMFB1	Subclone of pFR15 in pGS72	This work
pFR1501 to pFR1504	*Hin*dIII deleted derivatives of pFR15	This work
pFR1506 to pFR1509	*Bgl*II deleted derivatives of pFR15	This work
Phages		
fFR*nodC*	M13*mp8* carrying intra-*nodC* fragment of *R. meliloti* 41	Rodríguez-Quiñones *et al.* 1987
fMG*nodD*	M13*mp8* carrying intra-*nodD* fragment of *R. meliloti* 41	Rodríguez-Quiñones *et al.* 1987
pBH*hsnB*	M13*mp18* carrying intra-*hsnB* fragment of *R. meliloti* 41	Rodríguez-Quiñones *et al.* 1987

[a]Indigenous *Rhizobium* plasmids are designated as proposed by Casse *et al.* (1979).

enzyme was from Boehringer. All enzymes were used according to the manufacturer's recommendations. ^{32}P-dATP was purchased from the Isotope Institute of Budapest (Hungary) or from Amersham (England).

Molecular cloning and DNA isolation procedures. Total genomic DNA, large and mini-scale plasmid, cosmid and single-stranded M13 preparations, and bacterial transformation were as described (Rodríguez-Quiñones *et al.* 1987). Construction of a genomic library of RS1051 was done in the cosmid vector pVK102 as described (Rodríguez-Quiñones *et al.* 1987). About 2,000 tetracycline-resistant, kanamycin-sensitive transductants were selected. The clone bank was tested by complementation of auxotrophic mutations located at different sites on the chromosome of *R. meliloti* 41.

Preparation of pFR15 derivatives. Plasmid pMFB1 was obtained by direct cloning of the electroeluted (Maniatis *et al.* 1982) 15.1-kb *Xho*I fragment from pFR15 in the vector pGS72. Deleted derivatives were generated after partial digestion of pFR15 with either *Hin*dIII (pFR1501–pFR1504) or *Bgl*II (pFR1505–pFR1509) as described by Buikema *et al.* (1983).

Tn*5* transposon mutagenesis. Random mutagenesis was done according to Ditta (1986).

Hybridization. DNA fragments were transferred from agarose gels onto nitrocellulose filters (Schleicher and Schuell, BA85, 0.45 μm) by the method of Southern (1975). Intra-*nodC*, -*nodD*, and -*hsnB* gene probes were labeled as described (Rodríguez-Quiñones *et al.* 1987). Plasmid pID1 was either nick translated (Maniatis *et al.* 1982) or digested and labeled by the procedure of Feinberg and Vogelstein (1983). The 1.45-kb *Rt-efn* probe was labeled by the procedure of Feinberg and Vogelstein (1983). Hybridization conditions were as described (Rodríguez-Quiñones *et al.* 1987).

Plant tests and isolation of bacteria from nodules. Nodulation tests were done on *Trifolium pratense* (red clover) and *Trifolium repens* (white clover) in petri dishes (Rolfe *et al.* 1980) or in test tubes (Kondorosi *et al.* 1977). Plants were screened for nodules at 3-day intervals beginning 7 days after inoculation. Each test was repeated three or more times, and at least one of them was carried out in test tubes. At least 25 parallels were included for each sample. Nodulation of siratro (*Macroptilium atropurpureum*) and alfalfa (*Medicago sativa*) were assayed as described by Kondorosi *et al.* (1977) and pea (*Pisum sativum*) as described by Beynon *et al.* (1980). Bacteria were isolated from nodules as described by Kondorosi *et al.* (1982).

RESULTS

Identification and characterization of the Sym plasmid. *R. l.* bv. *trifolii* RS1051 carries three plasmids of molecular weight 190, 280, and approximately 470 MDa. To partially or totally remove these plasmids, heat-curing experiments were done, and the plasmid profile of 100 single colonies was determined in each case. Plasmid profile alterations were detected in 62 instances. These include a large deletion (ranging from 80 to 100 MDa) in the smallest plasmid (pRtrRS1051*a*) in 57 clones, a very large deletion (about 150 MDa) in three clones (RS1041, RS1042, and RS1045), and total curing of plasmid pRtrRS1051*a* in two clones (RS1043 and RS1044). Figure 1 shows the plasmid profile of some of these derivatives. All the clones with altered plasmid profile were Nod$^-$ when tested in both red and white clover, indicating that *nod* genes were present in pRtrRS1051*a*.

To determine if all the essential genes for clover nodulation are located in pRtrRS1051*a*, the indigenous RS1051 plasmids were labeled with Tn*5* (see Materials and Methods) and mobilized to a plasmidless strain of the distantly related *A. tumefaciens*. With the help of plasmid pJB3JI, we could transfer plasmids pRtrRS1051*a* and pRtrRS1051*b* to *A. tumefaciens* GMI9017. Low-frequency transfer of pRtrRS1051*a* was also achieved in the absence of helper plasmid, thereby demonstrating that this plasmid is self-transmissible. *Agrobacterium* transconjugants harboring plasmid pRtrRS1051*a* elicited Fix , small white nodules, and/or swelling ("pseudonodules") that appeared in 80% of the tested plants (red and white clover) with a delay of about 1 wk.

To provide further evidence that the symbiotic functions are carried by pRtrRS1051*a*, we compared the hybridization pattern of the wild-type strain RS1051 with its cured and deleted derivatives. In a previous paper we reported the hybridization pattern of strain RS1051 with the intragenic *nod* probes of *R. meliloti* 41 (Rodríguez-Quiñones *et al.* 1987). The single bands that appear when RS1051 total DNA is hybridized against *nodC* and *hsnB* are missing in all Nod$^-$ in which pRtrRS1051*a* is partially or totally lost (data not shown). However, one of the two bands that appear in RS1051 when the *nodD* probe is used remains even in the Nod$^-$-cured derivatives (Fig. 2). This clearly indicates that one of the *nodD* copies is located in a replicon other than the pSym. Two bands are also present when pID1 is used as a *nif* probe against RS1051 total DNA digested with either *Bgl*II, *Hind*III or *Eco*RI, whereas hybridization signal is completely absent in the Nod$^-$-deleted derivatives (data not shown).

Isolation of cosmid clones that carry the RS1051 nodulation region and reconstruction experiments. The entire RS1051 clone bank was introduced into RS1043 (a RS1051 derivative lacking the whole pSym) and into RS1019 (a Nod$^-$ derivative with a deletion in the pSym). The transconjugants were selected in a minimal medium MM supplemented with tetracycline and used as inoculants on clover. Nodules were found in 80% of the plants, from which

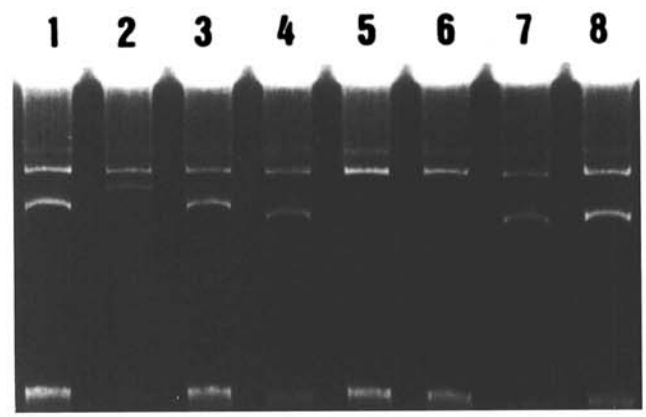

Fig. 1. Plasmid profiles of *Rhizobium leguminosarum* bv. *trifolii* RS1051 and deleted derivatives. Lane 1, RS1038; lane 2, RS1051; lane 3, RS1039; lane 4, RS1040; lane 5, RS1041; lane 6, RS1042; lane 7, RS1011; lane 8, RS1012.

bacteria were reisolated on TY-rich medium and then checked for tetracycline resistance. Plasmid DNA was isolated from several of these clones and used to transform *E. coli* HB101. Cosmids pFR14 and pFR15 were extracted from the transformants. Both cosmids were remated into RS1043 and RS1019 and the transconjugants used to inoculate plants. In both cases plants (white and red clover) were fully nodulated. Nodules were Fix$^-$ and in the case of RS1043 appeared with a slight delay of 3–4 days, as compared with the wild type (Fig. 3A). Clover nodulation like that induced by RS1051 was achieved when the cosmids were introduced into strain 300 of *R. l.* bv. *viceae*. *A. tumefaciens* GMI9017 carrying pFR14 or pFR15 induced smaller, white nodules and/or swellings, namely pseudonodules, that appeared with a 10- to 14-day delay in 75–100% of the tested plants (Fig. 3A). Bacteria were routinely reisolated from nodules and checked for tetracycline resistance and plasmid profile to ensure the presence of pFR14 or pFR15 in the corresponding recipient. No difference was observed between red and white clover plants.

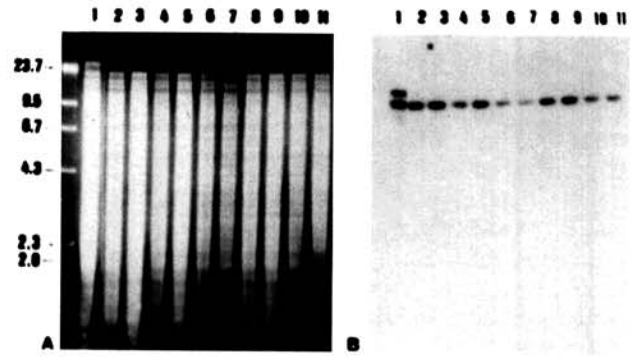

Fig. 2. A, *Eco*RI-digested total DNA from *R. l.* bv. *trifolii* strains. Lane 1, RS1051; lane 2, RS1042; lane 3, RS1043; lane 4, RS1044; lane 5, RS1017; lane 6, RS1019; lane 7, RS1020; lane 8, RS1022; lane 9, RS1023; lane 10, RS1030; lane 11, RS1033. **B**, Hybridization with fMG*nodD*.

Clover nodulation was not found when pFR14 or pFR15 was introduced into *R. meliloti* or *Rhizobium* sp. MPIK3030.

When cosmids pFR14 and pFR15 were mapped with four restriction enzymes (Fig. 4), it was shown that they had overlapping inserts that shared a 14-kb region. The approximate location of the *nodC*, *nodD*, and *hsnB* (=*nodE*)-like genes was obtained by hybridization of blots containing single, double, and triple digestion combinations (with *Hin*dIII, *Eco*RI, *Bgl*II, and *Xho*I) against the intra-*nod* probes (Fig. 4). The *nif* probe did not hybridize with pFR14 or pFR15 DNAs.

To further define the limits of the *nod* region, several pFR15 derivatives were obtained. Plasmid pMFB1 (Fig. 4) carries the 15.1-kb *Xho*I fragment from pFR15 inserted in the mobilizable broad host range cosmid vector pGS72. When introduced in the Nod$^-$ RS1051 derivatives or in *A. tumefaciens* GMI9017, pMFB1 failed to restore white and red clover nodulation, whereas in *R. l.* bv. *viceae* 300, nodulation was the same as that induced by pFR15. Therefore, the leftmost region of pFR15, which is missing in pMFB1, contains genetic information necessary for nodulation and is functionally conserved in *R. l.* bv. *viceae*.

Similar tests were carried out for the pFR15-deleted derivatives obtained after partial digestion with either *Bgl*II or *Hin*dIII (Fig. 4). The results are summarized in Table 2. Plasmid pFR1504 behaves identically to pFR15, as was expected because it carries the 14-kb region that is shared in pFR14 and pFR15. In the RS1051 Nod$^-$ derivatives, pFR1508 behaves like pFR15 and pFR1504 on both red and white clover (Fig. 3A), thereby restricting to 11.45 kb the essential *nod* region of *R. l.* bv. *trifolii*. However, when the host is white clover and the recipient is *R. l.* bv. *viceae*, pFR1508 causes reduced nodulation. Nodules and swellings appear after a long delay (about 10 days), are found in less than 40% of the plants, and occur mainly on the root tips and axils. Bona fide nodulation could not be detected in white clover inoculated with *Agrobacterium* transconjugants carrying pFR1508.

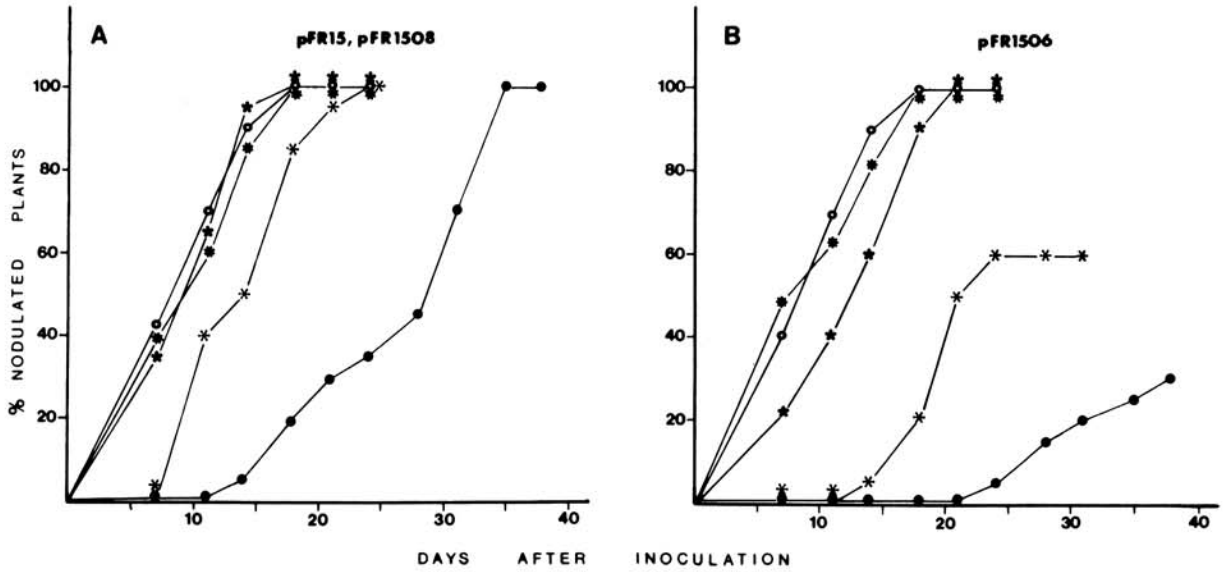

Fig. 3. Kinetics of nodulation induced on red clover by *R. l.* bv. *trifolii* RS1051 (open circle) and by transconjugants of RS1019 (dark star), RS1043 (light star), *R. l.* bv. *viceae* 300 (asterisk), and *A. tumefaciens* GMI9017 (closed circle), carrying plasmids pFR15 (**A**) and pFR1506 (**B**). Data obtained with plasmid pFR1508 are statistically indistinguishable from those on panel A.

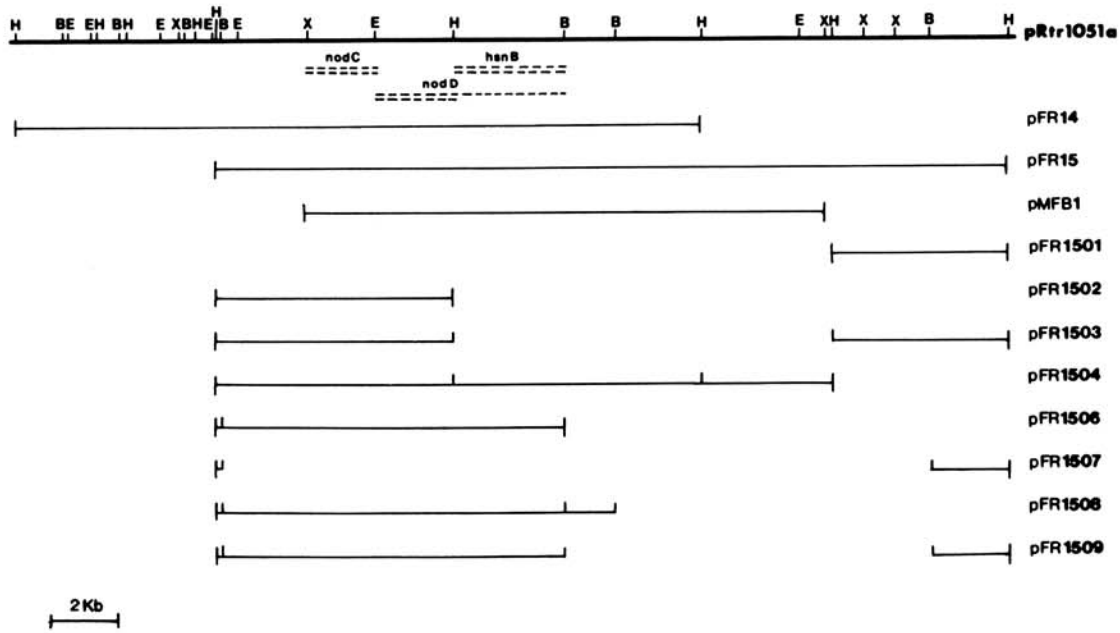

Fig. 4. Physical map of the *R. l.* bv. *trifolii* RS1051 *nod* cosmids pFR14 and pFR15 and derivatives of pFR15. The double dotted lines represent strong homology with the intra-*nod* probes; the single dotted line represents weak homology.

Table 2. Symbiotic phenotype induced on red and white clover by RS1051 cosmid clones in different recipients

	Strain[a]			
Plasmid	**RS1019**	**RS1043**	**Rle300**	**AtGMI9017[b]**
pMFB1	−(−)	−(−)	++(++)	−(−)
pFR15, pFR1504	++(++)	+(+)	++(++)	+(+)
pFR1508	++(++)	+(+)	++(+/−)	+(−)
pFR1506, pFR1509	+(+)	+/−(+/−)	++(+/−)	+/−(−)

[a]++, wild-type nodulation; +, slightly reduced efficiency of nodulation (see text); +/−, reduced nodulation (see text). Symbols in brackets refer to nodulation on white clover.
[b]Pseudonodules.

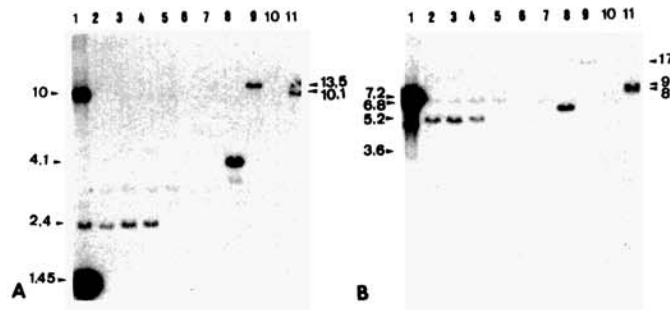

Fig. 5. Southern hybridization of the *Rt-efn* 1.45-kb *Bgl*II probe to *Bgl*II (**A**) and *Hin*dIII-digested DNA (**B**) from strains of *R. l.* bv. *trifolii*: lane 1, RS1051; lane 2, RS1019; lane 3, RS1022; lane 4, RS1033; lane 5, RS1034; lane 6, RS1023; lane 7, RS1043; lane 8, *R. l.* bv. *viceae* 300; lane 9, *Rhizobium* sp. MPIK3030; lane 10, *A. tumefaciens* GMI9017; lane 11, *R. meliloti* AK631. B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; X, *Xho*I.

Plasmid pFR1506 is only different from pFR1508 in that the rightmost 1.45-kb *Bgl*II fragment is lacking. When introduced into RS1019, pFR1506 (and pFR1509) elicited complementation, but nodulation occurred with a 3- to 4-day delay in 80–100% of the plants (Fig. 3B), and the average number of nodules per plant appeared to be slightly less than the wild type. In the RS1043 background, pFR1506 induced a greatly decreased complementation; nodulation experienced a 8- to 10-day delay, and only 40–60% of the plants were nodulated (Fig. 3B). Nodules were significantly smaller, and nodule number was reduced by approximately 50%. In no case was any difference between red and white clover nodulation observed. In red clover, a drastically reduced "nodulation" efficiency was also observed in *A. tumefaciens* GMI9017 carrying pFR1506 (Fig. 3B). No difference was observed between pFR1508 and pFR1506 in either red or white clover when the recipient is *R. l.* bv. *viceae* 300, indicating functional conservation of the 1.45-kb region in this species.

The 1.45-kb *nod* locus is reiterated in *R. l.* bv. *trifolii* RS1051 and structurally conserved among other Rhizobia. Plasmid pFR1508 was digested with *Bgl*II, and the 1.45-kb fragment was separated, labeled, and used as a probe against digested genomic DNA of RS1051 and several of its Nod$^-$ derivatives. Three hybridizing bands appeared in the wild-type DNA when digested with either *Bgl*II, *Hin*dIII (Fig. 5), or *Eco*RI (not shown). One of those bands (2.4-kb *Bgl*II, 5.2-kb *Hin*dIII, and 13.0-kb *Eco*RI hybridizing bands) remained present in three (RS1019, RS1022, and RS1033) out of 11 Nod$^-$ derivatives analyzed. Figure 5 shows the result with *Bgl*II and *Hin*dIII in six of the Nod$^-$ derivatives. Transconjugants of RS1022 and RS1033, carrying pFR15, pFR1508, and pFR1506, were checked on clover and showed a behavior identical to that obtained with RS1019. Strains RS1023 and RS1034 do not contain, like RS1043, any copy of the *nod* locus, and their corresponding transconjugants carrying pFR15, pFR1508, and pFR1506 behaved like the RS1043 ones when tested on clover.

Strong hybridization to the 1.45-kb region was found in *R. l.* bv. *viceae* 300 (Fig. 5). Fragments homologous to 1.45-kb *nod* locus were also found in *Rhizobium* sp. MPIK3030 and in *R. meliloti* AK631. In this latter strain two bands were observed. No homology was detected in *Agrobacterium*.

Tn*5* mutagenesis of the nodulation region of *R. l.* bv. *trifolii* RS1051. To more precisely delimit the nodulation regions, plasmid pFR1508 was used as a target for Tn*5* mutagenesis. Plasmid DNA was extracted from the Tn*5*-containing clones, digested with the appropriate enzyme combinations, and the blots were hybridized against intra-*nodC*, *nodD*, and *hsnB* probes, thus allowing a more precise localization of these genes (Fig. 6). The symbiotic phenotype of all Tn*5* mutants was studied in several recipients; the results are summarized in Figure 6.

Mutations R19 to K101 map on the *nodC*-hybridizing fragment and to the left of *nodD*. As expected, these mutations cause a clear Nod$^-$ phenotype in RS1019 and RS1043 but have no effect on the capability of pFR1508 to extend the host range of *R. l.* bv. *viceae* to red clover.

Mutations F24 and F33 lie on the *nodD*-hybridizing fragment but have little effect when present in RS1019 or RS1043. Nodules appear in 90–100% of the plants with a delay of no longer than 3–4 days, and the number of nodules per plant is slightly reduced to 50–75% of the wild-type level. However, in *R. l.* bv. *viceae*, mutations F24 and F33 elicit, even at best, extremely poor red clover nodulation: nodules are poorly developed in no more than 30% of the plants, after a long delay (about 15 days), and the number of nodules per plant is much below normal. In contrast, when white clover is the plant host, the nodulation induced by *R. l.* bv. *viceae* carrying F24 and F33 is not worse than with pFR1508.

Mutations on the right side of pFR1508 (from 9 to F13) result in a Nod$^+$-delayed phenotype that is not complemented by *R. l.* bv. *viceae nod* genes. On this basis, these mutants can be classified as *hsn*. This result also confirms the location of the *hsnB* (=*nodE*)-hybridizing band between F43 and F31. All the mutations in this region induce in *R. l.* bv. *trifolii* RS1019 and *R. l.* bv. *viceae* 300 a similar delayed phenotype in red clover, whereas a more drastic effect is observed in RS1043. In the former recipients, the delay on red clover is 6–8 days, yet 100% of the plants are nodulated. In RS1043, nodulation is induced in only 40–60% of the plants, with half the normal average number of nodules per plant and with nodules appearing after a 12–15 day delay. For RS1019 and RS1043, the delays are somewhat longer when white clover is the host, and are much longer for *R. l.* bv. *viceae*.

Mutation 17 and those located in the 1.45-kb *Bgl*II fragment identically reproduce the symbiotic behavior of pFR1506. Mutations F65, F64, F95, 13, and 14 map within the 1.45-kb region. Thus, these results indicate that the *nod* locus extends somewhat to the left of the 1.45 *Bgl*II region. Because this region severely influences the efficiency of clover nodulation, we will use the term *Rt-efn* to refer to this region (Fig. 6). All mutations in this region were functionally complemented by *R. l.* bv. *viceae*.

In *Agrobacterium* the results on red clover (Fig. 6) were comparable to those in *R. l.* bv. *trifolii*, indicating that all the genetic information that was found to be necessary for *R. l.* bv. *trifolii* is also required for *Agrobacterium*. Additionally, all Tn*5* mutations on the right of K101 were tested in the heterologous hosts alfalfa, pea, and siratro. Nodulation was not detected on any of the hosts when these Tn*5*-mutated plasmids were present in either RS1019 or RS1043.

DISCUSSION

In the last few years substantial progress has been made in the understanding of the molecular basis of the nodulation process. However, the available data correspond to a relatively small number of strains that have been used as a

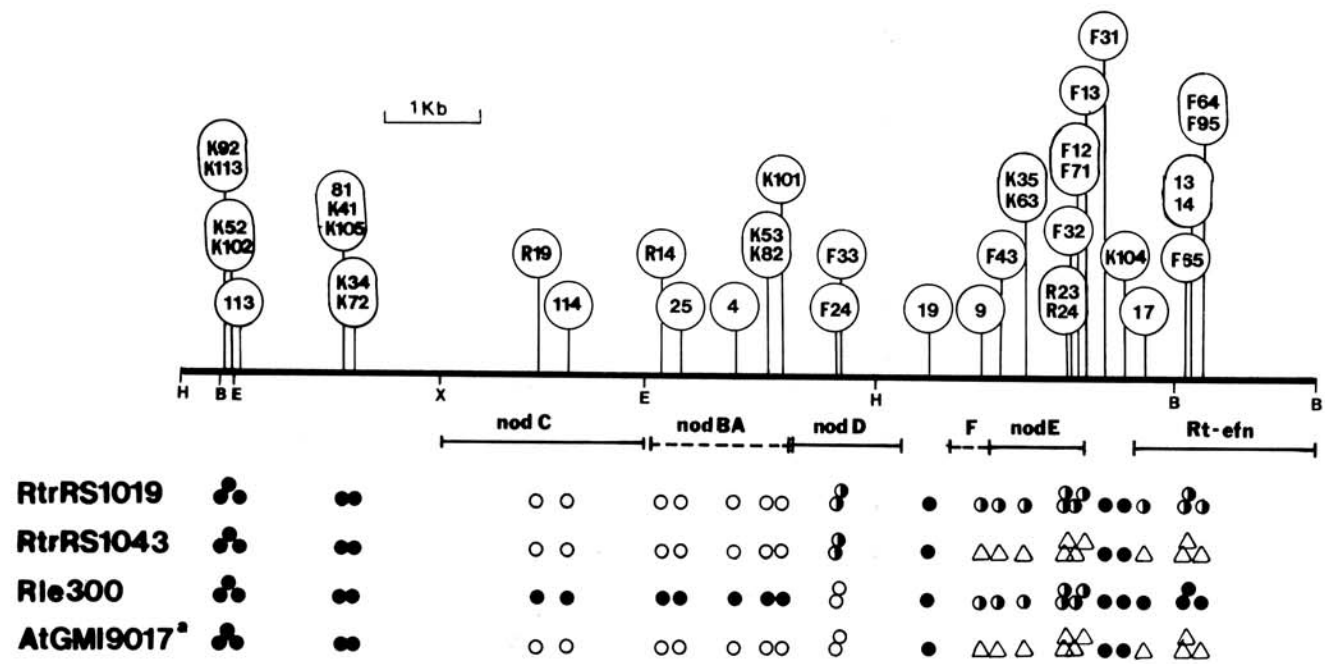

Fig. 6. Location of Tn*5* insertions on pFR1508 and position of the *nodC*-, *nodD*-, *nodE*-, *nodAB*-, and *nodF*-like genes and *Rt-efn* region. The location of *nodC*, *nodD*, and *nodE* (solid lines) was determined by hybridization; the location of *nodAB* and *nodF* (dashed lines) is by comparison with other strains of *Rhizobium* (see text). Below, symbiotic phenotypes induced on red or white clover by *R. l.* bv. *trifolii* RS1019 and RS1043 transconjugants, and on red clover by *R. l.* bv. *viceae* 300 and *A. tumefaciens* GMI9017 transconjugants. •, wild-type-like nodulation; ◑, slightly reduced nodulation (see text); △, very reduced nodulation (see text); O, Nod$^-$. AtGMI9017, pseudonodules. B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; X, *Xho*I.

target for such studies. There is not sufficient evidence to guarantee that the proposed models are workable in all the strains of a given species. In this paper we have identified the Sym plasmid of strain RS1051 of *R. l.* bv. *trifolii* and subsequently characterized its nodulation region. Some differences in the *nod* gene structure have been found as compared with the well-studied strain ANU843.

The identification of the pSym was first approached by elimination of the native plasmids and further analysis of the resulting phenotype on plants. Heat treatments have been successfully used for this purpose, although in some cases megaplasmids have been difficult to eliminate (Banfalvi *et al.* 1981; Rosenberg *et al.* 1981). In our study the curing phenomenon showed scarce reproducibility; only in one out of 13 experiments (i.e., the analysis of 1,300 colonies) did we obtain curing or deletion of plasmids. However, we studied plasmid profiles and not nodulation ability. Deletions of less than 20 MDa would not be seen in a 200-MDa plasmid. Soberón-Chávez *et al.* (1986) have described Nod$^-$ derivatives of *R. phaseoli* isolated after heat treatment that do not show detectable change in the plasmid profile. The fact that all the plasmid profile-altered derivatives had changes in only one plasmid and were found to be Nod$^-$ might suggest the presence of a "hot spot" for deletion or rearrangement rather than a random phenomenon. Such hot spots have also been observed in other strains (Banfalvi *et al.* 1981; Meade *et al.* 1982) and proposed to be insertion sequences. So far, native insertion sequences that preferentially transpose into the *nod-nif* region have been characterized in two strains of *R. meliloti* (Ruvkun *et al.* 1982; Dusha *et al.* 1987).

To exclude the possibility that only some genes essential for nodulation are present in pRtrRS1051*a*, a more direct approach was undertaken. The mobilization of the indigenous RS1051 plasmids into a distantly related plasmid-free *Agrobacterium* strain resulted in nodule development only in those cases where pRtrRS1051*a* was transferred. In the absence of the helper plasmid, low frequency of self-transmissibility was observed for pRtrRS1051*a*.

Further characterization of the *nod* region was made possible by the isolation of clones pFR14 and pFR15 from a RS1051 gene library. As previously reported for ANU843 (Schofield *et al.* 1984), we found all essential *nod* genes to be clustered in a DNA region of 14 kb. This region enabled *Agrobacterium* and *R. l.* bv. *viceae* to nodulate clover, but failed to extend the host range of *R. meliloti* and *Rhizobium* sp. MPIK3030. The result with *R. meliloti* is in agreement with previous reports (Fisher *et al.* 1985). However, the capability of *R. l.* bv. *trifolii* ANU843 *nod* genes to allow *Rhizobium* sp. NGR234, a strain very closely related to MPIK3030, to nodulate clover has been described (Schofield *et al.* 1984; Bassam *et al.* 1986).

We demonstrate that the pFR1508 1.7-kb region located to the right of mutation 17 affects the efficiency of nodulation. This region is reiterated on the RS1051 pSym, and our results indicate that the presence of at least two copies is necessary for a successful nodulation. When only one copy is present, nodulation displays a 3- to 4-day delay (e.g., in RS1043 carrying pFR1508 or RS1019 carrying pFR1506). When none of the copies is present, nodulation is drastically reduced, as it happens with RS1043 carrying pFR1506. We have termed this region *Rt-efn* by analogy with the region described in *R. meliloti* 41 (Putnoky and Kondorosi 1986) as they are phenotypically similar. However, they are structurally and, most probably, functionally different. The *Rt-efn*-hybridizing fragment of *R. meliloti* 41 and the *efn*-containing fragment of *R. meliloti* 41 are different. In addition, a *nodD* gene (*nodD2*) is harbored by the *efn* region of *R. meliloti* 41 (Göttfert *et al.* 1986). The phenotype of our *Rt-efn* region differs from those of either the ANU843 regions III, IV, or V. The *Rt-efn* region works equally in both red and white clover and plays no apparent role in host range determination; it is structurally conserved in *Rhizobium* sp. MPIK3030, *R. meliloti* 41 (where it is also reiterated), and structurally as well as functionally conserved in *R. l.* bv. *viceae*. The *Rt-efn* region seems to correspond by both location and phenotype to the recently described *R. l.* bv. *viceae nodL* gene (Surin and Downie 1988), although no *nodL* reiterations were detected nor strong homology in species other than *R. l.* bv. *trifolii*. If it were so, *Rt-efn* could be ascribed as corresponding to the ANU843 region IV (where a *nodL* gene has been found), even though the phenotype of both regions seems to differ.

By subcloning analysis we have found the left 2.6-kb region of pFR15 to be necessary for nodulation of *R. l.* bv. *trifolii*. This region maps in a position similar to ANU843 region II (Djordjevic *et al.* 1985) and, like it, is functionally conserved in *R. l.* bv. *viceae*. However, whereas mutations in the ANU843 region II or in the analogous *R. l.* bv. *viceae* region containing *nodIJ* lead to a nodulation-delayed phenotype (Djordjevic *et al.* 1985; Evans and Downie 1986), the lack of this region (as in pMFB1) results in a Nod$^-$ phenotype. Interestingly, Tn*5* mutants located in the 2.6-kb region showed no effect. It is possible that the RS1051 region II extends between mutations K72 and R19, being located immediately downstream of *nodC*, as it happens in *R. l.* bv. *viceae*.

Furthermore, by deletion analysis of pFR15 we have also found that only 11.45 kb is necessary for efficient red clover nodulation. Nevertheless, efficient white clover nodulation induced by *R. l.* bv. *viceae* or *Agrobacterium* requires an additional region of DNA present in pFR1504 and not in pFR1508. Because this region seems to be needed only in a heterologous background, it could be suggested that in *R. l.* bv. *trifolii* that region is functionally reiterated.

The region located between mutations 19 and F31 responds both by hybridization and by symbiotic phenotype to what has been reported for the ANU843 region III. By comparison with the *nod* organization of other strains of *R. leguminosarum* and according to physical location and phenotype, mutations 9 and F43 must be located on the *nodF* gene, in the same way that mutations from R14 to K101 must lie on the *nodAB* genes (Fig. 6). Djordjevic *et al.* (1985) found that region III mutants show a markedly poor nodulation on white clover, little effect on subterranean clover, and altered host range properties, now being able to weakly nodulate peas. We did not find any extended host range ability on siratro, alfalfa, or peas, but a delayed nodulation was found in both red and white clover. The effect on subterranean clover has not been examined. We have additionally found that the mutants in this area induce a more impaired nodulation in RS1043 than in RS1019 or *R. l.* bv. *viceae*. It is possible that high *Rt-efn* gene(s) dosage is necessary for the normal functioning of the region III.

However, the participation of other gene(s), present in pRtrRS1051*a* and therefore absent from RS1043, interacting positively with those of region III, cannot be excluded.

By hybridization analysis we also show that RS1051 carries two copies of *nodD*, one of them located in a replicon other than the pSym. In *R. l.* bv. *trifolii* ANU843 and *R. l.* bv. *viceae*, containing a single *nodD* gene, a *nodD* mutation where three *nodD* copies are present, a single mutation does not unduly impair nodulation (Göttfert *et al.* 1986; Honma and Ausubel 1987). In *R. l.* bv. *trifolii* RS1051 mutations in the *nodD* copy cloned in pFR1508 have no more effect than a slight delay in the nodulation. However, when the *nodD*-mutated pFR1508 plasmid is transferred to *Agrobacterium*, clover nodulation is completely lost. This strongly suggests that, at least in the absence of the former one, the second *nodD* copy is functional and substantially contributes to the nodulation event. On the other hand, as it was found in ANU843 (Spaink *et al.* 1987), the RS1051 *nodD* copy contained in pFR1508 does not behave as a common *nod* gene, because its mutation cannot be effectively complemented by wild-type *R. l.* bv. *viceae* when the host is white but not red clover.

Note added to the proof: By using the 1.7-kb *Bgl*II fragment of plasmid pRt288 (Djordjevic *et al.* 1986) as an internal *R. l.* bv. *trifolii* ANU843 *nod* region II (*nodIJ*) probe, the corresponding genes were localized adjacent to the *nodC* in *R. l.* bv. *trifolii* RS1051. Hybridization with the 0.4-kb *Cla*I-*Pst*I fragment of plasmid pJJ179 (*nodL* probe) resulted in detection of homology to the DNA fragment identified as *Rt-efn* in this work.

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