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Conserved Nodulation Genes in *Rhizobium meliloti* and *Rhizobium trifolii*

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Plasmids which contained wild-type or mutated *Rhizobium meliloti* nodulation (*nod*) genes were introduced into Nod⁻ *R. trifolii* mutants ANU453 and ANU851 and tested for their ability to nodulate clover. Cloned wild-type and mutated *R. meliloti nod* gene segments restored ANU851 to Nod⁺, with the exception of *nodD* mutants. Similarly, wild-type and mutant *R. meliloti nod* genes complemented ANU453 to Nod⁺, except for *nodCII* mutants. Thus, ANU851 identifies the equivalent of the *R. meliloti nodD* genes, and ANU453 specifies the equivalent of the *R. meliloti nodCII* genes. In addition, cloned wild-type *R. trifolii nod* genes were introduced into seven *R. meliloti* Nod⁻ mutants. All seven mutants were restored to Nod⁺ on alfalfa. Our results indicate that these genes represent common nodulation functions and argue for an allelic relationship between *nod* genes in *R. meliloti* and *R. trifolii*.

Rhizobium is a genus of bacteria that are able to establish symbiotic nitrogen-fixing root nodules with plants, primarily in the family *Leguminosae*. *Rhizobium* spp. are largely defined by host-plant range within the *Leguminosae* (12). In homologous (nodule-productive) combinations of bacteria and plants, microscopic studies reveal that bacteria attach to plant cells, the root hairs of the host curl markedly, and host cells are invaded by way of infection threads (2, 15, 22). In heterologous (nonnodulating) combinations of bacteria and host, host root hairs may show partial deformation but no markedly curled root hairs (32). Genetic studies of nodulation by fast-growing *Rhizobium* strains have demonstrated a series of loci required for nodulation (*nod* genes) which are linked to nitrogenase (*nif*) genes on very large symbiotic (pSym) plasmids (1, 10, 17, 19, 24, 29). Mutations in these *nod* genes result in the failure of nodule development at early stages. In *R. meliloti* 1021, Nod⁻ mutants have been isolated which fail to curl root hairs (16). By genetic and sequence analysis, the mutations causing these mutants apparently map to four genes (16; T. T. Egelhoff, R. F. Fisher, T. W. Jacobs, J. T. Mulligan, and S. R. Long, DNA, in press).

In crosses between *R. leguminosarum* and *R. trifolii*, host-range selectivity is cotransferred with other nodulation loci (9, 10, 14), suggesting that *nod* and host-range genes are either identical or closely linked in these species. In this study, we report that several nodulation genes in *R. meliloti* are functionally replaceable by a cloned *nod* gene DNA fragment of *R. trifolii* and that *R. meliloti* clones likewise complement two *R. trifolii* Nod⁻ mutants. These complementations are not accompanied by transfer of host plant selectivity, in contrast to the studies between more closely related species. The complementation of each *R. trifolii* mutant by *R. meliloti* DNA fragments maps to a specific physical location in the cloned fragments, indicating an allelic relationship between the genes in the two organisms.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. Transposon Tn*5* mutagenesis was used to generate mutated versions of the *R. meliloti* 1021 *Eco*RI nodulation fragment cloned in pRK290. Corresponding mutant derivatives of *R. meliloti* 1021 were obtained by homogenotization (16, 27).

The locations of the Tn*5* insertions are shown in Fig. 1. (Corresponding plasmids bear the 8.7-kilobase (kb) *Eco*RI fragment, with transposon Tn*5* in the indicated location, cloned into the *Eco*RI site of pRK290.) *R. trifolii* wild-type strain ANU843 and Nod⁻ strains ANU851, ANU453, and ANU845 have been described by Schofield et al. (29) and Djordjevic et al. (9). The Sym-plasmid-cured fast-growing *Rhizobium* sp. strain ANU265 has been described by Morrison et al. (21). Recombinant plasmids pRt032 and pRt587 have been described by Shine et al. (30) and Schofield et al. (29), respectively.

Conjugations. Plasmids were routinely maintained in *Escherichia coli* HB101. pRK290-based plasmids were transferred into *Rhizobium* recipients by using pRK2013 as a helper plasmid by the triparental conjugation technique (8). *E. coli* was counterselected with minimal sucrose medium, and *Rhizobium* containing pRK290 was selected with 10 μg of tetracycline (Tc) per ml. Plasmids were visualized by the direct lysis method of Eckhardt (11) with the modifications of Rosenberg et al. (25).

Construction of pRtRF101. Recombinant plasmid pRt587, containing the wild-type *R. trifolii* 14-kb *Hin*dIII *nod* gene fragment in vector plasmid pBR328, was cut with *Hin*dIII; the insert and pBR328 vector fragments were separated on a 0.6% low-melting-temperature agarose gel and ligated by the method of Crouse et al. (7) with *Hin*dIII-digested pWB5a (a pRK290-derivative plasmid containing a polylinker) which was a generous gift of W. J. Buikema (Harvard University, Cambridge, Mass.). The ligation mixture was used to transform competent *E. coli* HB101 cells to Tc^r.

Nodulation assays. Seeds of alfalfa (AS13R; Ferry Morse) or clover (Dutch White clover; Agway Seeds) were sterilized by ethanol and Clorox washes, soaked in several changes of sterile water, and planted on nitrogen-free agar slopes (20). Bacteria were grown to the stationary phase in selective TY (3) medium, collected by centrifugation, washed, and added to plants at approximately 10^9 cells per plant. Nodulation phenotype was scored visually at 2.5 weeks and again at 4 weeks.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source or reference
R. meliloti		
1021	Wild-type, Nod$^+$ Fix$^+$ on alfalfa, Sm^r	
J162	1021 *nodC162*::Tn*5* Nod$^-$ Sm^r Nm^r	16
J169	1021 *nodC169*::Tn*5* Nod$^-$ Sm^r Nm^r	16
J8B4	1021 *nodC8B4*::Tn*5* Nod$^-$ Sm^r Nm^r	16
J2B2	1021 *nodB2B2*::Tn*5* Nod$^-$ Sm^r Nm^r	16
J5B7	1021 *nodA5B7*::Tn*5* Nod$^-$ Sm^r Nm^r	16
J4C4	1021 *nodA4C4* ::Tn*5* Nod$^-$ Sm^r Nm^r	16
J9B7	1021 *nodD9B7*::Tn*5* Nod$^-$ Sm^r Nm^r	16
R. trifolii		
ANU843	Wild type, Nod$^+$ Fix$^+$ on white and subterranean clovers	23
ANU845	Sym plasmid-cured derivative of ANU843 Nod$^-$	9
ANU851	ANU843 *nod-851*::Tn*5* Nod$^-$	9
ANU453	ANU794 *nod-453*::Tn*5* Nod$^-$	9
Rhizobium sp. strain ANU265	Sym-plasmid-cured derivative of ANU240 Nod$^-$	21
E. coli HB101	F$^-$ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rps-120*(Sm^r) *xyl-5 mtl-1 supE44*	5
Plasmids		
pRmJ30	pLAFR1 + 8.7-kb *Eco*RI fragment from *R. meliloti* nodulation region, Tc^r	16
pRt587	pBR328 + 14-kb *Hin*dIII fragment from *R. trifolii* nodulation region, Ap^r Cm^r	29
pRt032	pKT240 + 14-kb *Hin*dIII fragment from *R. trifolii* nodulation region, Km^r	30
pRtRF101	pWB5a + 14-kb *Hin*dIII fragment from pRt587 containing *R. trifolii* nodulation region, Tc^r	This report
pWB5a	pRK290 containing polylinker in *Eco*RI site, Tc^r	W. J. Buikema[a]
pRmJ162	pRK290 + 1021 *nod-162*::Tn*5* 14.4-kb *Eco*RI fragment, Tc^r Nm^r	16
pRmJ170	pRK290 + 1021 *nod-170*::Tn*5* 14.4-kb *Eco*RI fragment, Tc^r Nm^r	16
pRmJ160	pRK290 + 1021 *nod-160*::Tn*5* 14.4-kb *Eco*RI fragment, Tc^r Nm^r	16
pRmS6B7	pLAFR1 + 1021 *nod-6B7*::Tn*5* 14.4-kb *Eco*RI fragment, Tc^r Nm^r	16
pRmS9B7	pLAFR1 + 1021 *nod-9B7*::Tn*5* 14.4-kb *Eco*RI fragment, Tc^r Nm^r	16

[a] W. J. Buikema, Harvard University, Cambridge, Mass.

RESULTS

Introduction of *R. trifolii nod* genes into *R. meliloti* mutants. We have confirmed that when pRt032 is introduced into a Sym-plasmid-deleted *R. trifolii* strain, ANU845, or into the Sym-plasmid-deleted broad-host-range strain ANU265, it confers the ability to nodulate clover (29; this report). We wanted to determine whether the *R. trifolii nod* genes were capable of complementing *R. meliloti* Tn*5*-induced Nod$^-$ mutants to Nod$^+$. It was necessary to insert the *R. trifolii nod* genes into another broad-host-range vector, since the kanamycin resistance-neomycin resistance (Km^r-Nm^r) Tn*5* insertions already present in the mutant recipients would not permit selection of the pRt032 Km^r marker. We therefore recloned the *R. trifolii nod* genes borne on the 14-kb *Hin*dIII fragment into a pRK290 derivative as pRtRF101, which permits selection of transconjugants by Tc^r. When pRtRF101 was subsequently introduced into *R. trifolii* ANU845 and ANU265, it induced the formation of nodules on clover (data not shown), thus retaining the properties of its parent plasmid, pRt032 (29).

TABLE 2. Nodulation by *R. meliloti* strains

Strain	Site of Tn*5* insertion: distance (bp) from right end of 8.7-kb gene[a] *Eco*RI fragment	Nodulation response:			
		No plasmid		With pRtRF101	
		Alfalfa	Clover	Alfalfa	Clover
1021	None	+	−	+	−
162	555 (*nodC*)	−	−	+	−
169	1,231 (*nodC*)	−	−	+	−
8B4	1,595 (*nodC*)	−	−	+	−
2B2	1,984 (*nodB*)	−	−	+	−
5B7	2,394 (*nodA*)	−	−	+	−
4C4	3,075 (*nodA*)	−	−	+	−
9B7	3,922 (*nodD*)	+/−	−	+	−

[a] Gene location is as determined by Egelhoff et al. (in press). bp, Base pairs.

To test the behavior of these genes in *R. meliloti*, plasmid pRtRF101 was introduced into wild-type strain 1021 and seven derivatives containing Tn*5* insertions in the 8.7-kb *Eco*RI *nod* gene fragment (Fig. 1). These Tn*5* insertions render the strains Nod$^-$ on alfalfa (16). The pRtRF101 transconjugants of these mutant strains, however, were Nod$^+$ on alfalfa (Table 2). Nodules formed by complemented Nod$^-$ mutants appeared morphologically and functionally the same as those induced by the parental strain 1021. This suggests that the *nod* genes interrupted by insertion of Tn*5* into the *R. meliloti* 8.7-kb *Eco*RI fragment are functionally equivalent to those located on the *R. trifolii* 14-kb *Hin*dIII fragment. Despite the fact that the 14-kb *Hin*dIII fragment present in pRtRF101 contains all of the Sym-plasmid-encoded information necessary for the formation of nodules on clover (29), none of the *R. meliloti* transconjugants was able to nodulate clover (Table 2).

Functional relationship of *R. meliloti* and *R. trifolii nod* loci. Plasmid pRmJ30, bearing the 8.7-kb *Eco*RI fragment of *R. meliloti* in pRK290, restores the nodulation phenotype to *R. trifolii* Nod$^-$ mutants ANU851 and ANU453 (Table 3). We wished to test whether this was due to substitution of an independent *R. meliloti* nodulation pathway for the normal *R. trifolii* nodulation pathway or to the presence of individual loci on pRmJ30 which were allelic equivalents of those mutated in the *R. trifolii* strains. If the former case were

TABLE 3. Clover nodulation by *R. trifolii* strains containing *R. meliloti* clones[a]

R. meliloti plasmid	Nodulation by recipient *R. trifolii* strain		
	ANU843 (wild type)	ANU851 (Nod$^-$)	ANU453 (Nod$^-$)
None	+	−	−
pRmJ30	+	+	+
pRm162 (*nodCII*::Tn*5*)	+	+	−
pRm170 (*nodCI*::Tn*5*)	+	+	+
pRm160 (*nodA*::Tn*5*)	+	+	+
pRm6B7 (*nodA*::Tn*5*)	+	+	+
pRm9B7 (*nodD*::Tn*5*)	+	−	+

[a] All transconjugants failed to nodulate alfalfa (at least five trials).

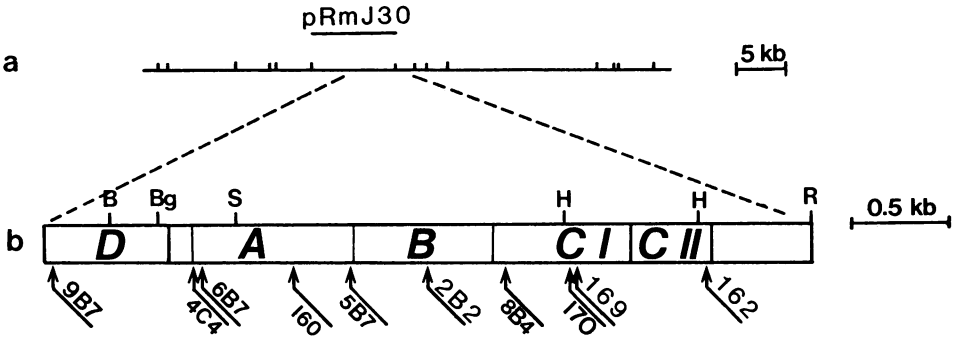

FIG. 1. Map of a region of the *R. meliloti* 1021 megaplasmid containing the *nod* and *nif* genes. (a) *Eco*RI sites (vertical lines) and the 8.7-kb *Eco*RI fragment which was subcloned as pRmJ30 are indicated. (b) An expanded representation of the *nif*-proximal portion of the 8.7-kb *Eco*RI fragment containing the common *nod* genes. Restriction sites for *Bam*HI (B), *Bgl*II (Bg), *Sst*I (S), *Hin*dIII (H), and *Eco*RI (R) are indicated. The sites of individual Tn*5* insertions discussed in the text are indicated by the arrows. Common *nod* genes have been determined by complementation and sequence analysis (16). Those relevant to this study are D and CI/CII, indicated in the boxes.

true, all Nod$^-$ mutations in the *R. meliloti* strains should prevent complementation of the *R. trifolii* mutant strains. If the latter case were true, mutations in some but not all positions should render pRmJ30 unable to complement particular *R. trifolii* mutations.

Several pRmJ30 derivatives bearing a Tn*5* insertion in the 8.7-kb *Eco*RI fragment were introduced into strains ANU851 and ANU453. The ability to complement specific Tn*5* mutations in *R. trifolii* mapped to specific regions of the *R. meliloti* 8.7-kb *Eco*RI fragment (Table 3). All wild-type and mutant pRmJ30 derivatives complemented strain ANU851 to Nod$^+$, with the exception of pRm9B7; therefore, strain ANU851 contains the mutated equivalent of the *R. meliloti* gene inactivated by Tn*5* insertion at a site, 3,924 base pairs from the right end of the 8.7-kb *Eco*RI fragment. This genetic locus has been designated *nodD* (Egelhoff et al., in press). Similarly, all wild-type and mutant pRmJ30 derivatives complemented strain ANU453 to Nod$^+$, except for pRm162, which lies in *R. meliloti* mutant group *nodC*. Thus, ANU453 bears a mutation equivalent to the *R. meliloti* gene inactivated by Tn*5* insertion at a site 553 base pairs from the right end of the 8.7-kb *Eco*RI fragment (*nodC*, region II).

DISCUSSION

Host selectivity at early stages of infection is a striking feature of *Rhizobium*-plant interactions. In this paper we report that a series of Nod$^-$ mutants are complemented, without change of host-plant selectivity, by cloned fragments from heterologous *Rhizobium* spp. This phenomenon has previously been demonstrated with complementation of *R. meliloti* point or deletion mutants by indigenous *R. leguminosarum* (1, 17) and *R. trifolii* (9) plasmids.

We showed that transposon Tn*5* Nod$^-$ mutations covering the *nifHDK*-proximal 3 kb (Fig. 1) of the 8.7-kb *Eco*RI fragment and likely to be in several different *R. meliloti* nodulation genes (16; Egelhoff et al., in press) were complemented by a cloned fragment of *R. trifolii* DNA. Similarly, the *R. meliloti* DNA fragment in pRmJ30 restored nodulation to two *R. trifolii* Nod$^-$ mutants, and this complementation was prevented by transposon mutations only in specific positions within clone pRmJ30. Our findings thus suggest a functional equivalence of individual genes in both nodulation regions. Hybridization data (28) and preliminary sequence comparisons (26, 31; Egelhoff et al., in press; J. Watson, personal communication) indicate significant sequence homology in this region. In addition to this structural gene homology between different species, regulation of nodulation genes may be similar, since they function across species boundaries. This presents an interesting contrast to the *nif* loci, which are highly homologous in different *Rhizobium* spp. but fail to function across species boundaries (6).

The individual Nod$^-$ mutations in the 8.7-kb *Eco*RI fragment were complemented by heterologous *R. trifolii* DNA while maintaining the parental selectivity for alfalfa. It therefore appears that none of the genes thus far identified in this region is required specifically for alfalfa nodulation but that these genes represent common nodulation functions. Such functions may interact with highly conserved structures or functions in many or all legume (or even nonlegume) plant cells. Whether the *R. meliloti* alleles of these genes are completely species neutral, however, is not known. Hirsch et al. (13) have reported that *Agrobacterium tumefaciens* strains bearing pRmJ30 form nodules at low frequency on alfalfa. This transconjugant stimulates the formation of abnormal lateral roots on white clover but has no effect on other legumes tested. It therefore appears that factors that influence host selectivity may be coded for in the 8.7-kb *Eco*RI fragment.

In *R. leguminosarum*, *R. trifolii*, and *R. phaseoli*, the host range can be extended to new host plants by transfer of plasmids or cloned *nod*-region DNA fragments of other *Rhizobium* spp. (9, 10). This fact indicates that host selectivity is a positive function and acts as a dominant trait among these closely related *Rhizobium* spp. Similarly, when pRtRF101 is introduced into two strains lacking Sym plasmids (ANU845 and ANU265), thereby permitting the formation of nodules on clover, it acts as a dominant, positive effector. However, transfer of intact *R. trifolii* plasmids (9) or cloned *R. trifolii nod* gene fragments (this report) into *R. meliloti* cells does not extend host range. *R. meliloti* is a fast-growing strain but is less closely related to *R. trifolii* than are *R. leguminosarum* and *R. phaseoli* (4). It is possible that the more distant *R. meliloti* contains genes which restrict host range (18). Such negative host-range determinants may also operate in other *Rhizobium*-plant systems.

Functional complementation tests are a useful adjunct to sequence comparison, since they may reveal genes involved in nodulation whose phenotype is not as clear on one host as on another. For example, *R. meliloti nodD*, in which mutations give a Nod$^+$/Nod$^-$ phenotype on alfalfa (mutant 9B7), is required for restoring nodulation to strain ANU851, a nonleaky Nod$^-$ *R. trifolii* mutant (Table 3). Whether this leaky phenotype in *R. meliloti* reflects a difference in the

behavior of the plant hosts or other factors is not known. It is possible that the sets of nodulation genes in different *Rhizobium* spp. are overlapping but not completely identical.

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