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Genetics

Conservation of nodulation genes between *Rhizobium meliloti* and a slow-growing *Rhizobium* strain that nodulates a nonlegume host

(*Parasponia Rhizobium*/symbiosis/interspecies complementation)

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ABSTRACT ***Parasponia*, a woody member of the elm family, is the only nonlegume genus whose members are known to form an effective nitrogen-fixing symbiosis with a *Rhizobium* species. The bacterial strain RP501 is a slow-growing strain of *Rhizobium* isolated from *Parasponia* nodules. Strain RP501 also nodulates the legumes siratro (*Macroptilium atropurpureum*) and cowpea (*Vigna unguiculata*). Using a cosmid clone bank of RP501 DNA, we isolated a 13.4-kilobase (kb) *Eco*RI fragment that complemented insertion and point mutations in three contiguous nodulation genes (*nodABC*) of *Rhizobium meliloti*, the endosymbiont of alfalfa (*Medicago sativa*). The complemented *R. meliloti nod* mutants induced effective nitrogen-fixing nodules on alfalfa seedlings but not on siratro, cowpeas, or *Parasponia*. The cloned RP501 nodulation locus hybridized to DNA fragments carrying the *R. meliloti nodABC* genes. A 3-kb cluster of Tn*5* insertion mutations on the RP501 13.4-kb *Eco*RI fragment prevented complementation of *R. meliloti nodABC* mutations.**

Biological nitrogen fixation is the process by which microorganisms carry out the enzymatic reduction of atmospheric nitrogen to ammonia. Species of the Gram-negative genus *Rhizobium* carry out nitrogen fixation in symbiotic association with a plant. Almost exclusively, the host plant is a legume.

Particular *Rhizobium* strains develop specific associations with members of the Leguminoseae. Some strains are highly host specific, while others are promiscuous and can interact with several host plants in the legume family (1).

The experiments described here concern the anomalous association between one strain of *Rhizobium* and a nonlegume, *Parasponia rigida*, a member of the family Ulmaceae. The infection pathway in this association differs markedly from the typical legume infection process, which involves either the invasion of curled root hairs via infection threads and the intracellular release of bacteria or the invasion of the root via epidermal "cracks" and subsequent passive spread of bacteria via host cell division (2). In the *Parasponia* symbiosis, infecting bacteria penetrate the epidermis and outer cortical layers of the root via intercellular spaces generated by cell divisions in the epidermis and cortex. The epidermal cell divisions are induced by the bacteria and, occasionally, even root hair cells, which become swollen and distorted but not curled, divide, an event peculiar to this infection process (3). Infection threads are initiated within the cortex, and bacteria are retained within these threads, where they fix nitrogen. This latter stage contrasts dramatically with the events of legume nodule development where bacteria are liberated from infection threads and differentiate as nitrogen-fixing bacteroids within the plant cell, separated from the plant cell cytoplasm only by a plant-generated membrane.

As a first step in identifying the genetic basis for the unique capacity of the *Parasponia Rhizobium* to fix nitrogen in symbiotic association with a nonlegume, we have sought to clone the genes from this *Rhizobium* that are required for the nodulation of *Parasponia* or legume host plants. In particular, we are interested in answering the question of whether the same *Rhizobium* genes are involved in the nodulation of *Parasponia* and legume hosts.

Rhizobium genes required for nodulation (*nod* genes) have previously been identified and cloned from the fast-growing *Rhizobium* strains *R. meliloti* (4), *R. trifolii* (5), and *R. leguminosarum* (6); at least one of these *nod* genes is functionally and structurally conserved between these comparatively closely related strains (7).

To clone *nod* genes from the *Parasponia Rhizobium* strain RP501, we adopted a strategy of cross-species complementation using *R. meliloti* nodulation mutants. This strategy was adopted because nodulation mutants of *Parasponia Rhizobium* were not available and it was based on the assumption that, in spite of significant differences in the nodulation process between different *Rhizobium* species, some nodulation functions might nevertheless be conserved. Thus, a cloned bank of *Parasponia Rhizobium* DNA was constructed in a broad host range mobilizable cosmid vector and conjugated into nodulation-deficient mutants of *R. meliloti*. As described in this paper, we made the unexpected discovery that nodulation genes affecting early events in the nodulation process are functionally, structurally, and organizationally conserved between two widely divergent *Rhizobium* species.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Bacterial strains and plasmids used in these experiments are listed in Table 1. Bacterial conjugations were performed as described (12).

Media. LB, TY, M9-sucrose, and Nod media have been described (8).

DNA Biochemistry. Total DNA of *Parasponia Rhizobium* strain 501 was prepared as described (8). Restriction endonucleases were purchased from Boehringer Mannheim or Bethesda Research Laboratories and were used according to manufacturers instructions. Agarose gel electrophoresis was performed as described (13). Southern blotting and hybridizations were performed essentially under conditions described by Ruvkun and Ausubel (14), with the following modifications: hybridizations were carried out at 42°C in the

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Abbreviation: kb, kilobase(s).

Table 1. Bacterial strains and plasmids

Strains	Relevant markers	Source or ref.
E. coli		
HB101	*hsd 20Sr⁻, recA13, ara14, proA2 lacY1, galK2, rpsL20(str-r), xyl-5, mtl-1, lam⁻*	H. Boyer
HB101(1am)	*hsd20r, recA13, ara14, proA2, lacY1, galK2, rpsL20 (str-r), xyl-5, mtl-1, supE44(lamcI857)*	F. J. deBruijn
DK1	*(lac)x74-del, galU, galK, rpsL(str-r) (Srl-recA)306-del*	D. Kurnit
R. meliloti		
Rm1021	*str-r*	8
Rm1126	Rm1021::Tn*5*, *str-r, nm-r, nod⁻, hac⁻*	8
Rm1027	Rm1021::Tn*5*, *str-r, nm-r, nod⁻, hac⁻*	8
Gy833	Point mutant, *nodC*	7
Gy1823	Point mutant, *nodAB*	7
TJ1A3	*str-r, nm-r, nodA*::Tn*5*	9
TJ2B2	*str-r, nm-r, nodB*::Tn*5*	9
TJ170	*str-r, nm-r, nodC*::Tn*5*	9
TJ8A2	*str-r, nm-r, nodC*::Tn*5*	9
Rhizobium Strain 501		J. Tjepkema
Agrobacterium tumefaciens A136	*rif-r*, C58 cured of Ti plasmid	D. Garfinkel
Plasmid		
pLAFR1	*IncP*, repRK2, *Tc-r, cos*	10
pRK2013	*repcolE1, nm-r*	11
pEK12	*ap-r, nodABC* of Rm41 cloned in pBR322	7
pPRC6	*tet-r*, pLAFR1 with 21.3-kb insert	This work
pPRC6.1	*tet-r*, subclone of pPRC6, 13.4 *Eco*RI insert	This work
pPRC6.2	*tet-r*, opposite orientation, same insert as pPRC6.1	This work
Phage λ::Tn*5*	467: b221 rex::Tn*5* *cI857* Oam Pam	N. Kleckner

presence of 50% formamide/10% dextran sulfate. Nick-translations were performed according to Maniatis *et al.* (15).

Restriction fragments for subcloning were isolated from agarose gels by using a glass powder purification protocol (16) and were ligated to digested vector fragments with T4 DNA ligase.

Construction of a Cosmid Gene Library from RP501. Total RP501 DNA was partially digested with *Eco*RI and fractionated on sucrose gradients as described (13). Fractions containing DNA fragments in the 21-kilobase (kb) to 35-kb size range were pooled and coprecipitated with pLAFR1 DNA (a broad host range cosmid vector) that had been digested to completion with *Eco*RI. The mixture was redissolved in a volume calculated to result in a final concentration of 400 μg of insert DNA per ml and 100 μg of vector DNA per ml. Subsequent to ligation with T4 DNA ligase, the mixture was packaged according to the method of Hohn (17). The packaged ligation product was used to infect *Escherichia coli* strain HB101. After 2 hr of growth at 37°C, the cells were plated on LB plates containing 7.5 μg of tetracycline per ml and incubated for 2 days at 37°C. Two thousand tetracycline-resistant (Tc^r) colonies were picked and inoculated into 96-well microtiter plates as described (13). Approximately 80% of the cosmid clones examined by gel electrophoresis contained inserts. In addition to storing the clone bank in microtiter plates, ≈1600 Tc^r colonies were scraped directly from Petri dishes and frozen *en masse* in LB medium containing 3% (vol/vol) glycerol/tetracycline (10 μg/ml). These mixed cultures were used as the donor inoculum for mass matings involving the whole gene library.

Transposon Tn*5* Mutagenesis. Preparation of λ::Tn*5* lysates and transposon Tn*5* mutagenesis of plasmid pPRC6 were carried out essentially as described (18). Subsequent to infection with λ::Tn*5*, ≈12,000 kanamycin-resistant (Km^r) HB101/pPRC6 colonies were pooled, and plasmid DNA was prepared by the method of Birnboim and Doly (19). This plasmid DNA was then used to transform *E. coli* strain DK1, and Km^r transformants were selected on LB kanamycin plates. Plasmid DNA was prepared from 60 Km^r transformants, and the location of Tn*5* was determined by restriction endonuclease analysis using *Eco*RI, *Xho* I, and *Sal I*.

Nitrogenase Assays. Inoculation of plants with *Rhizobium* and acetylene reduction assays were performed as described (8).

Microscopy. Three- or 4-week-old nodules were excised from alfalfa roots, fixed, and prepared for light microscopy as described (20).

RESULTS

***Parasponia Rhizobium* Contains Nodulation Genes Which Complement *R. meliloti* nod⁻ Mutants.** To determine whether *Parasponia Rhizobium* strain 501 (RP501) contains nodulation genes functionally homologous to those already identified in *R. meliloti*, we attempted to obtain functional complementation of *R. meliloti* nod⁻ mutants with cloned *Parasponia Rhizobium* DNA fragments. A gene library of RP501 DNA was constructed in the cosmid vector pLAFR1 by using a partial *Eco*RI digest of RP501 DNA, and the library was mobilized *en masse* from *E. coli* strain HB101 into the *R. meliloti* nod⁻ mutant strains Rm1126 and Rm1027. *R. meliloti* strains Rm1126 and Rm1027 both contain mutations in the *nodC* gene (21), one of three so-called "common nodulation" genes (7), and they are incapable of initiating nodule development on alfalfa (5). Strains containing *nodC* mutations fail to curl root hairs. Because root hair curling is ordinarily associated with successful infection of alfalfa, it is believed that the *nodC* product acts early in the nodulation process (22). Strain Rm1126 contains an endogenous insertion sequence (IS-1) in *nodC* and strain Rm1027 contains a complex *nodC* insertion involving both transposon Tn*5* and phage μ sequences (13).

About 800 Tc^r *R. meliloti* Rm1126 and Rm1027 transconjugants containing the RP501 library were mass inoculated in two groups of ≈400 colonies each onto alfalfa plants grown aseptically in test tubes on nutrient agar slants. Twenty-nine and 38 plants were inoculated with Rm1126 and Rm1027 transconjugants, respectively. The formation of nodules served as a selection for cloned RP501 sequences that complemented the nodulation defects in the *R. meliloti* hosts.

Twenty-three of 29 alfalfa plants inoculated with Rm1126 transconjugants were nodulated after 5 weeks, and 28 of 38 plants inoculated with Rm1027 transconjugants were nodulated. The parental strains (Rp501, Rm1027, Rm1126) did not nodulate plants (0 of 30 plants), nor did the Rp501 clone bank in *E. coli* when tested on 30 plants. Bacteria were isolated from several surface-sterilized nodules formed by the Rm1126 and Rm1027 transconjugants, and plasmid DNA was prepared from individual isolates and subjected to restriction endonuclease digestion. A single plasmid DNA species, pPRC6, which contained a 13.4-kb *Eco*RI insert, was found to be common to all of these isolates. [Plasmid pPRC6

also contained a 6.7-kb insert, which we later demonstrated to be a partial duplication of the vector pLAFR1 (data not shown).] When ^{32}P-labeled pPRC6 DNA was used to probe *Eco*RI digests of RP501 DNA by using the Southern blotting and hybridization technique (14), a 13.4-kb genomic fragment was labeled, verifying that no rearrangement of insert DNA took place during the cloning. pPRC6 DNA was used to transform *E. coli* strain HB101, and the plasmid was subsequently conjugated back into Rm1126. Single Rm1126 transconjugants carrying pPRC6 acquired the capacity to nodulate alfalfa plants.

To verify that the cloned RP501 13.4-kb *Eco*RI fragment contained the DNA sequences responsible for complementing *R. meliloti* strains Rm1126 and Rm1027, the 13.4-kb *Eco*RI insert fragment in pPRC6 was subcloned in both orientations in pLAFR1 (resulting in plasmids pPRC6.1 and pPRC6.2). Both subclones complemented Rm1027 in nodulation assays. This latter result suggests that the *Parasponia Rhizobium* nodulation gene or genes were being expressed from an endogenous *Parasponia Rhizobium* promoter in *R. meliloti*. A restriction map of the 13.4-kb *Eco*RI fragment cloned in pPRC6 is shown in Fig. 1.

Characteristics of Nodules Elicited by *R. meliloti* Containing *Parasponia Rhizobium* Nodulation Genes. Rm1027/pPRC6 and Rm1126/pPRC6 induced root hair deformations similar to wild-type *R. meliloti*; shepherd's crooks were elicited at the same frequency observed in infections with wild-type *R. meliloti*. The parental strains Rm1126, Rm1027, and RP501 did not elicit any noticeable response (root hairs were not curled nor were they swollen or distorted).

Nodules elicited by Rm1027/pPRC6 and Rm1127/pPRC6 were completely effective and reduced acetylene at levels comparable to those of nodules elicited by the wild-type *R. meliloti* strain Rm1021. Light microscope examinations showed that nodules elicited by infection with Rm1126 transconjugants were indistinguishable from wild-type-induced alfalfa nodules in overall structure and internal anatomy (Fig. 2). The cylindrical shape and distinct zonation into meristematic, early symbiotic, and late symbiotic regions is apparent in longitudinal sections of nodules induced by both wild-type and Rm1126 transconjugants. Five nodules elicited by the transconjugants were examined, and all exhibited the wild-type nodule morphology.

Structural Conservation of Nodulation Genes. In addition to genetic data, we also obtained structural evidence that indicated evolutionary conservation between the common *nod* region of *R. meliloti* and the *nod* genes carried on pPRC6. In *R. meliloti*, evidence strongly suggests that the so-called "common" nodulation genes, *nodABC*, constitute a single operon with transcription initiated at *nodA* (23). We isolated ^{32}P-labeled DNA fragments from plasmid pEK12, which carries *R. meliloti nodABC* (24), and we used these labeled fragments to hybridize to nitrocellulose filters prepared by the Southern blotting technique, which contained restriction endonuclease-digested pPRC6 DNA. The results of these hybridizations are shown in Fig. 3. A 1.0-kb *Sph* I fragment, which carries most of *R. meliloti nodC*, hybridized to a 1.9-kb *Sal* I fragment of pPRC6 and to an overlapping 5.2-kb *Xho* I fragment. A 2.0-kb *BamHI/Sal* I fragment from pEK12, which includes *nodA* and *nodB* and ≈100 base pairs from the beginning of *nodC*, hybridized to a 1.3-kb *Sal* I fragment and to an 8.8-kb *Sal* I fragment of pPRC6 (which consists of 6.6 kb of DNA from Rp501 and 2.2 kb of vector DNA) and to overlapping 5.2-kb *Xho* I and 2.4-kb *Xho* I fragments. Probes showed no hybridization to vector sequences. This pattern of hybridization, in which *nodC* and *nodAB* of *R. meliloti* show homology to different contiguous restriction fragments of the *Parasponia Rhizobium nod* locus, indicates conservation of more than one of these genes and also suggests conservation of the organization of this symbiotic operon.

Localization of RP501 Nodulation Genes on pPRC6 by Tn*5* Mutagenesis. The 13.4-kb *Eco*RI fragment of pPRC6 was subjected to Tn*5* mutagenesis as described by de Bruijn *et al.* (18). Tn*5* insertions in the fragment were mapped unambiguously by reconciling *Sal* I and *Xho* I patterns of digestion of purified plasmid DNAs. Each of 14 separate Tn*5* insertions distributed across the fragment as well as 4 insertions in the vector were assessed for their capacity to complement the mutant *nodC R. meliloti* strain Rm1027. A correlated physical and genetic map showed that Tn*5* insertions that prevented complementation of *nod* mutants were clustered in a 3-kb region that overlapped the structurally conserved sequences defined by DNA hybridization (Fig. 1). It is notable that in *R. meliloti*, the *nodC* gene spans ≈1 kb of DNA sequence, while the *nodABC* operon covers ≈3 kb of sequence. Because most Tn*5* insertions exhibit a polar effect on downstream sequences (25, 26), it is possible, in view of the hybridization results described above and the fact that insertions within a 3-kb region prevented complementation, that the Tn*5* mutation ml-13 is exerting a polar effect on a downstream *Parasponia Rhizobium nodC*-like gene contained within a larger *Parasponia Rhizobium* nodulation operon.

To specify further the identity of the cloned genes from *Parasponia Rhizobium*, we carried out experiments in which pPRC6 was used to complement separate mutations in *R. meliloti nodA*, *nodB*, and *nodC* genes. *R. meliloti* strains

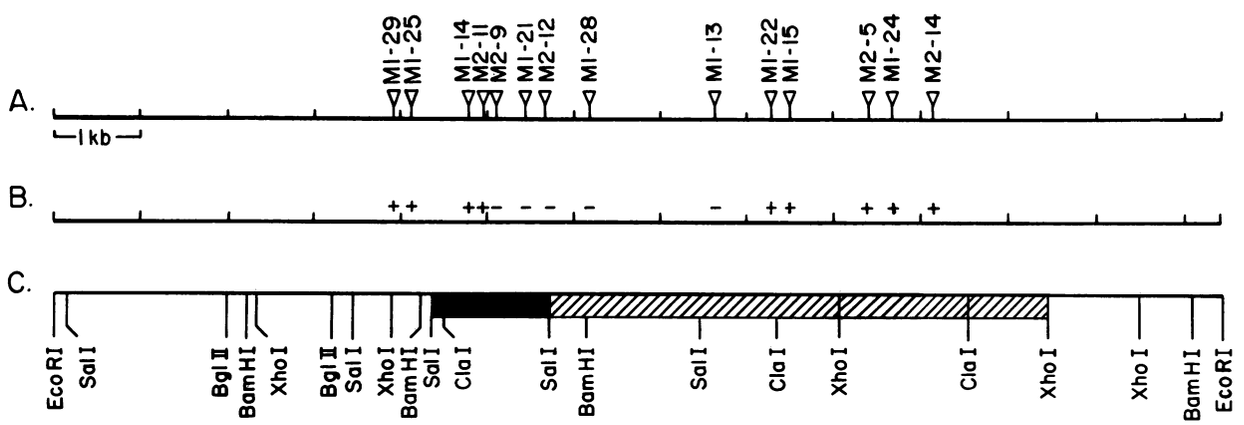

FIG. 1. Physical and genetic map of Rp501 nodulation locus. Physical and genetic map of the 13.4-kb *Eco*RI fragment from *Rhizobium* strain 501, which complements *R. meliloti nod* mutants. (*A*) Mapped locations of Tn*5* insertions in pPRC6. (*B*) Nodulation phenotypes when plasmids carrying Tn*5* insertions are used to complement Rm1027. (*C*) Restriction map of pPRC6. Black box indicates homology to a *nodC* probe from Rm1021. Shaded region shows homology to an Rm1021 probe that carries *nodAB*.

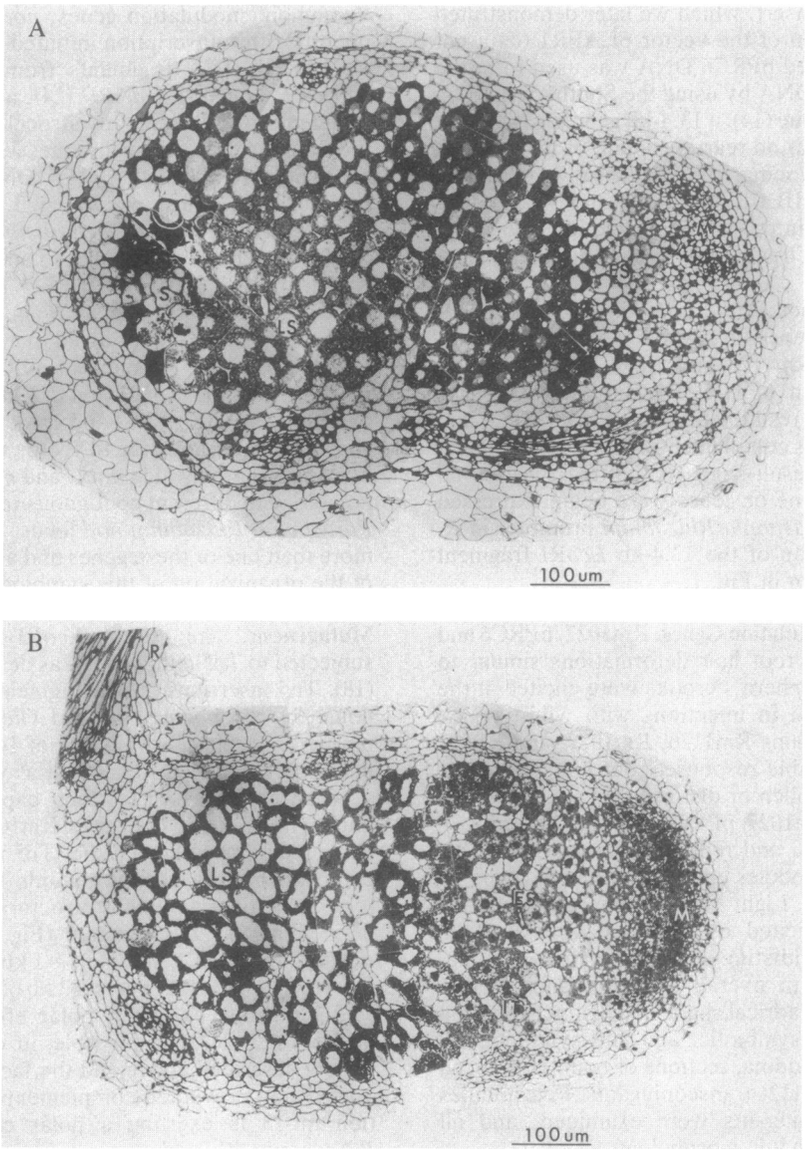

FIG. 2. Sections of nodules induced by (*A*) wild-type Rm1021 showing characteristic distinct zonation into meristematic (M), early symbiotic (ES), late symbiotic (LS), and senescent (S) zones; (*B*) Rm1126/pPRC6 transconjugants. Zonation patterns are the same as in wild-type nodules. R, root.

TJ2B2, TJ170, and TJ1A3 contain Tn*5* mutations in *nodA*, *-B*, and *-C*, respectively, while GY1823 and GY833 are point mutations of *nodA* or *nodB* and *nodC*, respectively. pPRC6 conferred the capacity to nodulate alfalfa on each of these strains, indicating that functions analogous to those provided by the *nodABC* genes of *R. meliloti* are supplied by the *Parasponia Rhizobium* locus.

DISCUSSION

We have shown that cross-species complementation is a valuable technique for cloning functionally conserved symbiotic genes from *Parasponia Rhizobium*. These genetic data demonstrate functional conservation between nodulation loci in fast- and slow-growing *Rhizobium* strains. We have also shown structural conservation between the common *nod* region of *R. meliloti* and the locus carried on pPRC6. Strikingly, it appears likely that the genes *nodABC*, which are responsible for early nodulation functions, are conserved substantially between divergent *Rhizobium* strains with very different host specificities. This conservation of the *nod* operon indicates that all three *nod* genes in concert may provide basic functions essential for all *Rhizobium*-induced nodules.

The results presented here are congruent with unpublished data obtained recently by other workers. K. Scott and co-workers (K. Scott, personal communication) have demonstrated substantial homology at the level of the amino acid sequence between a nodulation gene of *R. trifolii* and a nodulation gene of a slow-growing strain of *Rhizobium* from *Parasponia* ANU289. J. D. Noti and co-workers have achieved complementation of Rm1027 and Rm1126 with cosmid clones of cowpea *Rhizobium* strain IRC78 (J. D. Noti, personal communication). Thus, in addition to conservation of common *nod* genes between fast-growing *Rhizobium* strains, evidence is accumulating to indicate that these symbiotic genes are also found in the genomes of other slow-growing *Rhizobium* strains, regardless of the host specificity of these strains.

Slow-growing rhizobia differ significantly from fast-growing strains with respect to breadth of host range, growth rate, endogenous antibiotic resistance markers, and their capacity to fix nitrogen *ex planta* (27). In fast-growing rhizobia, symbiotic genes (*nif* and *nod*) are generally borne on large

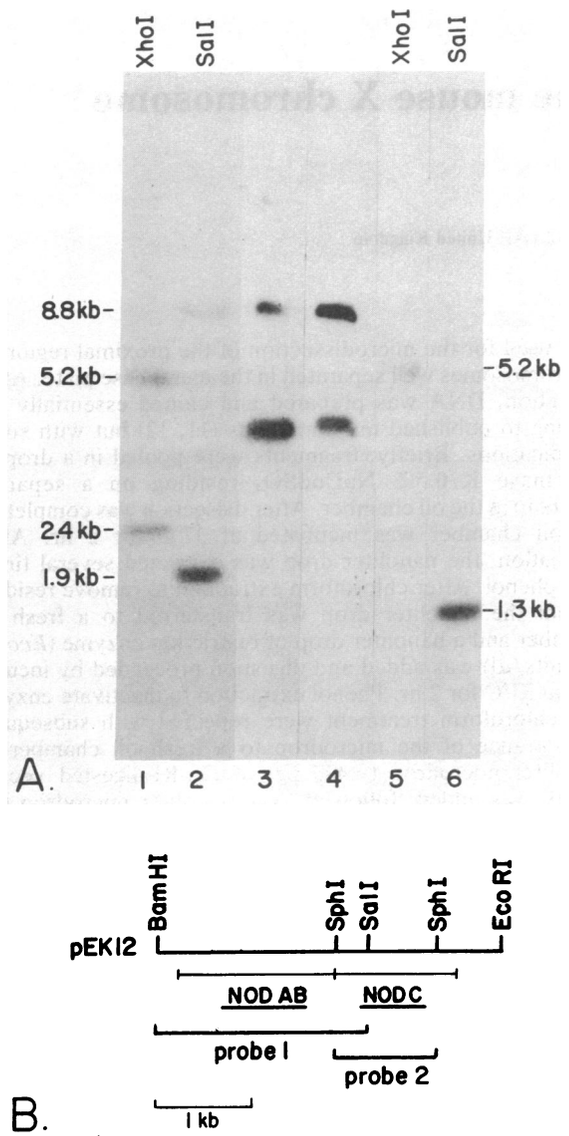

FIG. 3. Conservation of nodulation genes. Southern hybridizations of *R. meliloti nodABC* probes to restriction digests of pPRC6 DNA. (*A*) pPRC6 DNA digested with *Xho* I (1, 5) and *Sal* I (1, 6) probed with nick-translated probe 1 (lanes 1–3) and probe 2 (lanes 4–6). Lanes 3 and 4 contain (uncut) a *Bam*HI/*Sal* I subclone of pEK12. (*B*) Map of pEK12 (7), showing fragments that were used as hybridization probes.

endogenous plasmids (28). Slow-growing rhizobia do not possess "Sym" plasmids, and it is assumed that symbiotic genes are located on the chromosome. Despite these differences, and despite some variations in the infection process (2), it appears that some nodulation functions may be conserved between fast- and slow-growing rhizobia. The functions of nodulation gene products and their relationship to events in the infection process are not known.

Parasponia Rhizobium strain 501 has the capacity to nodulate legume hosts as well as the nonlegume host *Parasponia* (29). Since the infection process leading to development of *Parasponia* nodules is markedly different from the infection process in legumes [cowpeas are invaded via infection threads in curled root hairs (30)], we are interested in determining whether the same genes are involved in nodulation of legume and nonlegume hosts, or if "elm-specific" or "legume-specific" nodulation genes exist. Further experiments are necessary to elucidate the role of the nodulation region cloned in pPRC6 in its natural background.

Advantages of the strategy we used to identify this nodulation locus include eliminating the necessity, initially, of working with a slow-growing strain of *Rhizobium*, Rp501, and a slow-growing plant, *Parasponia*. Cross-species complementation, in which recipient strains carry known symbiotic defects, could be useful in the identification of other conserved symbiotic functions. Conservation of the entire *nodABC* operon implies that this operon has a basic and essential function in *Rhizobium* nodulation of many host types.

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