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Molecular characterization of nodulation genes of Rhizobium fredii strain USDA193

Neela Ramakrishnan Soman

Iowa State University

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Molecular characterization of nodulation genes of Rhizobium fredii strain USDA193

by

Neela Ramakrishnan Soman

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Major: Genetics

Members of the Committee:

Approved: In Charge of Major Work

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Iowa State University

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1986
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CONCLUSIONS
GENERAL INTRODUCTION

Bacteria of the genus *Rhizobium* can infect and nodulate legume roots, establishing a nitrogen fixing symbiosis. The process of symbiotic nitrogen fixation and the steps leading to it are highly complex and are influenced by genetic factors of both the host and the endosymbiont. Other factors such as nutrition and environmental conditions can also influence the efficiency of this symbiosis.

*Rhizobium*-legume symbiosis results in the formation of root nodules, specialized structures in which the invading bacteria fix atmospheric nitrogen into a form which the plant can assimilate and use for protein biosynthesis (for a review see Verma and Long, 1983; Vincent, 1980). On a global basis, this association may reduce 20 million tons of atmospheric nitrogen to ammonia per annum which amounts to about 70-80% of all biologically fixed nitrogen per year (Burris, 1980).

*Rhizobium*-legume attachment is highly specific. One of the early structural responses of the host upon infection is the curling of the root hairs (Mac) which may be caused by the inhibition of cell growth at the attachment site (Bauer, 1981). The curled root hair entraps the adhering *Rhizobium*. 
enrich nutrients for its survival and form a microenvironment where cell wall degrading enzymes are formed (Callaham, 1979; Verma, 1982). The host plant may also play a role in controlling the mode of infection by the bacteria as well as the morphology and intracellular organization of the infected cell. All bacterial attachments do not lead to infection. Very few root hairs to which infective rhizobia attach eventually become infected probably due to the transient susceptibility of the root hairs to infection by the rhizobial symbiont (Bhuvaneswari et al., 1980; 1981). Following the specific attachment of Rhizobium with its host, infection proceeds in a controlled fashion. Tubular structures called infection threads develop from the site of bacterial entry and grow towards the base of the infected root-hair cells and cortical cells which become meristematic prior to or after the infection and restrict the bacterial growth within an extracytoplasmic compartment. Rhizobia enter the root cortex as the thread branches and traverses the walls of the plant cells in the developing nodule meristem. The bacteria are finally released, into the cells of meristem, from the tips of infection thread branches but remain enclosed in a membrane envelope (peribacteroid membrane) derived from the
host plasmalemma (Newcomb, 1981; Verma and Long, 1983). There they differentiate into bacteroids that synthesize nitrogenase and convert atmospheric nitrogen into ammonia.

Based on the pattern of nodule development, two basic types of nodule growth have been observed (Sprent, 1979), determinate and indeterminate nodules. In determinate nodules, bacteria spread by division of preinfected cells and are generally restricted to the central cells. The nodule assumes a spherical shape with few infection threads and no persistent meristems. In indeterminate (meristematic) nodules, infection of new cells occur via infection threads, with a persistent meristem located at the tip of the nodule. Determinate nodules are generally found in tropical legumes such as soybeans, phaseolus beans, cowpeas and siratro while indeterminate nodules are observed in temperate legumes like peas, beans, clovers and medics.

Although rhizobia infect legumes generally via root hairs, the phenomenon is not universal. In a number of tropical legumes, rhizobia appear to invade by penetrating the intercellular spaces between cells near the root surface (Chandler 1978; Chandler et al., 1982; Tsien et al. 1983). This type of bacterial invasion has been referred to as "crack entry", although the actual process of bacterial
entry has not been resolved yet (Chandler, 1978; Chandler et al., 1982).

Symbiotic genes of Rhizobium are generally present on plasmid DNA (Nuti et al., 1979; Prakash et al., 1981; Broughton et al., 1981; Masterson et al., 1982; 1985; Noel et al., 1984; Schofield et al., 1984; Downie et al., 1983). In R. meliloti, the nod and nif genes are present on a megaplasmid (Banfalvi et al., 1981; Rosenberg et al., 1981; Long et al., 1982). Slow growing strains of Rhizobium, designated as Bradyrhizobium japonicum, have not been shown to carry nif sequences on plasmid DNA (Masterson et al., 1982; Haugland and Verma, 1981). Another exception is the R. fredii strain USDA194 which does not carry nod or nif genes on the plasmid DNA (Masterson et al., 1985). Using transposon mutagenesis and other molecular biology techniques, several genes involved in nodulation (Downie et al., 1983; Kondorosi et al., 1984; Long et al., 1982; Schofield et al., 1983) and nitrogen fixation (Corbin et al., 1983; Downie et al., 1983; Forrai et al., 1983; Prakash et al., 1981; Rosenberg et al., 1981; Scott et al., 1982; Szeto et al., 1984; Zimmerman et al., 1983) have been identified and several have subsequently been sequenced (Egelhoff et al., 1985; Jacobs et al., 1985; Rossen et al., 1984; Torok et al., 1984). Such studies have identified a
region coding for four nodulation (nod) genes, nod A, -B, -C and -D genes, with nod D read divergently from nod ABC (Djordjevic et al., 1985; Egelhoff et al., 1985; Jacobs et al., 1985; Downie et al., 1985; Kondorosi et al., 1984). Mutations in nod A, -B and -C genes failed to induce root hair curling (Hac") and nodule formation while mutation in nod D resulted in a delayed nodulation (Nod\textsuperscript{d}) phenotype (Djordjevic et al., 1985; Downie et al., 1983; Jacobs et al., 1985; Kondorosi et al., 1984). These four genes referred to as "common" nod genes (Kondorosi et al., 1984) are functionally interchangeable among R. trifolii, R. leguminosarum and R. meliloti (Banfalvi et al., 1981; Kondorosi et al., 1984; Fisher et al., 1985; Djordjevic et al., 1985). Moreover, an adjacent region which controls the host specificity of nodulation (hsn) has been identified in R. meliloti (Kondorosi et al., 1984; Truchet et al., 1985), in R. leguminosarum (Downie et al., 1983; Gotz et al., 1985) and in R. trifolii (Djordjevic et al., 1985; Schofield et al., 1984).

Regulation and expression of nodulation genes remain essentially unknown since nod transcripts and their products from free-living Rhizobium could not be isolated (Kondorosi et al., 1984). Fusion of the nod genes to strong E. coli promoters (Schmidt et al., 1984) or use of an E. coli in
vitro transcription/translation system only produced gene products corresponding to \textit{nod} C and \textit{nod} D (Downie et al., 1985). In vitro transcriptional and translational gene fusion studies suggest that with the exception of \textit{nod} D gene, all \textit{nod} genes are induced by a plant factor present in the plant exudate (Mulligan and Long, 1985; Innes et al., 1985; Rossen et al., 1985; Shearman et al., 1986). Mulligan and Long (1985) also observed that the degree of induction of \textit{nod} C by plant factor was much higher in the presence of additional copies of \textit{nod} D, suggesting that \textit{nod} D may be a regulator of \textit{nod} C. The \textit{nod} DABC gene products of \textit{R. meliloti} have been identified to be 33 kDa (kilodaltons), 21 kDa, 28 kDa and 44 kDa, respectively, using in vitro, maxicell and minicell \textit{E. coli} expression systems and both \textit{nod} A and \textit{nod} C proteins have been purified (Egelhoff and Long., 1985; John et al., 1985). Antibodies raised against purified \textit{nod} C gene product inhibited nodulation by 50\% (John et al., 1985). In \textit{Agrobacterium tumefaciens}, \textit{vir} C gene required for infection of host plants was found to be induced by a plant signal (Okker et al., 1984). Stachel et al. (1985b and 1986) showed that when \textit{A. tumefaciens} is co-cultivated with plant cells, the expression of \textit{vir} B, -C, -D, -E, -G and \textit{pin} F of the \textit{vir} regulon is induced to high levels. A functional \textit{vir} A locus was shown to be essential
for induction of other vir loci (Stachel et al., 1986). Furthermore, they have identified the plant molecules acetosyringone (AS) and a-hydroxyacetosyringone (OH-AS) which specifically activate vir gene expression (Stachel et al., 1985a). In Rhizobium, an analysis of R. meliloti nod gene promoters has identified conserved sequences of 47 bp with subsequences of 7 bp, 5 bp and 25 bp, located about 200-240 bp upstream from the translational start codons of nod ABC and one each in front of nod E, -F and -G genes (Rostas et al., 1986). This conserved sequence has been shown to be present in several other Rhizobium species located adjacent to nod genes. It is possible that in Rhizobium several operons are activated either directly or indirectly by a plant signal. Attempts at isolating and characterizing the plant factor involved are currently in progress.

Rhizobium and Agrobacterium belong to the same family Rhizobiaceae. The endosymbiont rhizobia and the plant pathogenic Agrobacterium share many similar characteristics. The symbiotic functions of fast-growing Rhizobium strains are generally coded by Sym plasmids whereas the tumor inducing ability of A. tumefaciens is controlled by Ti plasmids. Hooykaas et al. (1982) demonstrated genetic relatedness between Rhizobium and Agrobacterium by comparing
the chromosomal linkage maps of *Rhizobium* and *Agrobacterium*. Prakash and Schilperoort (1982) reported a high degree of homology between *Rhizobium* Sym plasmids and *Agrobacterium* Ti plasmid by DNA hybridization studies. Hadley and Szalay (1982) demonstrated the presence of DNA sequences homologous to the right border region of T-DNA in diverse *Rhizobium* species. Studies with *Rhizobium* fredii plasmid DNA suggest that the observed homology of the Sym plasmid with T-DNA and vir-DNA of *A. tumefaciens* could be due to the presence of an insertion sequence (IS66) homologous present in both species (see Section I). The presence of insertion sequences in different *Rhizobium* strains (ISRI in *R. lupini*, ISRm1 in *R. meliloti*, RSRja and RSRjB in *B. japonicum*) have been reported (Priefer et al., 1981; Ruvken et al., 1982; Kaluza et al., 1985).

Recently, a novel group of fast-growing rhizobia that nodulate soybeans was isolated from root nodules and soil collected in the People's Republic of China (Keyser et al., 1982). These isolates share many characteristics with other fast-growers. Since the host specificity of these isolates is similar to that of the slow-growing soybean rhizobia, they were grouped together with the slow-growers as *Rhizobium japonicum*. Most of these fast-growing strains form nitrogen fixing nodules on the genetically unimproved
Chinese cultivar Peking and Fix- nodules on commercial North American cultivars. To distinguish between slow-growing and fast-growing rhizobia, Jordan (1982) has created a new genus Bradyrhizobium to include all of the slow-growing rhizobia. The fast-growing soybean bacteria have been reclassified as Rhizobium fredii (Scholl and Elkan, 1984). Hybridization studies using R. meliloti nif and nod gene sequences as probes, homologous sequences were identified on the Sym plasmid DNA from R. fredii strains (Masterson et al., 1985; Prakash and Atherly, 1984). The nif and nod sequences were found to be reiterated on the Sym plasmids in R. fredii strains USDA193 and USDA205 (Prakash and Atherly, 1984).

Explanation of dissertation format. My research work for this doctoral dissertation is presented in different sections for clarity and convenience for publication. In Section I of this dissertation is reported for the first time the presence of an insertion sequence homologue in different R. fredii plasmid DNA. The major part of my research work towards a Ph.D degree focuses on the characterization of nodulation genes in R. fredii strain USDA193 (presented in Sections II and III). In Section II is reported the identification of nodulation genes from R. fredii strain USDA193 and their expression in different
Rhizobium strains, thus extending the host range for nodulation by these Rhizobium strains. Section II also reports for the first time the ability of a nod D gene carrying fragment to confer upon heterologous bacteria the ability to nodulate soybeans in the absence of nod ABC genes. In Section III, I report the identification of a new functional gene (host specificity gene) on a 2.8 kb EcoRI fragment using site-specific mutagenesis and functional complementation studies.

Section I reports the results of homology studies using R. fredii DNA, A. tumefaciens T-DNA right border region and an insertion sequence IS66. The data presented in Fig.2 were contributed by the second author, R. K. Prakash. My contribution as senior author relates to the remaining data as well as the interpretation of the results.

Section II describes a detailed work on the nodulation genes of R. fredii strain USDA193. The nod clones pPA1, pPA2 and pPA3 were constructed by the second author, R. K. Prakash. All the microscopic works were carried out by the third author, S. Shantharam. The fourth author, N. M. Duteau has provided the pSym cured strain of R. fredii, IA728. As senior author, I have contributed towards the identification of nod A, -B, -C and -D genes by hybridization as well as by functional complementation
studies, transfer of the nod fragments into different strains looking for nodulation ability on soybean plants, execution of all the plant tests involved including those required for microscopic works, extension of the host range for nodulation by transferring a 2.8 kb DNA fragment into different rhizobia and finally interpretation of all the data involved.

In Section III, I have carried out further research on the characterization of nodulation genes of USDA193 by site-directed transposon mutagenesis and "omega" insertional mutagenesis of the 2.8 kb nod gene fragment looking for functional regions involved in nodulation.

Towards the end of this dissertation, I have included additional data related to the work already described in the previous sections as well as novel observations (see summary and discussion) in Appendices.
SECTION I. CONSERVATION OF IS66 HOMOLOGUE OF OCTOPINE TI PLASMID DNA IN RHIZOBIUM FREDII PLASMID DNA
Conservation of IS66 homologue of octopine Ti plasmid DNA in Rhizobium fredii plasmid DNA

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SUMMARY

DNA sequences homologous to the T-DNA region of the octopine-Ti plasmid from Agrobacterium tumefaciens are found in various fast-growing Rhizobium fredii strains. The largest fragment (BamHI fragment 2) at the right-boundary region of the 'core' T-DNA hybridizes to more than one plasmid present in R. fredii. However, one smaller fragment (EcoRI fragment 19a) adjacent to the 'core' T-DNA shows homology only with the plasmid carrying the symbiotic nitrogen-fixation genes (pSym). Hybridization data obtained with digested R. fredii USDA193 pSym DNA suggest that the homology is mainly with two HindIII fragments, 1.7 kb and 8.8 kb in size, of the plasmid. The 1.7 kb HindIII fragment also hybridizes to two regions of the virulence plasmid of A. tumefaciens, pAL1819, a deletion plasmid derived from the octopine Ti plasmid, pTiAch5. Hybridization studies with an insertion element IS66 from A. tumefaciens indicate that the 1.7 kb HindIII fragment of R. fredii plasmid, homologous to the T-DNA and the virulence region of Ti plasmid, is itself an IS66 homologue.
INTRODUCTION

Rhizobium and Agrobacterium belong to the same family, the Rhizobiaceae. Both show similarities in their ability to interact with higher plants and to induce localized cell proliferation. Rhizobia are gram-negative bacteria which fix nitrogen in a symbiotic association with leguminous plants resulting in the development of specialized structures called root nodules. On the other hand, oncogenic strains of A. tumefaciens form crown-gall tumors in dicotyledonous plants after infection at a wounded site. However, the mode of infection of both Rhizobium and Agrobacterium is quite different. Rhizobium behaves in a host-specific manner, whereas Agrobacterium often shows a broad host range. Agrobacterium is considered to be closely related to most of the fast-growing rhizobia (R. leguminosarum, R. trifoli, R. phaseoli, R. meliloti, etc.), but more distantly to some of the slow-growing strains (Bradyrhizobium japonicum) (Heberlein et al., 1967; Zurkowski and Lorkiewicz, 1976). The tumor-inducing (Ti) plasmids of different virulent strains are reported to contain highly conserved DNA sequences (Chilton et al., 1978; Depicker et al., 1978; Drummond and Chilton, 1978;
Hepburn and Hindley, 1979; Ooms et al., 1980; Perry and Kado, 1982; Thomashow et al., 1981; 1980). Transfer, integration, and expression of a segment of these conserved sequences, called T-DNA, forms the molecular basis of crown gall tumor formation. In nopaline Ti plasmids, the T-DNA is present as a contiguous DNA segment, whereas in octopine Ti plasmids, T-DNA is separated into TL-, TC- and TR-DNAs by two imperfect 24 base repeats lying within the T-DNA. Another pair of imperfect 24 base repeats border the T-DNA sequences. The TL-DNA segment carries the genes necessary for tumorigenesis (Garfinkel et al., 1981; Koekman et al., 1979; Ooms et al., 1982a; Ooms et al., 1982b). The core T-DNA (or TL-DNA) of octopine plasmids comprises the region between Smal fragment 17 and EcoRI fragment 19a. Another region in the Ti plasmid called the virulence (vir) region is essential for T-DNA transfer and integration (Garfinkel and Nester, 1980; Hille et al., 1982; Holsters et al., 1980; Klee et al., 1983; Ooms et al., 1980).

Several Rhizobium strains are reported to contain large plasmids ranging in size from 90-1000 Mdals (Benyon et al., 1980; Nuti et al., 1977; Prakash et al., 1981). In most of the fast-growing Rhizobium strains, the genetic information for plant host range and nodule formation (Brewin et al., 1980a; Brewin et al., 1980b; Higashi, 1967; Hirsch et al.,
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1980; Johnston and Beringer, 1975; Johnston et al., 1978; Rosenberg et al., 1981; Ruvkun et al., 1982; Zurkowski and Lorkiewicz, 1976), nitrogen fixation (Nuti et al., 1979; Prakash et al., 1981), hydrogenase activity (Brewin et al., 1980b), conjugal transfer and bacteriocin production (Hirsch, 1979) are carried by one or more of these indigenous plasmids. Homologies between large plasmids of R. leguminosarum 1001, R. trifoli 5a, and R. phaseoli 3622 and regions of both octopine and nopaline Ti plasmids have been observed earlier although no homology with the T-DNA region could be detected (Prakash and Schilperoort, 1982). Recently, the presence of DNA sequences exhibiting considerable homology with the right-border region of the T-DNA has been reported in diverse Rhizobium species (Hadley and Szalay, 1982).

In this paper, we report that the observed homology of the right-border region of T-DNA and the virulence region of the octopine A. tumefaciens Ti plasmid with the R. fredii plasmid DNA is due to the presence of an insertion element IS66 sequence.
MATERIALS AND METHODS

**Bacterial strains and chemicals** The bacterial strains used in this study are listed in Table 1.

The restriction endonucleases were purchased from New England Biolabs. All the required chemicals were purchased from Sigma Chemicals and Co.

**Isolation of plasmid DNA** The plasmid DNA from Rhizobium and Agrobacterium strains were isolated as described by Hirsch et al. (1980). The DNA was separated by centrifugation in a CsCl-ethidium bromide gradient at 35,000 rpm for 40 h and purified by dialyzing against low TE buffer (10 mm Tris, 1 mm EDTA, pH 7.5). The smaller plasmids from *E. coli* bacteria were isolated by rapid boiling method as described by Holmes and Quigley (1981).

**Isolation of total DNA** The Rhizobium strains were grown in 10 ml TY broth (10 g/l tryptone, 3 g/l yeast-extract, and 0.1% CaCl₂) at 30°C overnight with shaking. Cells were harvested and washed with 0.1% sarkosyl and then with high TE buffer (50 mm Tris, 20 mm EDTA, pH 8.0). Cells were treated as described by Haugland and Verma (1981) with the following modifications. After treatment with protease (1 mg/ml) and SDS (1%), the lysate was sheared
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<td>USDA 110</td>
<td>Nod⁺ Fix⁺(sg)</td>
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<td><em>R. fredii</em></td>
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<td>&quot;</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<td>Machida et al., 1984.</td>
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<tr>
<td>pTA66E32gi</td>
<td>Cm⁺ Tet⁺ Ap⁺</td>
<td>(IS66 clone)</td>
<td></td>
</tr>
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</table>

*Nod,* ability to nodulate; *Fix,* in planta nitrogen fixation (with the exception of USDA 191, all others are *Fix⁻* except on the cultivar Peking); *fg,* fast-growing strain; *sg,* slow-growing strain; *Km-*kanamycin; *Spc-*spectinomycin; *Ap-*ampicillin; *Cm-*chloramphenicol; *Tet-*tetracycline.
Table 1 (Continued)

<table>
<thead>
<tr>
<th>Bacterium or plasmid</th>
<th>Phenotype Relevant properties</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAL1819</td>
<td>Km(^r), Spc(^r), Ap(^r) (R(^r) of vir region)</td>
<td>Hille et al., 1982.</td>
</tr>
<tr>
<td>pPNAl</td>
<td>Ap(^r), Tet(^r) (1.7kb R.fredii clone)</td>
<td>This study.</td>
</tr>
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and extracted three times with phenol (saturated with high TE) and two times with chloroform:isoamyl alcohol (24:1) mixture. The DNA was precipitated with two volumes of isopropanol and dissolved in high TE. The DNA was further purified by passing through a CsCl-ethidium bromide gradient.

**In-well lysis method** To isolate the low-mobility megaplasmid DNA from *Rhizobium*, we modified the procedure described earlier (Eckhardt, 1978). Overnight-grown cells were diluted 100 fold in TY medium and grown for 16 h at 30°C without aeration. About 2 X 10^7 cells were washed with 0.5 ml of 0.1% sarkosyl solution in high TE buffer (pH 8.0) and suspended well in 40 µl lysozyme mixture composed of lysozyme (5 mg/ml), RNase1 (1 µ/ml) and 25% sucrose in TB buffer (89 mM Tris base, 2.56 mM disodium EDTA, 89 mM boric acid, pH 8.2). The cell suspension was loaded immediately into the wells of a horizontal gel. The horizontal gel for plasmid DNA isolation was prepared by constructing two long wells in a 5 mm-thick 0.7% horizontal (25 x 20 cm) agarose gel. One well was constructed by placing an insert in the agarose to obtain a 3 mm-wide slot. The other wells (the sample wells) were prepared by inserting a 1 mm-wide comb. The slot and the comb wells were separated from each other.
by about 3 mm. Once the gel was polymerized, the 3 mm-wide slot filler (the back side of a comb) was first removed and filled with TB buffer containing 0.5% sucrose, 0.5% agarose, and 1% SDS. When this slot solidified, the 1 mm-wide comb was removed, and each well was loaded with bacterial cells. The cell suspension inside the slot was kept at room temperature for 10 min for lysozyme action and then overlaid with TB buffer containing 1% SDS and 5% sucrose. Electrophoresis was performed for 30 min at 5 mA and 6-8 h at 150 V. After ethidium bromide staining (0.50 mg/l) of the gel, the DNA was visualized under UV light.

Agarose gel electrophoresis and southern hybridization

Digested DNA was separated on a 0.7% agarose (Sigma) gel followed by ethidium bromide (0.1%) staining for 1 h. The gel was photographed under UV light using a polaroid MP-4 camera with type 665 film. Lambda DNA digested with HindIII and EcoRI restriction enzymes was used as a size standard. DNA in the gel was denatured, neutralized, and transferred to Gene Screen under the conditions described in New England Nuclear Catalog No. NEF 972. The plasmids pAL1819 and pRAL3910 were labelled with $^{32}$P-dCTP by nick translation reaction (BRL catalogue No. 8160 SB). Hybridization conditions were as described by Prakash and Atherly (1984).
Construction of nod and nif clones

A genomic bank of the pSym from *R. fredii* strain USDA193 (pRjaUSDA193) was constructed in the cosmid vector pVK102 (Knauf and Nester, 1982). By colony hybridization using *R. meliloti* nod and nif gene sequences as probes, bacterial clones carrying nod and nif genes were selected (Ramakrishnan et al., 1986).

Cloning of USDA193 plasmid sequence homologous to the TL-DNA border region

By colony hybridization using the EcoRI fragment 19a of T-DNA as probe, the cosmid clone carrying the 1.7 kb HindIII fragment was selected from the genomic bank of pRjaUSDA193. The cosmid clone was cleaved with HindIII, and the 1.7 kb band was cut out from a 0.8% low melting temperature gel and electroeluted in 1 X TB buffer. The 1.7 kb fragment was then subcloned into the vector pBR322 and checked by further hybridization to the EcoRI fragment 19a of the Ti plasmid.
RESULTS

Hybridization of T-DNA fragments to the plasmid DNA from R. fredii strains. It has been shown recently that the plasmid DNA and chromosomal DNA from several fast-growing and slow-growing Rhizobium strains show homology with the BamHI fragment 2 and the HindIII fragment 1 of T-DNA (see Fig. 1a for the restriction map of T-DNA) from the octopine Ti plasmid of A. tumefaciens (Hadley and Szalay, 1982). To determine whether similar homology exists with the plasmid DNA from different R. fredii strains, we hybridized the BamHI fragment 2 of T-DNA to intact plasmid DNA separated by gel electrophoresis.

Fig. 2A shows the separation of R. fredii plasmid DNA by horizontal gel electrophoresis. Adapting the procedure described in materials and methods, we could detect a megaplasmid in all the fast-growing R. fredii strains in addition to the large plasmid already identified by using the alkaline denaturation procedure (Masterson et al., 1982). The size of the megaplasmid from R. fredii is nearly identical to the megaplasmid present in R. meliloti (Figure not shown) which is about 1000 Mdal (Burkardt and Burkardt, 1984). The genes for symbiotic nitrogen fixation have been
Fig. 1. Physical maps of the T-DNA and vir-DNA of the octopine Ti-plasmid, pTiAch5

(a) A physical map of the T-DNA region of the octopine Ti-plasmid, pTiAch5. \( T_L \) and \( T_R \) regions represent the left and right regions of the T-DNA. BS stands for border sequence. The darkened box represents the region of homology with the IS66 element.

(b) A physical map of the vir-region of the octopine Ti-plasmid, pTiAch5. The hatched boxes represent regions of homology with the nopaline Ti-plasmid, pTiC58 and the open boxes show the vir-operon structure. The darkened boxes represent the regions homologous to the IS66 element.
Fig. 2. Agarose gel electrophoresis of lysates from *Rhizobium*

(A) Lanes 1-5 represent the plasmid profiles of *R. fredii* strains USDA201, 194, 206, 191 and 193 respectively.

(B) Autoradiograph of (A) using the probe BamHI fragment 2 of T-DNA from the octopine Ti-plasmid, pTiAch5.
shown to be located on the megaplasmid in _R. meliloti_ (Banfalvi et al., 1981). However, in _R. fredii_ strains, these genes are present on a plasmid of molecular weight about 190 Mdal (Masterson et al., 1982).

It is evident from Fig. 2B that the BamHI fragment 2 of T-DNA hybridized not only to the megaplasmid but also to the pSym of _R. fredii_ strains. The BamHI fragment 2 also hybridized to the smallest plasmid present in USDA206. However, it did not show any homology with the plasmid DNA from _R. fredii_ strain USDA194. No hybridization to chromosomal DNA was detected in any of the lanes probably due to the low amount of chromosomal DNA present.

As shown in Fig. 1a, the T-DNA is classified into left (TL-) and right (TR-) region. The EcoRI fragment 19a which overlaps with the BamHI fragment 2 represents the border sequence of TL-DNA. This region has been found to be necessary for the integration of T-DNA into the plant genome (Joos et al., 1983; Ooms et al., 1982b; Peralta and Ream, 1985; Ream and Peralta, 1985; Shaw et al., 1984). Since the BamHI fragment 2 hybridized with the _R. fredii_ plasmid DNA, it was of interest to see if sequences homologous to the EcoRI fragment 19a are present in different _R. fredii_ plasmid DNA. With the EcoRI fragment 19a as probe,
hybridization was detected only with the plasmid carrying the \textit{nif} and \textit{nod} genes, even after long exposure (data not shown).

For precise location of the regions homologous to the TL-DNA border on the plasmid from \textit{R. fredii}, blots of HindIII digested plasmid DNA from several strains were hybridized with a $^{32}$P-labelled EcoRI 19a clone, pRAL3910. Fig. 3B illustrates the hybridization pattern. Distinct hybridization was observed with two HindIII fragments of size 8.8 kb and 1.7 kb, respectively, with \textit{R. fredii} strains (weak hybridization in lanes 2 and 3 was due to short exposure time). With strain 205 strong hybridization to multiple bands (12 kb, 9.5 kb, 8.8 kb and 1.7 kb) was observed. Strain 201 also showed a multiple hybridization pattern.

\textbf{Hybridization of the insertion element IS66 to \textit{R. fredii} plasmid DNA} The EcoRI fragment 19a representing the border region of TL- and TR- DNA of the Ti plasmid has been reported to contain an insertion sequence, IS66. IS66 is a 2548 bp long element with a 20 bp terminal inverted repeat at each end and present in at least three copies in octopine Ti plasmids of \textit{A. tumefaciens} (Machida et al., 1984). To confirm that the homology of EcoRI fragment
Fig. 3. Hybridization of $^{32}$P-labeled pRAL3910 and pTA66E32gi to plasmid DNA from R. fredii strains

(A) Agarose gel electrophoresis of HindIII digested plasmid DNA from different R. fredii strains. Lanes 1-4 represent the plasmid DNA from strains USDA193, 191, 201, and 205 respectively.

(B) and (C). Autoradiographs of the Southern blot hybridizations to (A) using pRAL3910 (B) and IS66 clone pTA66E32gi (C), respectively, as probes. The IS66 clone contains the 3.7 kb EcoRI fragment of pTiA66 T-DNA carrying the IS66, cloned into the EcoRI site of the vector, PBR325. (B) and (C) represent hybridization to the same blot. First, the blot was hybridized to the $^{32}$P-labeled pRAL3910 and autoradiographed (B); then the blot was stripped of the probe by repeated washings of the membrane with 0.5 N KOH at room temperature with constant agitation (16-20 h). The membrane was air-dried and hybridized to the IS66 probe and autoradiographed again (C). Hybridization conditions were as outlined in materials and methods.
19a with \textit{R. fredii} plasmids is due to the presence of IS66-like sequences in the plasmid genome, we used the IS66 clone as a radioactive probe and hybridized it to HindIII-digested blot of \textit{R. fredii} plasmid DNA. Fig. 3C shows the hybridization result. With the exception of the strain USDA205, the IS66 clone hybridized more strongly with the 1.7 kb HindIII fragment of \textit{R. fredii} plasmid DNA than with the 8.8 kb HindIII fragment.

Hybridization of the 1.7 kb HindIII fragment of \textit{R. fredii} plasmid to the virulence region of Ti plasmid

Hybridization analysis using the IS66 element probe revealed that the octopine Ti plasmid contains one homologue of the insertion sequence in the junction between TL-DNA and TR-DNA (IS66t) and two homologues (IS66v1 and IS66v2) in the \textit{vir} region (Machida et al., 1984). To determine if the 1.7 kb HindIII fragment of \textit{R. fredii} plasmid, homologous with the IS66 sequence, hybridizes to the corresponding sequences in the \textit{vir} region of the octopine Ti plasmid, we first subcloned the 1.7 kb HindIII fragment in the vector pBR322, designated it as pPNAl, radioactively labelled it and hybridized it to the BamHI-digested plasmid, pAIL1819. The R' plasmid pAIL1819 is a cointegrate of the R plasmid, R772 and the virulence
Fig. 4. Hybridization profile of plasmid DNA

(A) Agarose gel electrophoresis of plasmid DNA.
Lane 1: pPNAI digested with HindIII; lane 2: pAL1819 digested with BamHI and lane 3: pRAL3910 digested with EcoRI.

(B) Autoradiograph of the Southern blot to (A) using $^{32}$P-labeled EcoRI fragment 19a of T-DNA from pTiAch5 as probe.

(C) Autoradiograph of the Southern blot to the virulence plasmid pAL1819 digested with BamHI followed by hybridization using $^{32}$P-labeled pPNAI as probe. The probe was isolated by minipreparation as described in materials and methods.

(D) Hybridization pattern of HindIII digested pRjaUSDA193 using $^{32}$P-labeled pAL1819 as probe.
region of the octopine Ti plasmid, pTiAch5 (Hille et al., 1982). The hybridization pattern is shown in Fig. 4C. The 1.7 kb HindIII fragment hybridized to two BamHI bands, 14.2 kb and 6.3 kb of the virulence plasmid pAL1819. Hybridization to a 36 kb BamHI band was due to the vector hybridizing to the R plasmid sequences. Fig. 4B (lane 2) shows the hybridization pattern using the EcoRI fragment 19a of T-DNA as probe. The probe hybridized to two BamHI fragments of size 14.2 kb and 6.3 kb, of the virulence plasmid (lane 2). Since EcoRI fragment 19a was used as probe, no hybridization to the 36 kb band was seen. The virulence region of the octopine Ti plasmid, which hybridizes both to the 1.7 kb HindIII fragment of R. fredii plasmid DNA and to the EcoRI fragment 19a of T-DNA, has been reported to contain two copies of the insertion element IS66 (Machida et al., 1984).

The data presented in Fig. 3 and Fig. 4 strongly suggest that the 1.7 kb HindIII fragment of R. fredii plasmid DNA is indeed an IS66 homologue.

Fig. 4D shows cross hybridization of the virulence plasmid pAL1819 against the HindIII-digested R. fredii USDA 193 plasmid DNA. The virulence plasmid strongly hybridized to two more HindIII fragments of R. fredii USDA193 plasmid
Fig. 5. Hybridization of $^{32}\text{P}$-labeled pPNAl plasmid to the total DNA from Rhizobium strains

(A) HindIII restriction pattern of total DNA from different Rhizobium strains, USDA193, 191, 201, 205, 194 and 110 respectively (lanes 1-6).

(B) Autoradiograph of blot hybridization to (A) using $^{32}\text{P}$-labeled pPNAl as probe.
DNA, of size 7.8 kb and 3.9 kb, respectively, in addition to the 1.7 kb HindIII fragment. These two DNA fragments represent sequences homologous to other regions of the virulence plasmid pAL1819.

Hybridization of the 1.7 kb HindIII fragment of R. fredii plasmid to the total DNA of R. fredii To investigate if more than one copy of the insertion sequence of the 1.7 kb HindIII fragment were present in R. fredii strains, we hybridized the 1.7 kb clone pPN1 to the HindIII-digested total DNA from R. fredii strains. The results are shown in Fig. 5B. The 1.7 kb HindIII fragment which hybridized strongly with the plasmid DNA from R. fredii strain USDA193 (data not shown), showed weak signal with the total DNA from this strain (5B; lane 1, after long exposure time). DNA from the strains USDA191, USDA205 and USDA194 showed additional bands hybridizing with the 1.7 kb HindIII fragment of R. fredii (lanes 2, 4 and 5).

Hybridization to multiple bands detected with the total DNA from R. fredii strains USDA191 and USDA205 indicates the presence of more than one copy of the 1.7 kb HindIII fragment in their genome (lane 2). A 2.5 kb HindIII fragment of the total DNA from USDA193 and USDA201 strains also hybridized to the probe (lanes 1 and 3). This band was
absent in the plasmid DNA blot (Fig. 3B) suggesting its chromosomal origin. As with nif and nod genes (Masterson et al., 1985), no hybridization was detected with the plasmid DNA from R. fredii strain USDA194 (Fig. 2B, lane 2). However, there seems to be multiple copies of the 1.7 kb and the 8.8 kb HindIII fragments on the chromosomal DNA of USDA194, since the probe hybridized comparatively strongly with its total DNA (Fig. 5B, lane 5).

We also included one slow-growing strain of R. japonicum USDA110 in our study. Because no plasmid is yet reported to be present in this strain, we used the HindIII digested total DNA for hybridization to the 1.7 kb HindIII fragment of R. fredii. Hybridization to multiple bands was observed after long exposure (lane 6).

Localization of the insertion sequence in the region involved in symbiotic nitrogen fixation Cosmid clones carrying the nif and nod genes of the plasmid from R. fredii strain USDA193 were digested with restriction enzymes and the blot hybridized using the EcoRI fragment 19a of the Ti plasmid as probe. Fig. 6B shows the hybridization pattern indicating that insertion sequences are located on a 4.9 kb EcoRI fragment carrying the nif DH genes of R. fredii strain (lane 6) (Masterson et al., 1985). The larger signals in
Fig. 6. Restriction profiles of different plasmid clones (A) and the corresponding autoradiograph of the Southern blot using $^{32}$P-labeled pRAL3910 as probe (B).

Overlapping cosmid clones of the plasmid from *R. fredii* strain USDA193 representing the 6.8 kb HindIII fragment carrying nod D gene and flanking sequences (lanes 1-3); HindIII digested sequences homologous to the vir-region of the octopine Ti-plasmid, pTiACH5 (lanes 4-5) and EcoRI digested fragments carrying the 4.9 kb nif region and adjacent regions (lanes 6-7).
lanes 6 and 7 were due to vector-vector hybridization. Lane 7 shows an overlapping cosmid clone carrying flanking sequences but not the 4.9 kb nif fragment. No hybridization other than to the vector DNA was observed in lane 7. With nod clones of R. fredii USDA193 strain, weak hybridization was observed with a 7.6 kb HindIII band (lanes 1 and 2). Preliminary mapping of pRjaUSDA193 indicated that the 7.6 kb HindIII fragment is adjacent to a 6.8 kb HindIII fragment (Masterson and Atherly, 1986). The 6.8 kb HindIII fragment was shown to carry the nod D gene of R. fredii strain USDA193 (Ramakrishnan et al., 1986).
DISCUSSION

We have found that sequences homologous to the right-border region of TL-DNA are conserved in different fast-growing strains of *R. fredii*. Two different HindIII fragments, 8.8 kb and 1.7 kb in size, show considerable homology with the TL-DNA border region. Interestingly, we observed that the 1.7 kb HindIII fragment also hybridizes with the virulence region of the Ti plasmid, suggesting that copies of DNA sequences homologous to the 1.7 kb fragment of plasmid DNA of fast-growing *R. fredii* strains are present both in the T-DNA region as well as in the virulence region of the Ti plasmid of *A. tumefaciens*. The T-DNA and virulence region of Ti plasmid have been shown to be essential for tumorigenesis in *Agrobacterium*. In octopine Ti plasmids, the TL-DNA region alone is sufficient for tumor formation when the virulence region is supplied either in cis or in trans. During tumor formation, the T-DNA is excised and integrated into the plant genome. The border regions flanking the T-DNA are believed to play a role in the formation of a circular intermediate during the excision process (Zdena et al., 1985).

In *Rhizobium*, which is closely related to
Agrobacterium, the actual steps involved in the nodulation process are still unknown. However, it is clear that both bacterial and plant genes are required for effective symbiosis. The genes involved in symbiotic nitrogen-fixation process are often present on large indigenous plasmids of *R. fredii* strains (Masterson et al., 1982), with the exception of strain USDA194, in which symbiotic genes are found to be coded either by a megaplasmid or by the chromosomal DNA (Masterson et al., 1985).

Recently, it was reported (Machida et al., 1984) that sequences homologous to an insertion element IS66 are present in the T-DNA region as well as in the virulence region of the Ti plasmid, pTiB6806. One copy of this element is present in the region between TL- and TR- DNA, and two copies were located in the virulence region of the wild type Ti plasmid. The IS66 element was originally identified as an insertion in the T-DNA region of a mutant plasmid, pTiA66, which resulted in slow growth of crown gall tumors. Results of cross hybridization experiments indicated that the 1.7 kb HindIII fragment of *R. fredii* plasmid hybridized to regions of the Ti plasmid, which correspond to the IS66 homologues of the wild type plasmid and that the 1.7 kb fragment is indeed a homologue of IS66
element. The T-DNA region and the virulence region of the octopine Ti plasmid have been shown to be conserved in Ti plasmids of different A. tumefaciens strains (Chilton et al., 1978; Depicker et al., 1978). Considerable DNA homology between Ti plasmid DNA and DNA from other strains such as A. rhizogenes (Drummond and Chilton, 1978; Engler et al., 1981; Hepburn and Hindley, 1979; Thomashow et al., 1981; White and Nester, 1980) and several fast- and slow-growing Rhizobium strains (Hadley and Szalay, 1982; Prakash and Schilperoort, 1982) have been reported in the literature. Therefore, it is possible that some of the conserved sequences are due to insertion sequence IS66 present within these species.

Hybridization results of plasmid and total DNA from different Rhizobium fredii strains indicate that one or more copies of the 1.7 kb HindIII fragment may be present on the chromosome of some of the strains. In USDA194, all such sequences are exclusively present on the chromosome. Interestingly, the symbiotic genes are known to be present only on the chromosome of this particular strain. It might be possible that the DNA segments coding for functional genes, originally present on the chromosome, were excised and maintained as an extrachromosomally replicating plasmid DNA molecule through evolution. One possible role of the
insertion-like sequences may be to mediate such an excision process. On the other hand, they might be involved in some translocation events occurring within the chromosome or in the plasmid itself, generating divergence within the same species.

The results presented in Fig. 6 (lanes 6 and 7) show that the insertion sequence of IS66 is located near the nif genes of the sym plasmid of the strain USDA193. The 4.9 kb EcoRI fragment of USDA193 plasmid DNA, hybridizing with the insertion sequence IS66, is a reiterated segment of nif D and H (Prakash and Atherly, 1984). One may attribute the reiteration of the nif genes observed in this strain to the presence of such insertion like sequences in the vicinity of these genes. Recent evidences point out that the occurrence of insertion-like sequences in different Rhizobium strains is not very rare. Insertion element ISRm1, which transposes preferentially into nif genes has been found in R. meliloti (Ruvkun et al., 1982). Another insertion element ISRI was isolated from R. lupini (Priefer et al., 1981). However, it is not clear whether these elements are similar to the IS66 element.

Clustering of insertion sequences around the nif region of Rhizobium genome seems to be a general phenomenon. Recently, two different repeated sequences (RSRja and
RSRjB), similar to insertion elements, were discovered in the Bradyrhizobium USDA110 genome located near the nif region (Kaluza et al., 1985). However, there is no evidence that these elements are functionally involved in symbiotic nitrogen fixation.

The precise role of the conserved sequences present in Agrobacteria or rhizobia is not yet known. The T-DNA border plays an important role in tumor formation in Agrobacterium (Perry and Kado, 1982). The deletion of T-DNA border region reduced oncogenicity dramatically and upon reintroduction of this region, oncogenicity was fully restored (Caplan et al., 1985; Holsters et al., 1980). But, deletion of the left border region of T-DNA did not affect the tumor-forming ability of the Ti plasmid (Joos et al., 1983). Thus, the right border of the T-DNA plays an important role in tumorigenesis. In nopaline-type Ti plasmid, the right border repeat alone could restore virulence fully when introduced into an avirulent Ti plasmid having right border deletion (Wang and Nester, 1980) but in the octopine-type Ti plasmid, only partial activity was observed with the TL right border fragments that lacked sequences lying to the right of the repeat (Peralta and Ream, 1985), indicating the importance of the right side flanking sequences on border
repeat efficiency for T-DNA transmission. It was reported that two regions of the IS66 element show sequence homology with the right side region of the TL right border repeat or the TC-DNA (Machida et al., 1984). However, any homology of the IS66 element with the 25 bp terminus of T-DNA was not reported. We do not know if the IS66 homologue present in the T-DNA region (carrying the right inverted repeat and the left half of IS66) plays any role in tumorigenesis. Mutagenesis of the TC-DNA sequences would identify the regions directly involved in promoting border efficiency. A better explanation for the presence of IS66 homologues would be that such sequences are involved in the generation of genetic and structural variations in octopine Ti plasmids through evolution (Machida et al., 1984). Similarly, the IS66 homologues present in R. fredii strains may also play a role in the evolution of plasmids present in these strains.
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REFERENCES


Chilton MD, Drummond MH, Merlo DJ, Sciaky D: Highly


Eckhardt T: A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid 1:584-588, 1978


Hadley RG, Szalay AA: DNA sequences homologous to the T-DNA region of Agrobacterium tumefaciens are present in


Heberlein GT, Ley JD, Tijtgat R: Deoxyribonucleic acid homology and taxonomy of *Agrobacterium*, *Rhizobium* and *Chromobacterium*. J Bacteriol 94:116-124, 1967


Hirsch PR, Van Montague M, Johnston AWB, Brewin NJ, Schell


Kaluza K, Hahn M, Hennecke H: Repeated sequences similar to insertion elements clustered around the *nif* region of

Klee HJ, White FF, Iyer VN, Gordon MP, Nester EW:

Knauf VC, Nester EW: Wide host range cloning vector: a cosmid clone bank of an \textit{Agrobacterium} Ti plasmid. Plasmid 8:45-54, 1982


Masterson RV, Atherly AG: The presence of repeated DNA sequences and a partial restriction map of the \textit{pSym} of \textit{Rhizobium fredii} USDA193. Plasmid (in press), 1986


Peralta EG, Ream LW: T-DNA border sequences required for
crown gall tumorigenesis. Proc Natl Acad Sci USA 82:5112-5116, 1985


Ramakrishnan N, Prakash RK, Shantharam S, Duteau NM, Atherly AG: Molecular cloning and expression of R. fredii


Wang K, Herrera-Estrella L, Van Montagu M, Zambrisky P: Right 25 bp terminus sequence of the nopaline T-DNA is
essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. Cell 38:455-462, 1984


SECTION II. MOLECULAR CLONING AND EXPRESSION OF RHIZOBIUM FREDII USDA193 NODULATION GENES: EXTENSION OF HOST RANGE FOR NODULATION
Molecular cloning and expression of *Rhizobium fredii* USDA193 nodulation genes: Extension of host range for nodulation

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DNA hybridization using the cloned nodulation region of *R. meliloti* as probe revealed DNA homology with four *Hind*III fragments, 12.5 kb, 6.8 kb, 5.2 kb, and 0.3 kb in size, of the symbiotic plasmid pRjaUSDA193. Both hybridization and complementation studies suggest that the "common" nodulation genes, *nod* ABC and *nod* D genes, of *R. fredii* strain USDA193 are present on a 5.2 kb *Hind*III fragment and a 2.8 kb *Eco*RI fragment, respectively, of the *Sym* plasmid. Both fragments together could confer nodulation ability on soybeans when present in *Sym* plasmid-cured (*Sym") and wild type (*Sym*) *Rhizobium* strains or in Ti-plasmid-cured *Agrobacterium tumefaciens* strain. Furthermore, the 2.8 kb *Eco*RI fragment alone, with only *nod* D defined, was able to form nodule-like structures on soybean cv. "Peking". Microscopic examination of these nodules revealed bacterial invasion of the cells, probably, via root-hair penetration. None of the bacteria was surrounded by a peribacteroid membrane envelope. Bacterial strains harboring plasmids carrying the 5.2 kb and 2.8 kb *nod* fragments elicited root hair curling response on infection. These data suggest that genes responsible for host range determination and some of the early events of
nodulation may be coded by the 5.2 kb HindIII and 2.8 kb EcoRI fragments.
INTRODUCTION

Bacteria of the genus *Rhizobium* form symbiotic associations with leguminous plants and fix nitrogen in specialized structures called root nodules. This is a complex, multistage process involving induction of root-hair deformation (curling and/or branching), infection of root hairs, development and differentiation of root nodules, proliferation of bacteria within the host cells and finally the reduction of molecular nitrogen into ammonia (Bauer, 1981; Verma and Long, 1983; Vincent, 1980). Recently, a new group of fast-growing rhizobia that nodulate soybeans was isolated from mainland China (Keyser et al., 1982). These fast-growing soybean strains, previously known as *R. japonicum* have been redesignated as *R. fredii* (Scholla and Elkan, 1984). Studies of the nodulation process in fast-growing *Rhizobium* strains have demonstrated the presence of a series of genetic loci coding for nodulation (*nod*) and nitrogenase (*nif*) genes, on a very large symbiotic (*pSym*) plasmid (Banfalvi et al., 1981; Downie et al., 1983; Kondorosi et al., 1984; Long et al., 1982; Rosenberg et al., 1981; Schofield et al., 1983). The *nod* and *nif* genes of *R. fredii* strains have also been shown to be present on a large
plasmid (Masterson et al., 1982; Prakash and Atherly, 1984). The strain USDA194 is an exception, where the symbiotic genes are present either on a megaplasmid or on the chromosome (Masterson et al., 1985). R. fredii strain USDA193, used in this study, nodulates a highly inbred soybean cultivar "Peking" effectively and the North American cultivars of soybean ineffectively (Keyser et al., 1982).

Long et al. (1982) cloned a region of the symbiotic plasmid of R. meliloti 1021 (pRmSL26), which complemented two nodulation deficient mutants of R. meliloti. Transposon mutagenesis and DNA sequence analysis (Egelhoff et al., 1985; Jacobs et al., 1985) revealed a cluster of four nod genes, nod ABC and nod D, located on this fragment, with nod D read divergently from nod ABC. Mutations in nod A, -B, and -C genes failed to induce root hair curling (Hac\(^{-}\)) as well as nodule formation (Nod\(^{-}\)) while nod D mutation resulted in a leaky phenotype (Jacobs et al., 1985). These four nod genes referred to as "common" nod genes (Kondorosi et al., 1984) are functionally conserved in other Rhizobium species (Fisher et al., 1985). A 3.5 kb EcoRI-BamHI restriction fragment of pRmSL26 coding for the "common" nod genes was shown to hybridize to three EcoRI fragments; 11.3 kb, 5.3 kb, and 2.8 kb, respectively, of the symbiotic plasmid of R.
F. fredii strains USDA193 (Prakash and Atherly, 1984). The 5.3 kb EcoRI fragment of USDA193 and USDA205 contained a reiteration of the sequences present in the 2.8 kb EcoRI fragment (Prakash and Atherly, 1984).

It has been reported that modulation genes of Rhizobium species are expressed in A. tumefaciens and E. coli forming pseudonodules (Hirsch et al., 1984). However, in most cases, the nodule structures were ineffective and completely devoid of bacteria (Hirsch et al., 1984). Recently, Hirsch et al. (1985) reported nodule induction on alfalfa roots by A. tumefaciens and R. trifolii containing small segments of R. meliloti common modulation region. The transconjugants were Hac" and Inf" (infection thread) and bacteria were found only in the intercellular spaces. Expression of a 14 kb nod fragment of R. trifolii pSym plasmid in Lignobacter and Pseudomonas strains has also been reported (Plazinsky and Rolfe, 1985). The nodules initiated by Lignobacter transconjugants showed bacterial release into host cytoplasm. However, Pseudomonas transconjugants formed nodule-like structures, with few bacteria located within the intercellular spaces (Plazinsky and Rolfe, 1985).

In this study, we examined the genes encoded on R. fredii DNA homologous to the "common" nod region of R. meliloti 1021 by hybridization studies as well as by
functional complementation of R. meliloti mutants (Jacobs et al., 1985; Long et al., 1982). We also studied the infection process and the internal cytology of the nodules induced by the nod fragments in both homologous and heterologous backgrounds. Furthermore, we extended the host-range of different fast-growing rhizobia by transferring the nod region of R. fredii USDA193 into these strains. We report for the first time that the nod D gene carrying fragment can function independent of nod ABC genes to induce the formation of nodule-like structures.
MATERIALS AND METHODS

Plasmids and bacterial strains  The plasmids and bacterial strains used in this study are listed in Table 1.

Isolation of plasmid DNA  Plasmid DNA from \textit{R. fredii} strain was isolated by the method of Hirsch et al. (1980) and further purified as described by Masterson et al. (1985). Plasmid DNA from \textit{E. coli} strains was isolated by the rapid boiling method of Holmes and Quigley (1981). The DNA was further purified by repeated phenol extraction followed by extraction with chloroform:isoamyl alcohol (24:1) mixture. The DNA was precipitated with two volumes of ethanol at $-70^{\circ}C$ and dissolved in low TE buffer (10 mm Tris, 1 mm EDTA, pH 8.0).

Southern hybridization  Restriction enzyme digested DNA was separated on a 0.7% agarose gel by electrophoresis and transferred to Gene Screen as described in New England Nuclear Catalogue No. NEF 972. The plasmid DNA used as probe was labelled with $^{32}P$-dCTP by nick translation reaction (BRL Catalogue No. 8160 SB). Hybridization conditions were as described by Prakash and Atherly (1984).

Cosmid cloning  A cosmid clone bank of \textit{R. fredii} plasmid pRjaUSDA193 from strain USDA193 was constructed by
Table 1  Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. fredii USDA193</td>
<td>Wild type, Nod(^+), Nif(^+) on Peking and Nod(^+), Nif(^-) on North American cultivars</td>
<td>H. Keyser, USDA, Beltsville, MD.</td>
</tr>
<tr>
<td>IA728(pPA1)</td>
<td>Rif(^\text{R}), Tet(^\text{R})</td>
<td>This study.</td>
</tr>
<tr>
<td>IA728(pPA2)</td>
<td>, , ,</td>
<td>, , ,</td>
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<tr>
<td>IA728(pPA3)</td>
<td>, , ,</td>
<td>, , ,</td>
</tr>
<tr>
<td>IA47(pPA1)</td>
<td>Str(^\text{R}), Tet(^\text{R})</td>
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</tr>
<tr>
<td>IA47(pPA3)</td>
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</table>

\(^a\)Abbreviations: Nod\(^+\), nodulation; Nif\(^+\), fixation; Rif, rifampicin (200 μg/ml); Kan, kanamycin (100 μg/ml); Str, streptomycin (100 μg/ml); Amp, ampicillin (50 μg/ml); Tet, tetracycline (15 μg/ml); Nm, neomycin (100 μg/ml).
Table 1 (Continued)

<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Characteristics</th>
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<tr>
<td><strong>A. tumefaciens</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A136</td>
<td>Rif$^R$ derivative of C58, cured of Ti plasmid</td>
<td>M. D. Chilton, Ceiba Geigy, NC.</td>
</tr>
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<td>A136(pPA1)</td>
<td>Rif$^R$, Tet$^R$</td>
<td>This study.</td>
</tr>
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<td>A136(pPA3)</td>
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<tr>
<td><strong>R. leguminosarum</strong></td>
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<tr>
<td>248c(pPA1)</td>
<td>Rif$^R$, Tet$^R$</td>
<td>This study.</td>
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<td><strong>R. phaseoli</strong></td>
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<td>8401(pPA1)</td>
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<td>This study.</td>
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<td><strong>R. trifolii</strong></td>
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<td>This study.</td>
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<td>LPR5045(pPA3)</td>
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<td>162P17</td>
<td>Wild type, Nod&lt;sup&gt;+&lt;/sup&gt;, Nif&lt;sup&gt;+&lt;/sup&gt; on red clover and white clover, Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Nitragin, Co.</td>
</tr>
<tr>
<td>162P17(pPA1)</td>
<td>Rif&lt;sup&gt;R&lt;/sup&gt;, Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study.</td>
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<td>162P17(pPA3)</td>
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<td>, ,</td>
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<tr>
<td>R. meliloti</td>
<td>nodA::Tn5,Kan&lt;sup&gt;R&lt;/sup&gt;, Str&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Jacobs et al., 1985.</td>
</tr>
<tr>
<td>TJIA3</td>
<td>, ,</td>
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<tr>
<td>TJ2B2</td>
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<tr>
<td>TJ170</td>
<td>nodCl::Tn5, , ,</td>
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<td>TJ8A2</td>
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<td>TJ9B8</td>
<td>nodD::Tn5, , ,</td>
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</table>

Plasmids

<p>| pPA5             | Derivative of pRmSL26, Amp&lt;sup&gt;R&lt;/sup&gt; | Prakash and Atherly, 1984. |
| pPA4             | Clone of 5.2 kb HindIII nod fragment of pSym193 in pBR322, Amp&lt;sup&gt;R&lt;/sup&gt; | This study. |</p>
<table>
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<tr>
<th>Strain or Plasmid</th>
<th>Characteristics</th>
<th>Source</th>
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<td>pNA28</td>
<td>Clone of 2.8kb EcoRI nod fragment of pSyml93 in pACYC184, Tet</td>
<td>This study.</td>
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<td>pPA1</td>
<td>pVK101 derivative carrying 2.8kb EcoRI fragment, Tet</td>
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<tr>
<td>pPA2</td>
<td>Cosmid construct of pVK101 carrying 5.2kb HindIII fragment, Tet</td>
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</tr>
<tr>
<td>pPA3</td>
<td>pVK101 derivative carrying 5.2kb HindIII and 2.8kb EcoRI fragments of pSyml93, Tet</td>
<td></td>
</tr>
<tr>
<td>pRK2013</td>
<td>rep ColEI, Mm(^R), Kan(^R)</td>
<td>Ditta et al., 1980.</td>
</tr>
</tbody>
</table>
partial digestion with HindIII to generate DNA fragments in the size range of 20-30 kb. The digested DNA was then ligated (according to BRL Corp, Bethesda, MD, instructions) with HindIII digested broad host-range vector pVK102 (Knauf and Nester, 1982). The DNA was packaged into phage lambda as described by Hohn (1979) and the mixture used to infect E. coli strain HB101. About 100 tetracycline-resistant and kanamycin-resistant colonies were isolated. These colonies were analyzed by colony hybridization using a nick-translated probe, the 3.5 kb BamHI-EcoRI fragment from the plasmid pPA5 (Prakash and Atherly, 1984). This fragment contains the "common" nod region of R. meliloti and was isolated from an agarose gel by electroelution (Yang et al., 1979). Plasmid DNA from the cosmid clones was isolated by the method of Holmes and Quigley (1981).

Conjugation and plant nodulation assays For bacterial matings, $10^8$-$10^9$ cells from the log-phase were mixed on 0.45 μm nitrocellulose filters on solid TY media and incubated at 30°C for 48 h. The colonies were resuspended in 3-5 ml sterile water. The transconjugants were selected for by plating on TY plates containing rifampicin and tetracycline (200 μg/ml and 15 μg/ml, respectively). For complementation of R. meliloti mutants,
the transconjugants were selected on minimal media containing kanamycin (100 ug/ml), streptomycin (100 ug/ml) and tetracycline (15 ug/ml).

The bacterial strains used for plant inoculation were grown overnight at 30°C. 10^9 cells were used to inoculate surface-sterilized soybean (Glycine max L., cv. "Peking") seeds grown in the dark for 48 h. The inoculated seedlings were then planted in sterile growth pouches to which nitrogen-free nutrient solution was added and watered every two days. After 5 weeks, plants were harvested and nodules removed, surface sterilized with 70% ethanol, squashed, resuspended in sterile bacteroid isolation buffer (0.25 M mannitol, 0.25 M sorbitol, 2 mM CaCl_2, 2 mM KH_2PO_4, 2 mM Tris and 1 ml of horse serum., pH 7.4) or in distilled water, and streaked on minimal media plates containing the appropriate antibiotics. The plasmid profiles of the recovered bacteria were checked by using a modified Eckhardt procedure (Rosenberg et al., 1981).

**Complementation studies** Cosmid clones in E. coli coding for nod ABC and nod D genes were transferred to R. meliloti mutants (Jacobs et al., 1985) using the helper pRK2013 as described above. Selected transconjugants were used to inoculate 2-day old seedlings of alfalfa grown in agar slants containing nitrogen-free nutrient solution. The
plants were scored for nodulation after 5 weeks of growth.

**Light and electron microscopy**  
Surface-sterilized seeds of *Glycine max.* L., cv "Peking" were germinated in the dark for 48 h and transferred to sterile growth pouches for inoculation and incubation in growth chamber as described by Shantharam and Wong (1982). Samples were prepared for phase-contrast microscopy according to Heron and Pueppke (1984) including the appropriate controls. A minimum of 25 hand-cut cross sections of main root hair zone from four seedlings were examined systematically under the microscope (Leitz Dialux 2000 photomicroscope fitted with phase contrast optics).

The nodules or nodule-like structures formed by IA728 (pPA1), IA728 (pPA3), A136 (pPA1) and A136 (pPA3) transconjugants on soybean plants in growth pouches were subjected to transmission electron-microscopy according to Bal et al. (1982). At least 15-20 serial sections of nodules were used in this study.
RESULTS

*Cosmid cloning of nodulation genes.* To isolate the *nod* genes from *R. fredii* USDA193, we constructed a cosmid clone bank of the Sym plasmid, pRjaUSDA193, using the broad-host-range cosmid vector pVK102 (Knauf and Nester, 1982). By colony hybridization, using a 3.5 kb *BamHI-EcoRI* *nod* fragment of *R. meliloti* as probe, cosmid clones carrying DNA sequences homologous to the "common" *nod* region were identified (Prakash and Atherly, 1984). Fig. 1 shows the HindIII restriction digestion of the plasmid pRjaUSDA193 and the different cosmid clones and subsequent hybridization to pPA5, the *R. meliloti* *nod* clone. Four HindIII fragments, 12.5 kb, 6.8 kb, 5.2 kb and 0.3 kb in size, showed homology to the "common" *nod* region of *R. meliloti*. The 5.2 kb and 0.3 kb HindIII fragments were present on the same cosmid clone. However, the 12.5 kb HindIII and the 6.8 kb HindIII fragments were present on different clones, suggesting that the *nod* genes of *R. fredii* strain USDA193 are unlinked on the *pSym* plasmid. As previously reported (Prakash and Atherly, 1984), the 12.5 kb HindIII fragment (which corresponds to the 5.3 kb *EcoRI* fragment) and the 6.8 kb HindIII fragment (which corresponds to the 2.8 kb *EcoRI* fragment...
Fig. 1. Nod hybridization profile of pRjaUSDA193

(A) HindIII digested cosmid clones and the plasmid DNA pRjaUSDA193 from R. fredii USDA193. Lane 9 shows the HindIII restriction pattern of pRjaUSDA193, and lanes 1-8 show DNA from cosmid clones containing pRjaUSDA193 inserts of various size. The presence of both the 12.5 kb and 5.2 kb HindIII fragments in the cosmid clone in lane 6 is due to rearrangement of fragments during ligation (Masterson et al. unpublished data).

(B) Hybridization of $^{32}$P-labeled pPA5 clone of R. meliloti nod probe to the Southern blot of (A). HindIII digested lambda DNA was used as size standard. The letter 'V' represents the vector DNA pVK102, which is 23 kb in size.
fragment) cross-hybridize with each other and therefore represent reiterated *nod* fragments. The 5.2 kb and 0.3 kb *HindIII* fragments (which correspond to the 11.3 kb *EcoRI* fragment) do not hybridize to any other region of the *pSym* plasmid. Since the 5.2 kb *HindIII* fragment together with the 2.8 kb *EcoRI* fragment represent a conserved nodulation region of *R. meliloti* strain, these fragments were subcloned separately and in combination into the cosmid vector pVK102 and used for further study (see Table 1).

**Complementation and hybridization analysis** To determine the location of the conserved nodulation genes within the 5.2 kb *HindIII* and 2.8 kb *EcoRI* fragments of USDA193, we digested pPA5 with different restriction enzymes to release *nod ABC* and *nod D* genes separately, in accordance with the map of the 8.7 kb *EcoRI* fragment of pRmSL26 by Fisher et al. (1985). The DNA was blotted onto Gene Screen as described in materials and methods. The 5.2 kb *HindIII* fragment subcloned into pBR322 and the 2.8 kb *EcoRI* fragment cloned into pACYC184 were used as probes for hybridization. As evident from the data in Fig. 2, the 5.2 kb *HindIII* fragment hybridized to the fragments coding for *nod ABC* genes, while the 2.8 kb *EcoRI* fragment hybridized to the *nod D* gene fragment. To confirm the hybridization data, we
Fig. 2. Hybridization pattern of $^{32}$P-labeled pPA4 (A) and pNA28 (B) clones, respectively, to blots of restriction enzyme digested DNA of the plasmid clone, pPA5 (carrying "common" nod genes of \textit{R. meliloti} 1021). Lane 1 shows the BglII-HindIII digestion pattern, releasing vector (along with most of nod D), nod ABC and nod C, respectively. Lane 2 shows the BglII-EcoRI digestion pattern, releasing vector (+nodD) and nod ABC fragments. Lane 3 represents the BglII-BamHI digestion pattern, releasing nod D gene from the plasmid clone.
functionally complemented different $\text{Nod}^{-}$ mutants of $R$. meliloti 1021 with the cosmid clones pPA1 and pPA3 (Table 1). The former could complement mutations only in $\text{nod} \text{D}$ gene while the latter clone complemented all the mutations tested. Thus, the hybridization data and complementation data taken together, strongly suggest that the 2.8 kb EcoRI fragment codes for $\text{nod} \text{D}$ gene and that the 5.2 kb HindIII fragment codes for $\text{nod} \text{ABC}$ genes.

**Analysis of nod gene fragments of $R$. fredii USDA193 for nodulation ability**

We examined the nodulation ability of the nod fragments of $R$. fredii USDA193 by conjugal transfer of the different cosmid clones, pPA1, pPA2 and, pPA3, into pSym-cured $R$. fredii strain USDA193 (IA728) and also into Ti plasmid-cured strain of $A$. tumefaciens (A136) followed by inoculation of soybeans as described in materials and methods. We observed that pPA1 and pPA3 could confer nodulation ability to the plasmid-cured strains tested. We also transferred the two cosmid clones pPA1 and pPA3 into a deletion derivative of $R$. fredii USDA191 (IA47) and tested the transconjugants for nodulation ability. Results are presented in Table 2. In all instances very few nodules appeared (about 5/plant), were delayed in appearance (3-4 weeks post inoculation), medium in size and deformed in
shape when compared to the wild type nodules which normally appear in less than 10 days. The nodules were harvested after 4-5 weeks. From all the nodules induced by either pPA1 or pPA3, we were able to reisolate bacteria by growth on minimal media plates containing tetracycline (15 ug/ml). Tetracycline resistant colonies were further checked for rifampicin resistance to avoid the possibility of bacterial contamination.

To study the effect of sequences flanking the "common" nodulation genes on the nodulation ability of various strains carrying these regions on a plasmid, we transferred the cosmid clone pMA106 (6.8 kb HindIII fragment of R. fredii USDA193 and flanking sequences cloned in the cosmid pVK102) into plasmid-cured strains, R. fredii IA728 and A. tumefaciens A136, respectively, and tested them on soybean plants "Peking" (Masterson and Atherly, submitted). Surprisingly, no nodules were formed even after 5 weeks of growth post-inoculation.

**Root hair curling induction by cloned fragments**

Light microscopic examination of 5 days, 10 days and 15 days old root hairs of infected soybean cv. "Peking" revealed that the 5.2 kb HindIII fragment or the 2.8 kb EcoRI fragment alone could elicit the root hair curling response
Fig. 3. Light micrographs of soybean root hairs

(a) Marked root hair tip curling of soybean "Peking" leading to infection thread formation due to the inoculation of _Rhizobium fredii_ USDA193 (arrows indicate infection thread).

(b) Root hairs of soybeans which are uninoculated.

(c) Attachment of _Rhizobium fredii_ IA728 to the tip of soybean root hair.

(d) Marked root hair tip curling of soybean root hair due to the inoculation of IA728 (pPA1).

(e) Root hair tip curling and infection thread formation in soybean due to IA728 (pPA2).

(f) Root hair tip curling of soybean by IA728 (pPA3).
(Fig. 3d and e). *R. fredii* strain IA728 (pPA1) caused root hair curling and hypertrophy of root hairs (Fig. 3d). Bonafide infection threads were not observed. However, the transconjugant could initiate nodule-like structures after 3 weeks. Strain IA728 (pPA2) did not give rise to nodules but could still cause root hair tip curling and infection thread formation (Fig. 3e). Strain IA728 (pPA3) formed late nodules on "Peking" and caused both root hair tip curling and infection thread formation (Fig. 3f). *R. fredii* strain IA728 was included as a control. With this strain, we observed bacterial attachment to the root hair tip without curling, infection thread formation or nodule formation (Fig. 3b). Table 2 summarizes the symbiotic properties of the various transconjugants.

**Ultrastructure of soybean root nodules formed by nod fragments of *R. fredii* USDA193 in *R. fredii* and *Agrobacterium tumefaciens* background**

Wild type root nodule tissue of soybean formed by *R. fredii* strain USDA193 was examined under the light microscope as a reference standard. Fig. 4a shows its internal structure with densely infected cortical cells and a distinct host-cell nucleus. In contrast, the root nodules formed by IA728 (pPA1) and A136 (pPA1) transconjugants showed few infected cells and
Fig. 4. Ultrastructure of soybean root nodule

(a) Cross-section of the wild type root nodule of soybean caused by *R. fredii* USDA193.

(b) Electron micrograph of an ultra-thin section of soybean root nodule induced by IA728 (pPA1). The internal membranes have been broken down to form small vesicles (arrows). The few bacteria present, are not surrounded by peribacteroid membrane envelope and they are all localized in the peripheral cells of the cortical tissue which show cell wall thickening.

(c) Electron micrograph of soybean root nodule induced by IA728 (pPA3) in cross-section. Note the absence of peribacteroid membrane envelope and the presence of extensive rough endoplasmic reticulum.

(d) Light micrograph of the same nodule as in (c) in cross-section.

(e) Light micrograph of a cross-section of soybean root nodule formed by A136 (pPA3). Note very few bacteria invading the peripheral cells of the root nodule.

b-bacteria; cw-cell wall; m-mitochondria; n-nucleus; pc-peripheral cells; rer-rough endoplasmic reticulum; th-cell wall thickenings.
these were limited to the peripheral regions of the root nodule (Fig. 4b). Each infected cell contained a limited number of bacteria (about 4-6 bacteria/cell in a section). The bacteria were not surrounded by the peribacteroid membrane envelope (PME). The cytoplasmic matrix was disintegrated with numerous membranous vesicles dispersed inside the infected cells. The infected cells also showed thickening of the cell wall. No other cytoplasmic organelles were discernible.

As shown in Fig. 4c and 4d, the nodules formed by strain IA728 (pPA3) showed numerous infected cells with relatively few bacteria per cell. The nodule, at the ultrastructural level, showed bacteria in defined cavities but not surrounded by PME. The structural integrity of mitochondria was not well maintained. The cytoplasm showed whorls of endoplasmic reticulum (RE) and numerous polysomes. The root nodules formed by the transconjugant A136 (pPA3) showed internal cytology similar to that formed by IA728 (pPA1) (Fig. 4e) and, hence, not shown.

Extension of host-range for nodulation Since the
5.2 kb HindIII fragment in combination with the 2.8 kb EcoRI
fragment or the 2.8 kb EcoRI fragment alone, could confer
nodulation ability to pSym cured strains of R. fredii and Ti
Fig. 5. Soybean root nodules formed by wild type *R. fredii* strain USDA193 and the transconjugants, LPR5045 (pPA3) and 162P17 (pPA3), respectively, on cultivar "Peking"
Table 2  Symbiotic properties of the various transconjugants

<table>
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<tr>
<th>Bacterial strain</th>
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<th>Fix</th>
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<th>Hac</th>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>+</td>
<td>+</td>
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<td>-</td>
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<td>-</td>
<td>+</td>
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<tr>
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*Abbreviations: Nod, nodule formation; Fix, fixation; Roa, root adhesion; Hac, hair curling; Inf, infection thread formation.*
### Table 2 (Continued)

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plasmid-cured strain of Agrobacterium, it was of interest to see if the host range for nodulation could be extended by these nod fragments. For this purpose, we transferred the two cosmid clones (pPA1 and pPA3) into Sym plasmid-cured strains of R. leguminosarum, R. phaseoli and R. trifolii. The transconjugants were tested for their ability to nodulate cultivar "Peking". As presented in Table 2, the two cosmid constructs could confer nodulation ability to the pSym-cured strains. However, the frequency of nodulation was low when compared with that in R. fredii or Agrobacterium background (2-3 nodules/plant vs 5-6 nodules/plant). The nodules were of medium size and had a tumorous appearance and they appeared 3-4 weeks post inoculation. Occasionally, swellings of the roots were also observed. These "bumps" also contained bacteria. To rule out the possibility of artifacts, strains IA728 (pPA1) and IA728 (pPA3) were used to inoculate alfalfa seedlings (data not shown). The negative results further supported the fact that the genes carried on these fragments are specific for soybeans.

The expression of R. fredii nod genes was determined in the wild type, heterologous strain, by transferring the cosmid clones pPA1 and pPA3 to wild type R. trifolii 162P17.
These transconjugants, in turn, were used to infect soybean plants. We also tested *R. meliloti* transconjugants TJ9B8 (pPA1) and TJ9B8 (pPA3), used for the functional complementation study, for their ability to nodulate "Peking" soybean cultivar. Nodules occurred at a low frequency (2-3/plant), indicating that the genes carried on the two nod fragments of *R. fredii* strain USDA193 could indeed be expressed in a heterologous background even in the presence of wild type genes (The *R. meliloti* transconjugants were functionally equivalent to wild type strain, because the Tn5 mutations present in the genome could be functionally complemented by *R. fredii* nod fragments). In all the cases, bacteria could be reisolated from the nodules successfully for identification.
DISCUSSION

Nodulation genes in several fast-growing Rhizobium species have been shown to be clustered within a small region of plasmid DNA (Hombrecher et al., 1984; Kondorosi et al., 1984; Long et al., 1982; Schofield et al., 1984). However, our data suggest that the "common" nodulation genes of R. fredii strain USDA193 are not closely linked on the plasmid DNA. Although the precise location of these genes on the plasmid DNA is not certain, preliminary data suggest that nod D may be separated from nod ABC by about 65 kb (Masterson and Atherly, 1986). The 5.2 kb HindIII fragment and the 2.8 kb EcoRI fragment, homologous to the "common" nodulation region of R. meliloti bacteria, were found to carry the nod ABC and nod D genes, respectively.

The results presented show that cloned nodulation region of R. fredii strain USDA193 could confer nodulation ability to different pSym-cured strains. The few infected cells and the low level of bacterial population within each cell reflect a rather poor symbiosis. However, our experiments prove that short stretches of DNA could encode sufficient information necessary for some of the early events of infection. An unusual finding was that the 2.8 kb
EcoRI fragment, with only nod D identified, could elicit nodule formation. These nodules were nearly devoid of bacteria, except for the peripheral cells. There were no bacteria in the interior cells. The nodules formed by strain A136 (pPA3) transconjugant also showed a similar morphology. However, strain IA728 (pPA3) transconjugant could successfully invade the interior cells of the nodule tissue. Recently, it was reported that the "common" nodulation region of R. meliloti could induce nodulation of alfalfa (Hirsch et al., 1985). Bacteria were found only in the intercellular spaces and no root hair curling or infection thread were observed (Hirsch et al., 1985). Our results are also in agreement with their observations that bacterial attachment, root hair curling, infection thread formation, nodule morphogenesis and bacterial release might be independent events. The 5.2 kb HindIII fragment carrying nod ABC genes could elicit a root hair curling response, but failed to initiate nodulation. On the other hand, the 2.8 kb EcoRI fragment carrying nod D gene could elicit both functions with eventual bacterial release into the cells.

The functionally conserved "common" nodulation genes, nod ABC and nod D, seem to be responsible for the root hair curling phenomenon (Djordjevic et al., 1985; Downie et al., 1983; Hombrecher et al., 1984; Rossen et al., 1984).
Because both the 5.2 kb **HindIII** fragment and the 2.8 kb **EcoRI** fragment could elicit root hair curling response when individually present, it follows that the genes encoded on these two fragments can cause Hac\(^+\) phenotype, independent of each other. Since the two **nod** fragments do not cross-hybridize with each other, it is very unlikely that they contain similar genetic regions responsible for Hac\(^+\) phenotype.

As evident from Fig. 3, **R. fredii** strain IA728 (cured of its **Sym** plasmid) alone could attach to the host cell wall, indicating that bacterial chromosome plays an important role in the very early steps of Rhizobium-legume symbiosis. Results presented in Table 2 shows that this is a general phenomenon. A recent report proposed a role for 2-linked-B-D-glucans in the attachment of **A. tumefaciens** to plant cells (Puvanesarajah et al., 1985). The presence of 2-linked-B-D-glucans in **Rhizobium** has also been reported (Delf et al., 1983; Zvenhuizen et al., 1979). Microscopic data regarding the structure of nodules induced by both **nod** fragments (5.2 kb **HindIII** and 2.8 kb **EcoRI** in **R. fredii** as well as in **A. tumefaciens** background indicate that the chromosomal DNA may also be involved in the bacterial invasion of host cells. The **nod** fragments in homologous background could successfully invade the interior cells of
the root cortex (although at a low frequency), but when present in Agrobacterium background, the few bacteria observed were all found restricted to the peripheral cells. On the other hand, the transconjugant IA728 (pPA1) could not invade the interior cells. Hence, both chromosomal and plasmid encoded functions may be involved in the successful invasion of host cell by bacteria.

Soybeans inoculated with R. fredii or A. tumefaciens transconjugants developed nodules with intracellular bacteria, although no bonafide infection thread was visible. However, we did see infection thread-like structures on some root hairs after infection with IA728 (pPA3) and IA728 (pPA2) strains. Hence, the data argue for the usual mechanism of root hair penetration (for IA728 (pPa2) and IA728 (pPA3) strains, rather than for an intercellular or 'crack-entry' mode of invasion.

The nodules induced by all transconjugants cured of their pSym plasmids, were Fix". All were comparatively small, white inside and had a tumorous-like appearance. However, the nodules formed by R. trifolii (strain 162P17 with either PA1 or pPA3; see Table 1 and 2) transconjugants were pink inside. They all appeared between three and four weeks after inoculation. Compared to the wild type, the infected cells were very few. All the bacteria within the
nodules formed by reconstructed strains were devoid of a peribacteroid membrane envelope. The cytoplasm showed marked signs of degeneration. In spite of this, we could reisolate bacteria from all the nodules tested, and they still contained the same antibiotic resistance markers. Although strains carrying pPA1 cosmid could nodulate soybean, our results indicate that the presence of sequences adjacent to the 2.8 kb nod fragment, as in strain IA728 (pMA106), masks the nodulation ability of the 2.8 kb fragment.

The expression of R. fredii USDA193 pSym genes in other Rhizobium strains and in Agrobacterium tumefaciens to form ineffective nodules on soybean suggests that genetic information for host recognition may be contained within the two nod fragments of pSym193. Seemingly, the "common" nodulation genes of R. fredii USDA193 are responsible for inducing nodule initiation (Downie et al., 1983; Hirsch et al., 1985; Hirsch et al., 1984; Plazinski and Rolfe, 1985; Schofield et al., 1984). In R. leguminosarum strain TOM, a 2.0 kb DNA region closely linked to the genes required for root hair curling was reported to be responsible for the extension of host range of R. leguminosarum strains which normally do not nodulate cv. Afghanistan (Gotz et al., 1985). Our results further show that nod D gene carrying
fragment of USDA193, in the absence of **nod** ABC genes, could still initiate formation of nodule-like structures as well as extend the host range of other rhizobia to include soy beans as their host. The **nod** ABC genes may be involved in a later step or may act coordinately via a different pathway. The exact steps and factors involved in the nodulation process are yet to be defined. Since **nod** D gene product is widely believed to act as a regulatory factor (Egelhoff and Long, 1985; Rossen et al., 1985), it seems reasonable to suppose that the nodulation of soybean by pPAI transconjugants involves more than **nod** D gene. Presently, we are analyzing the 2.8 kb **EcoRI** fragment for possible functional genes, other than **nod** D gene.
ACKNOWLEDGEMENTS

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We thank Sharon Long for plasmid and bacterial strains and Dr. R. V. Masterson for critically reviewing the manuscript.
REFERENCES


Heron DS, Puappke SG: Mode of infection, nodulation


Hombrecher G, Gotz R, Dibb NJ, Downie JA, Johnston AWB, Brewin NJ: Cloning and mutagenesis of nodulation genes
from *Rhizobium leguminosarum* TOM, a strain with extended host range. Mol Gen Genet 194:293-298, 1984


Knauf VC, Nester EW: Wide host range cloning vectors: a cosmid clone bank of an *Agrobacterium* Ti plasmid. Plasmid 8:45-54, 1982


Masterson RV, Russel PR, Atherly AG: Nitrogen fixation
(nif) genes and large plasmids of *Rhizobium japonicum*. J Bacteriol 152:928-931, 1982


Rossen L, Shearman CA, Johnston AWB, Downie JA: The *nod D* gene of *Rhizobium leguminosarum* is autoregulatory and in the presence of plant exudate induces the *nod A, B, C* genes. EMBO J 4:3369-3373, 1985

Schofield PR, Djordjevic MA, Rolfe BG, Shine J, Watson JM:


Zenvenhuizen LPTM, Scholten-Koerselman HJ: Surface
carbohydrates of Rhizobium. I. B-1,2-Glucan. Antonie van Leeuwenhoek J Microbiol Serol 45:165-175, 1979
SECTION III. TRANSPOSON AND INSERTIONAL MUTAGENESIS OF THE NODULATION GENES OF RHIZOBİUM FREDII USDA193: IDENTIFICATION OF HOST SPECIFICITY GENE FOR NODULATION OF SOYBEAN
Identification of host specific gene for nodulation of soybean in *Rhizobium fredii* strain USDA193

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Department of Genetics
Iowa State University
Ames, Iowa.

ABSTRACT

Transposon mutagenesis studies of a 2.8 kb EcoRI fragment carrying the host specific nodulation genes of R. fredii strain USDA193 have identified two regions necessary for nodulation. One region codes for one of the "common" nod genes, nodD, and the second region codes for a new functional gene, nodN. Both functions have been shown to be essential for conferring soybean nodulation to pSym-cured strain of USDA193. Hybridization studies showed that the nodN sequences are conserved in different R. fredii strains.
INTRODUCTION

The Rhizobium-legume symbiosis is a multi-step process by which the bacteria invade the host cell wall and form specialized structures called root nodules in which they reduce the atmospheric nitrogen to ammonia (for a recent review see Verma and Long, 1983; Vincent, 1980). A novel group of fast-growing rhizobia that nodulate soybeans was isolated from People's Republic of China (Keyser et al., 1982) originally grouped together with the slow-growers as Rhizobium japonicum and recently redesignated as Rhizobium fredii (Scholla and Elkan, 1984). Most of the R. fredii strains fix nitrogen (Fix\(^+\)) on the genetically unimproved Chinese cultivar Peking but form Fix\(^-\) nodules on commercial North American cultivars. The genes involved in nodulation and nitrogen fixation have been located on large plasmids (pSym) in these strains (Masterson et al., 1985). USDA194 is an exception since sequences homologous to nod and nif genes of R. meliloti could not be located on the plasmid DNA (Masterson et al., 1985).

DNA hybridization and functional complementation studies have revealed that several genes such as nodABC genes involved in root hair curling and nodD involved in the
regulation of nod gene operons are highly conserved in
different Rhizobium species. These genes have been
designated as "common" nod genes (Kondorosi et al., 1984).
The genes involved in nodulation have mostly been identified
by transposon mutagenesis (Long et al., 1982; Djordjevic et
al., 1985a and b; Downie et al., 1983; Kondorosi et al.,
1984; Schofield et al., 1984). These genes have been
subcloned and subsequently transferred to different
recipient strains to identify the regions involved in
nodulation and host specific recognition (Downie et al.,
1983; Schofield et al., 1984). Such studies showed that the
DNA sequences involved in host specificity and nodulation
may be contained within a short region of about 10-14 kb.
Further analysis have identified a cluster of host specific
genes nodEFGH in R. meliloti (Rostas et al., 1986) and nodEF
and nodIJ in R. leguminosarum (Shearman et al., 1986). In
R. leguminosarum, nodD and a plant factor are required for
the induction of nodFE operon (Shearman et al., 1986).

We have previously reported the identification of nodA,
-B, -C and -D genes on two unlinked DNA fragments present on
the Sym plasmid, pRjaUSDA193 (Ramakrishnan et al., 1986b).
We also reported that the 2.8 kb EcoRI fragment carrying
nodD gene sequences could confer the ability to nodulate
soybeans to heterologous rhizobia such as R. leguminosarum,
R. phaseoli, R. trifolii and R. meliloti and also to Ti plasmid-cured strain of A. tumefaciens in the absence of nodA, -B and -C genes, suggesting that genes involved in host specificity and nodulation may be contained within the 2.8 kb EcoRI fragment. Appelbaum et al. (1985) has also reported the presence of nodD gene in the 2.8 kb EcoRI fragment of the Sym plasmid of R. fredii strain USDA191 which shows a similar restriction profile for the "common" nod gene carrying fragments (Masterson et al., 1985). By sequencing about 321 bp of nodD gene around the BamHI restriction site of the 2.8 kb EcoRI fragment, 80% homology between the predicted nodD gene polypeptides from USDA191 and R. meliloti 1021 was reported (Appelbaum et al., 1985). Since nodD gene product is widely believed to act as a regulator of nod gene expression (Innes et al., 1985; Mulligan and Long, 1985; Rossen et al., 1985; Shearman et al., 1986), it was of great interest to analyze further the 2.8 kb EcoRI fragment by site-specific transposon and in vitro insertional mutagenesis for other functional regions. Here, we report the identification of a region outside nodD gene sequences involved in soybean nodulation.
MATERIALS AND METHODS

Bacterial strains and plasmids The bacterial strains and plasmids used are listed in Table 1. R. fredii strains were routinely grown on TY medium (Beringer, 1974) and selected after conjugal matings on minimal media (0.6 g/l K$_2$HPO$_4$, 1 g/l D-glutamic acid, 1 g/l L-malic acid, 1 g/l ammonium sulfate, 15 g/l bacto-agar, adjust the pH to 5.95 and, after autoclaving add, 0.1% B-glycerophosphate, 0.001M CaCl$_2$ and 0.5 mM MgSO$_4$) containing appropriate antibiotics. E. coli strains were grown on LB medium (Beringer, 1974).

DNA isolation and manipulations Restriction enzymes and DNA ligase were purchased from New England Biolabs. Plasmid DNA isolation from E. coli and restriction digestions were as described by Maniatis et al. (1982). Total DNA from Rhizobium were isolated as described earlier (Ramakrishnan et al., 1986a).

Southern hybridization DNA hybridizations were carried out using Gene screen (New England Nuclear) as described earlier (Ramakrishnan et al., 1986a).

Tn5 mutagenesis For site specific Tn5 mutagenesis, we transformed the E. coli strain S605 (HB101 strain carrying Tn5 in the chromosome) with the plasmid pPA68, a
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<td>pSym cured derivative of USDA193, Nod⁻, Nif⁻, Rif⁺</td>
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<td>Rif⁺, Tet⁺</td>
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Abbreviations: Nod⁺, nodulation; Fix⁺, fixation; Rif, rifampicin (200 ug/ml); Km, kanamycin (100 ug/ml); Str, streptomycin (100 ug/ml); Spc, spectinomycin (100 ug/ml); Tet, tetracycline (15 ug/ml); Amp, ampicillin (50 ug/ml); Nm, neomycin (100 ug/ml).
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<td>pRK2013</td>
<td>rep CoLE1, Nmr^R, Km^R</td>
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pBR322 derivative carrying a 6.8 kb *HindIII* nod fragment of the plasmid pRjaUSDA193. The 6.8 kb insert carries the 2.8 kb *EcoRI* fragment as an internal fragment. The 2.8::Tn5 insertions obtained were subsequently cloned into the *EcoRI* site of the vectors, pACYC184, pBR322 and also into the broad-host range vector pVK101 (Knauf and Nester, 1982).

**Restriction mapping** To map the Tn5 insertions, the pACYC184 clones carrying the different Tn5 insertions were digested with *BamHI*, *SalI*, *EcoRI* and *HindIII* restriction enzymes and compared with the known restriction map of Tn5 (Jorgensen et al., 1979; Auerswald et al., 1981; Bruijn and Lupski, 1984).

**Insertional mutagenesis using "omega" fragment** The plasmid clone pPA1 carrying the 2.8 kb *EcoRI* fragment was digested with *BamHI* and ligated to *BamHI* digested pH45 plasmid DNA at 15°C overnight followed by transformation of HB101 strain (Prentki and Krisch, 1984). The transformants were screened for "omega" fragment insertion into the 2.8 kb fragment by plating on LB media containing tetracycline (15μg/ml), kanamycin (50μg/ml), streptomycin (50μg/ml) and spectinomycin (50μg/ml). The plasmid DNA was isolated by the rapid boiling method of Holmes and Quigley (1981). The *BamHI* digested DNA was run on a 0.7 % agarose gel to verify
insertion. The pVK101 (2.8:omega) clone was designated as pNA2DI.

**Bacterial matings** Rhizobia were grown in TY media at 30°C. All *E. coli* strains were grown in LB media at 37°C. pRK2013 was used as the helper strain for triparental mating (Ditta et al., 1980). $10^8$-$10^9$ cells in the log-phase were mixed on 0.45um nitrocellulose filters on TY plates and incubated at 30°C for 48 h. The bacteria were resuspended in 3 ml sterile water and the transconjunctants were selected on minimal media plates containing appropriate antibiotics. Tn5 insertions were scored for resistance to kanamycin (100 ug/ml), streptomycin (100 ug/ml) and neomycin (100 ug/ml).

**Nodulation tests** The bacterial cultures for inoculation of soybean cv., "Peking" were grown overnight in TY medium at 30°C. $10^9$ cells were resuspended in sterile water and used to inoculate surface-sterilized soybean (*Glycine max* L., cv. "Peking") seeds germinated in the dark for 48 h. The inoculated seedlings were grown in sterile growth pouches as previously described (Ramakrishnan et al., 1986b). The plants were scored for nodulation phenotype for a period of 5 weeks. Bacteria were isolated from the nodules and streaked on minimal media plates containing appropriate antibiotics. The plasmid profiles of the
isolated bacteria were verified by a modified Eckhardt procedure (Rosenberg et al., 1981).

**Root hair curling assay** Light microscopic examination of cross-sections of the main root hair zone from soybean cv. "Peking" seedlings inoculated with the strain IAN3 were carried out as described previously (Ramakrishnan et al., 1986b).
Restriction mapping of the 2.8 kb nod fragment  The 2.8 kb nod fragment cloned into the plasmid vector pACYC184 was digested with the restriction enzymes BamHI and SalI. Fig. 1 shows the restriction map of the 2.8 kb nod fragment.

Tn5 mutagenesis of the nod fragment  Transposon mutagenesis was carried out as described in materials and methods. Mutational hotspots were very rare in the 2.8 kb EcoRI fragment. The pBR322 derivative carrying the 6.8 kb HindIII fragment was used for Tn5 mutagenesis. Tn5 insertions within the 2.8 kb EcoRI fragment were cloned into the vector pACYC184. Fig. 2 shows the hybridization pattern of EcoRI digested plasmid clones using 32P-labeled 2.8 kb fragment. Using different restriction enzymes for digestion of the plasmid clones, all the insertions were precisely mapped. Fig. 1 shows the locations of the different insertions.

Localization of the nodD gene in the 2.8 kb EcoRI fragment  We have previously reported the identification of nodD gene in the 2.8 kb EcoRI fragment by both hybridization and functional complementation studies.
Fig. 1. Restriction map of the 2.8 kb EcoRI fragment of
pRjaUSDA193 showing Tn5 and "omega" insertions.
The predicted location of nodD and nodN are also
shown. The nodulation phenotype on "Peking" after
conjugation of the mutated fragments into wild type
USDA193 and pSym-cured strain IA728 are also
indicated.
† - Transposon mutation generated by site-specific mutagenesis
\( \Psi \) - Q\(_2\)-insertional mutation generated by in-vitro mutagenesis
+ - Nod\( ^+ \)
+\( ^d \) - Nod-delayed
- - Nod

R, B, S - Cleavage sites for EcoRI, BamHI and SalI
Fig. 2. Hybridization of $^{32}\text{P}$-labeled 2.8 kb EcoRI fragment to EcoRI digested plasmid clones of pNA28::Tn5
Fig. 3. Localization of **nodD** gene

(A) Restriction enzyme digested DNA of the cosmid pNA28 (pACYC184 clone of 2.8 kb **EcoRI** fragment), carrying a Tn5 insertion to the left of **BamHI** site (see Fig. 1). Lane 1: **SalI**-**EcoRI** digestion; lane 2: **EcoRI** digestion. Lane 3 shows **HindIII** digested lambda DNA run as size standard.

(B) Hybridization of (A) using $^{32}$P-labeled 3.5 kb **BamHI- EcoRI nod** fragment of *R. meliloti*, carrying **nodDABC** genes.
(Ramakrishnan et al., 1986b). Also, Appelbaum et al. (1985) has reported the sequence of the nodD gene present on the 2.8 kb EcoRI fragment of the Sym plasmid of R. fredii strain USDA191, a strain which shows a similar nod and nif hybridization pattern (Masterson et al., 1985). The restriction map of the 2.8 kb EcoRI fragment from the strain USDA193 (see Fig. 1) also agrees with that of the corresponding fragment from the strain USDA191 (Appelbaum et al., 1985). To further confirm the location of nodD gene around the unique BamHI site in the 2.8 kb fragment, we used a 32p-labeled 3.5 kb BamHI-EcoRI fragment from the plasmid pPA5 (see Table 1) carrying the "common" nod genes of R. meliloti strain 1021 as probe, to hybridize to restriction enzyme digested pACYC184 clone of 2.8 kb EcoRI fragment with a Tn5 insertion in close proximity to the BamHI site of the insert (see Fig. 1). Fig. 3B shows the hybridization result. The probe carryind nodA, -B, -C and -D genes hybridized to the 8.5 kb EcoRI fragment (2.8 kb EcoRI fragment + Tn5) in lane 2 and to the fragments carrying sequences around the BamHI restriction site in lane 1. The probe did not hybridize to the extreme right region of the 2.8 kb EcoRI fragment (0.6-0.7 kb SalI-EcoRI fragment).
Conjugal transfer of the mutated fragments into wild type R. fredii strain  

To mobilize the mutated fragments into wild type USDA193 strain for homogenotization, we recloned all the insertions into the EcoRI site of the suicide vector pBR322. The insertions were verified by restriction digestions of the plasmid clones followed by southern hybridization (data not shown). The plasmid clones were mobilized into the RifR derivative of the wild type R. fredii strain USDA193, as described in materials and methods. Probably due to the small size of the fragment used for mutagenesis, double recombination events were very rare. However, we found that insertions in the right hand region of the 2.8 kb EcoRI fragment (SalI-EcoRI fragment) gave rise to a mutant strain (IAN3) showing a delayed nodulation phenotype. Fig. 4 compares the number of nodules formed by the mutant and wild type strains as a function of the number of days after inoculation. The inset in Fig. 5 (5b) shows the root nodule formed by the strain IAN3.

Root hair curling assay  

Light microscopic examination of the root tips of "Peking" seedlings 10 days after inoculation with the bacteria IAN3 were carried out as described in materials and methods. Fig. 5a shows the root
Fig. 4. A graph comparing the number of nodules formed per plant vs the number of days after inoculation with the strains, USDA193 and IAN3
No. of Nodules Per Plant

USDA193
IAN3

No. of Days After Inoculation
hair curling (Hac) phenotype of this mutant.

Complementation of the delayed nodulation mutation

We transferred the cosmid clone pPA1 carrying the 2.8 kb EcoRI fragment into the mutant strain IAN3 and the transconjugant IAN3 (pPA1) was used to inoculate "Peking". Early nodulation characteristic of the wild type strain was observed suggesting that 2.8 kb EcoRI fragment could functionally complement the mutation in strain IAN3. Furthermore, we introduced a mutation in the nodD gene sequences by "omega" insertional mutagenesis as described in materials and methods and transferred the resulting cosmid pNA2D1 into the strain IAN3. The transconjugant was tested on plants as previously described. The mutation in strain IAN3 could be functionally complemented by the cosmid pNA2D1 indicating that the mutation in IAN3 is located outside the nodD gene sequences, in agreement with the restriction map shown in Fig. 1. We also tested the ability of the cosmid pEK10 carrying the hsn (host specific for nodulation) genes, nodEFG of *R. meliloti* (Torok et al., 1984; Rostas et al., 1986) to complement the mutation in the strain IAN3. However, the transconjugant IAN3 (pEK10) could not nodulate soybean. Also, hybridization study indicated that the hsn genes of *R. meliloti* did not carry DNA sequence homology
Fig. 5. Characterization of the delayed mutant strain IAN3

(a) Light micrograph of soybean root hair showing root hair curling due to inoculation of the strain IAN3.
(b) A root nodule formed on "Peking" due to inoculation of the strain IAN3.
with _R. fredii_ USDA193 plasmid (data not shown).

Transfer of cosmid clones carrying Tn5 and "omega" insertions into the strain IA728. We have earlier reported the ability of the 2.8 kb _EcoRI_ fragment of USDA193 to nodulate soybean when transferred into different rhizobia (Ramakrishnan et al., 1986b). To determine the phenotypic effect of mutations in this fragment on soybean nodulation, we transferred the cosmid clones carrying the various insertions into the strain IA728, a _pSym_-cured derivative of _R. fredii_ USDA193 and tested them on "Peking". The results are presented in Fig. 1. Insertions in the _nodD_ region as well as in the right hand region of the 2.8 kb _EcoRI_ fragment (represented as _nodM_ in Fig. 1) resulted in a Nod" phenotype with very few (1-2) white bumps appearing about 4 weeks after inoculation. The nodules formed in all other cases were similar in morphology to that formed by 2.8 kb _EcoRI_ fragment. An insertion in the extreme right hand side region also gave a Nod" phenotype. The results presented in Fig. 1 thus identify the presence of a new functional region coding for at least one gene involved in the nodulation of soybeans and has been designated as _nodM_.


Fig. 6. Hybridization of HindIII restriction enzyme
digested total DNA from different Rhizobium strains
using $^{32}$P-labeled right SalI-EcoRI fragment of the
2.8 kb EcoRI nod fragment.
Lanes 1-5: USDA193, USDA205, USDA194, 1781 and
USDA110.
Conservation of nodN gene sequences in Rhizobium fredii strains. To determine if the newly identified nodulation region from the strain USDA193 is conserved among different soybean nodulating Rhizobium strains, we purified the right SalI-EcoRI fragment from the 2.8 kb EcoRI fragment, carrying the nodN region and used it as a probe to hybridize to HindIII restriction enzyme digested total DNA from different Rhizobium strains. Fig. 6 shows the results of the hybridization. The probe hybridized to a 6.8 kb HindIII fragment of R. fredii USDA193, USDA205, USDA194 and 1781 (lanes 1-4). No significant homology was observed with the DNA from USDA110 under the high stringency conditions used for hybridization. We also included total DNA from heterologous strains such as R. leguminosarum 128C53, R. phaseoli DB176, R. trifolii 162P17, R. meliloti 102F28 and A. tumefaciens A277 (data not shown). However, we did not observe any hybridization to nodN gene probe.
DISCUSSION

We have previously reported the presence of the "common" nod genes, nodABC and nodD genes on 5.2 kb HindIII and 2.8 kb EcoRI fragments, respectively, of the Sym plasmid, pRjauUSDA193 (Ramakrishnan et al., 1986b). Conjugal transfer of these two nod fragments into pSym-cured derivatives of different rhizobia showed the ability of the 2.8 kb EcoRI fragment to nodulate soybeans (Ramakrishnan et al., 1986b). The only known gene on the 2.8 kb EcoRI fragment is the nodD gene. In R. meliloti, R. trifolii, and R. leguminosarum, the nodD gene is believed to play a regulatory role in combination with a plant cell factor in controlling the expression of other nodulation genes including the nodABC genes. Thus, we chose to analyze the 2.8 kb EcoRI fragment (using transposon mutagenesis as a tool) for functional regions involved in nodulation other than the nodD gene.

Earlier, we reported that R. fredii strains USDA201, USDA193 and USDA191 show similar hybridization pattern with R. meliloti nod probe containing nodABCD genes (Masterson et al., 1985). The results presented here further support this observation. Also, the restriction maps of the 2.8 kb EcoRI
fragments from the strains USDA193 and USDA191 are in close agreement. In addition, hybridization and functional complementation studies identifying the "common" nod genes, nodABC and nodD genes on 5.2 kb HindIII and 2.8 kb EcoRI fragments, respectively, of R. fredii USDA193 plasmid DNA agree with that reported for USDA191 nod fragments (Appelbaum et al., 1985; Ramakrishnan et al., 1986b). We report here that the nodD gene of the strain USDA193 is indeed located around the unique BamHI site in the 2.8 kb EcoRI fragment, as predicted from the sequence data available for the nodD gene of the strain USDA191 (Appelbaum et al., 1985).

All Tn5 insertions in the 2.8 kb EcoRI fragment were found to be localized in two regions. Attempts to replace the wild type sequences with the mutated fragments by homologous recombination events were largely unsuccessful, probably due to the small size of the fragment used. With single recombination events, it was difficult to judge the phenotypic effects of mutations on nodulation process. However, we obtained a mutant strain of USDA193, IAN3, showing delayed nodulation phenotype and having a Tn5 insertion in the right-hand region of the unique SalI site present in the 2.8 kb EcoRI fragment. This mutation could be functionally complemented by both the 2.8 kb EcoRI
fragment and a fragment carrying an in vitro constructed mutation in the \textit{nodD} gene. These data confirmed that the mutant phenotype of the strain IAN3 was due to the Tn5 insertion into a locus in the right SalI-EcoRI fragment and that it was neither due to a mutation generated by random integration during cointegrate formation nor due to a mutation in the \textit{nodD} gene.

Because the 2.8 kb EcoRI fragment alone could confer nodulation of soybeans to heterologous rhizobia, the host specific genes for soybean nodulation must be present on this fragment. Transfer of all the Tn5 insertions within the 2.8 kb EcoRI fragment into pSym-cured derivative of \textit{R. fredii} USDA193, followed by plant tests, showed the effect of the different mutations on host specific nodulation of soybeans. Our results indicate that both the \textit{nodD} gene and the newly identified \textit{nodN} region are necessary for soybean nodulation. DNA sequence analysis would provide more information about the number of genes involved and their direction of transcription. We do not know the exact role of these gene products in the nodulation process. Yet, we believe that they are involved in some of the early nodulation events, such as host recognition and infection.

The right SalI-EcoRI fragment of the 2.8 kb \textit{nod} fragment did not show any DNA homology with \textit{R. meliloti nod}
probes or the total DNA of heterologous strains. This suggests that these sequences may not be conserved in different rhizobia and that they may be specific for soybean nodulation. However, we could not detect any homology with the total DNA from *B. japonicum*. Hence, sequences identified in this study may be specific to *R. fredii* strains. Sequences involved in host specific nodulation have been identified in *R. meliloti* and *R. leguminosarum*, mutations in which generally led to a delayed nodulation phenotype (Rostas et al., 1986; Downie et al., 1985; Shearman et al., 1986). Such sequences were not generally conserved in different *Rhizobium* strains including *R. fredii* (Kondorosi et al., 1985; unpublished results). Thus, the genes involved in host specific nodulation events (hsn) may be specific to certain *Rhizobium* species. It is also possible that some of these genes are normally present in a repressed state in heterologous bacteria. In *R. trifolii*, Tn5 insertions in specific regions within a 14 kb *EcoRI* fragment carrying the host specificity genes resulted in mutants with altered host range ability (Djordjevic et al., 1985b). The phenomenon of host specific nodulation by rhizobia seems to be complex, even though in some strains, all genes necessary for nodulation lie in close proximity.

In *R. fredii* strains, the genes involved in nodulation
are on unlinked DNA fragments. The 2.8 kb nod fragment is reiterated, hybridizing to itself as well as to a 12.5 kb HindIII or 5.3 kb EcoRI fragment of pRjaUSDA193 (Prakash and Atherly, 1984). Our results indicate that nodN sequences are not present in the 12.5 kb HindIII fragment and that any sequence homology between the 2.8 kb EcoRI and the 12.5 kb HindIII fragments must be due to a reiteration of nodD gene. Appelbaum et al. (1985) has also shown the presence of a nodD-like gene in the 12.5 kb HindIII fragment, thus supporting our conclusion. It is not known whether the second copy of the nodD gene of R. fredii USDA193 is functional or not.

As reported previously, the nodules formed by pSym-cured strains carrying the 2.8 kb EcoRI fragment alone, were distinctly different from wild type nodules, with very few bacteria within the cells (Ramakrishnan et al., 1986b). The genes identified in this study may be involved in soybean specific nodulation since these genes could extend the host range of heterologous bacteria, enabling them to nodulate soybeans. It is possible that there are other genes involved in a similar function present elsewhere in the genome. Further studies indicate that regions linked to the nodABC genes of R. fredii USDA193 could code for soybean host specificity genes (manuscript in preparation). These
regions do not cross-hybridize with the 2.8 kb EcoRI fragment. Each cluster of the nod genes linked to the "common" nod genes seems to be capable of independent nodulation events, even though considerably less efficient, compared with that of the wild type. These findings would point towards the existence of alternative mechanisms for host cell recognition. Evolutionary events may account for the occurrence of host specificity genes widely scattered in the genome and associated with the functionally conserved "common" nod genes.

Recently, host specificity genes for siratro nodulation were shown to be linked to a nodD-like gene in the broad-host-range Rhizobium strain NGR234 (Bassam et al., 1986). NGR234 resembles R. fredii strains in that the nod genes are not clustered together but present on unlinked DNA fragments. Bachem et al. (1986) also reported the identification of host range determinants for siratro nodulation in the Rhizobium species MPIK3030, which is a derivative of NGR234. In both NGR234 and MPIK3030 strains, the host specificity genes are linked to the "common" nod genes as reported for R. meliloti (Kondorosi et al., 1985), R. leguminosarum (Downie et al., 1983, 1985; Hombrecher et al., 1984) and R. trifolii (Schofield et al., 1984; Djordjevic et al., 1985b). Our results show that soybean
host specificity genes, while not conserved in rhizobia, seem at least to be closely linked to the "common" nod genes, suggesting that the conserved genes may also be necessary for host range determination.
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REFERENCES


Bassam BJ, Rolfe BG, Djordjevic MA: Macroptilium atropurpureum (siratro) host specificity genes are linked to a nodD-like gene in the broad host range Rhizobium strain NGR234. Mol Gen Genet 203:49-57, 1986


Bruijn FJ, Lupski JR: The use of transposon Tn5 mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids - a review. Gene 27:131-149, 1984


Djordjevic MA, Schofield PR, Rolfe BG: Tn5 mutagenesis of Rhizobium trifolii host-specific nodulation genes results in mutants with altered host-range ability. Mol Gen Gen 200:463-471, 1985b


Jorgensen RA, Rothstein SJ, Reznikoff WS: A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. Mol Gen Genet 177:65-72, 1979


Knauf VC, Nester EW: Wide host range cloning vectors: a cosmid bank of an Agrobacterium Ti plasmid. Plasmid 8:45-54, 1982


Prakash RK, Atherly AG: Reiteration of genes involved in symbiotic nitrogen fixation by fast-growing Rhizobium

Prentki P, Krisch HM: In vitro insertional mutagenesis with
a selectable DNA fragment Gene 29:303-313, 1984

Ramakrishnan N, Prakash RK, Atherly AG: Conservation of
IS66 homologue of octopine Ti plasmid DNA in Rhizobium
freddi plasmid DNA. Plant Mol Biol (in press), 1986a

Ramakrishnan N, Prakash RK, Shantharam S, Duteau NM, Atherly
AG: Molecular cloning and expression of Rhizobium freddi
USDA193 nodulation genes: Extension of host range for
nodulation. J Bacteriol (submitted), 1986b

Rosenberg C, Boistard P, Denarie J, Casse-Delbart F: Genes
controlling early and late functions in symbiosis are
located on a megaplasmid in Rhizobium meliloti. Mol Gen

Rossen L, Shearman CA, Johnston AWB, Downie JA: The nodD
genome of Rhizobium leguminosarum is autoregulatory and in
the presence of plant exudate induces the nodA,B,C genes.
EMBO J 4:3369-3373, 1985

Rostas K, Kondorosi E, Horvath B, Simocsits A, Kondorosi A:
Conservation of extended promoter regions of nodulation
genes in Rhizobium. Proc Natl Acad Sci USA 83:1757-1761,
1986

Schofield PR, Ridge RW, Rolfe BG, Shine J, Watson JM:
Host-specific nodulation is encoded on a 14 kb DNA
fragment in *Rhizobium trifolii*. Plant Mol Biol 3:3-11, 1984


Shearman CA, Rossen L, Johnston AWB, Downie JA: The *Rhizobium leguminosarum* nodulation gene *nodF* encodes a polypeptide similar to acyl carrier protein and is regulated by *nodD* plus a factor in pea root exudate. EMBO J 5:647-652, 1986


Rhizobium-legume symbiosis is a very complex process, involving both plant and bacterial genes. The various steps leading to an effective symbiosis are still not well-understood. However, recent progress in the characterization of nodulation genes have contributed towards a better understanding of the basic phenomenon.

The research work reported in this dissertation attempts to identify the various genetic regions involved and their possible role in Rhizobium fredii-soybean symbiosis. In most of the Rhizobium strains, the symbiotic genes are found to be clustered within a short region of DNA. The genes involved in host specificity and nodulation are found to be coded on a 10 kb DNA fragment in R. leguminosarum, a 14 kb DNA in R. trifolii and less than 30 kb fragment in R. meliloti (Downie et al., 1983; Schofield et al., 1984; Truchet et al., 1985). Transposon mutagenesis studies and sequence analysis have identified several nodulation genes and most of these genes might be specifically turned on during symbiosis. In R. meliloti strain 41, a 47 bp conserved sequence in the 5' flanking regions of three transcriptional units coding for nodABC,
nodEFG, and nodH, has been identified recently (Rostas et al., 1986). It was also shown that these conserved sequences are necessary for the expression of nodulation genes. In *R. leguminosarum* also, sequences preceding nodA and nodF have been shown to be conserved and the amino acid sequence analysis of nodD protein has revealed a putative DNA-binding region (Shearman et al., 1986). These observations led them to propose that the nodD protein is activated by a plant factor (root exudate component) which then induces gene expression by binding to the highly conserved DNA sequences. The findings described in Sections II and III would favour this model of nod gene regulation. nodD gene of *R. fredii* strain has been shown to be present on the 2.8 kb EcoRI fragment of pRjaUSDA193 in Section II and more precisely, near the BamHI site of the 2.8 kb fragment, in Section III. The nodD carrying fragment was shown to be necessary for nodulation. Since nodABC genes involved in root hair curling are conserved in different *Rhizobium* strains and since these sequences are functionally interchangeable, it is very unlikely, that these genes are involved in host specificity of nodulation. The observations made in Section II that the 2.8 kb EcoRI fragment could extend the host range of heterologous bacteria suggest that the genes involved in host specificity
must be coded within this fragment. Transposon mutagenesis of the 2.8 kb fragment did reveal a second functional region which could code for a host specificity gene (Section III). Furthermore, these sequences were shown to be conserved in different soybean nodulating strains. Thus, in R. fredii, nodD protein activated by a plant factor may turn on the expression of other nod genes, such as nodABC involved in root hair curling functions (5.2 kb HindIII fragment), nodN (2.8 kb EcoRI fragment) responsible for host specificity, etc. Sequence analysis would reveal the presence of possible conserved regions preceding these nod genes.

In R. meliloti, the nod gene products have been characterized by maxicell and minicell analysis after achieving controlled overproduction of the proteins using suitable expression vectors (Egelhoff and Long, 1985; John et al., 1985). nodC protein of R. meliloti was proposed to be an integral membrane protein based on several evidences (John et al., 1985). In R. leguminosarum, amino acid sequence homology studies indicated that nodF protein may be similar to the acyl-carrier protein from E. coli and barley and hence proposed to be involved in an acyl transfer reaction (Shearman et al., 1986). None of the nod gene products of R. fredii strains have been characterized yet. It would be interesting to sequence the nodulation genes of
R. fredii strains and analyze the predicted amino acid sequences for homology to known protein molecules.

An interesting observation discussed in Section II is the ability of sequences flanking the 2.8 kb EcoRI fragment to repress nodulation. A transconjugant of the pSym-cured strain of R. fredii carrying a 6.8 kb HindIII fragment (the 2.8 kb EcoRI fragment is located within this fragment) was unable to nodulate soybeans (see Appendix III, Fig. 2). Transposon mutagenesis of the adjacent sequences would identify the regions involved in repression.

Some preliminary results point out the possibility of alternate mechanisms for nodulation (see Appendix V). Delayed nodulation (4 weeks after inoculation) was observed after inoculation of soybean with Sym plasmid-cured derivative of R. fredii strain into which the cosmid clone bank of pRjaUSDA193 was mobilized (unpublished results). Bacterial DNA could be reisolated from the nodules by the alkaline lysis method (Maniatis et al., 1982). Results presented in the Appendix V indicate that the bacteria carried the 5.2 kb HindIII fragment and flanking sequences. Since the 5.2 kb HindIII fragment alone could not induce nodulation of soybeans (Section II), it seems that sequences flanking the 5.2 kb HindIII fragment may code for regions involved in nodulation. It is possible that regulatory
molecules other than nodD could act in concert with plant factor to turn on nod gene expression. This would also suggest that there may be other genes responsible for host specificity. However, there are no evidences so far reported in support of this speculation.

Section I describes the identification and conservation of an insertion sequence homologue in the R. fredii genome. The putative insertion sequence was also found to be present near nif gene sequences of USDA193 strain. Recently, a spontaneous Fix" mutant of R. fredii was isolated after maintaining the wild type strain on TY plates for 27 months (Duteau NM, 1985). Hybridization studies using nif probe indicated that the nif hybridization profile of the mutant and wild type strains were strikingly different (Appendix VI, Fig. 1). This could arise as a result of spontaneous deletion near nif gene sequences or a possible genome rearrangement. The presence of putative insertion sequences in the vicinity of these genes would support such events. Hybridization data using an IS66 homologue as probe indicated that such sequences were not present in the genome of the mutant strain (Appendix VI, Fig. 2). These data support the occurrence of a deletion event which also included the IS66 sequence. Further characterization of the
mutant strain would precisely localize the genetic lesion responsible for the mutant phenotype.
REFERENCES


Callaham D: M.Sc. thesis. University of Massachusetts, Amherst, 1979


Duteau NM: Ph. D. thesis. Iowa State University, Ames,
Iowa, 1985


Hadley RG, Szalay AA: DNA sequences homologous to the T-DNA region of Agrobacterium tumefaciens are present in diverse Rhizobium species: Mol Gen Genet 188:361-369, 1982

Holmes DS, Qiugley C: A rapid boiling method for the

Hooykaas PJJ, Peerbolte R, Rosenberg-Tuink AJG, De Vries P, Schilperoort RA: A chromosomal linkage map of Agrobacterium tumefaciens and a comparison with the maps of Rhizobium spp. Mol Gen Genet 188:12-17, 1982


Mulligan JT, Long SR: Induction of Rhizobium meliloti nodC
expression by plant exudate requires \textit{nodD}. \textit{Proc Natl Acad Sci USA} 82:6609-6613, 1985


Prakash RK, Schilperoort RA: Relationship between nif plasmids of fast-growing \textit{Rhizobium} species and Ti plasmids of \textit{Agrobacterium tumefaciens}. \textit{J Bacteriol}


Rossen L, Shearman CA, Johnston AWB, Downie JA: The nodD gene of Rhizobium leguminosarum is autoregulatory and in the presence of plant exudate induces the nod A, B, C genes. EMBO J 4:3369-3373, 1985


Ruvken GB, Long SR, Meade HM, Van den Bos RC, Ausubel FM: ISRm1: A Rhizobium meliloti insertion sequence that transposes preferentially into nitrogen fixation genes.
J Mol Appl Gen 1:405-418, 1982

Schmidt J, John M, Kondorosi E, Kondorosi A, Weineke U,
Schroder G, Schroder J, Schell J: Mapping of the protein
coding regions of Rhizobium meliloti common nodulation
genes. EMBO J 3:1705-1711, 1984

Schofield PR, Djordjevic MA, Rolfe BG, Shine J, Watson JM:
A molecular linkage map of nitrogenase and nodulation
genes in Rhizobium trifolii. Mol Gen Genet 192:459-465,
1983

Schofield PR, Ridge RW, Rolfe BG, Shine J, Watson JM:
Host-specific nodulation is encoded on a 14 kb DNA
fragment in Rhizobium trifolii. Plant Mol Biol 3:3-11,
1984

Scholla MH, Elkan GH: Rhizobium fredii sp. nov., a
fast-growing species that effectively nodulates soybeans.

Scott KF, Hughes JE, Gresshoff PM, Beringer JE, Rolfe BG,
Shine J: Molecular cloning of Rhizobium trifolii genes
involved in symbiotic nitrogen fixation. J Mol Appl
Genet 1:315-326, 1982

Shearman CA, Rossen L, Johnston AWB, Downie JA: The
Rhizobium leguminosarum nodulation gene nodF encodes a
polypeptide similar to acyl carrier protein and is
regulated by nodD plus a factor in pea root exudate.
Sprent JI: The biology of nitrogen-fixing organisms. 


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APPENDIX I. RESTRICTION MAP OF THE IS66 HOMOLOGUE OF R. FREDII DNA
Fig. 1. Restriction map of the 1.7 kb HindIII fragment of
*R. fredii* strain USDA193 plasmid DNA, homologous to
the insertion sequence IS66 (see Section I)
APPENDIX II. MODIFIED (HORIZONTAL) ECKHARDT GEL ELECTROPHORESIS
Fig. 1. A model of the apparatus designed by R. K. Prakash for horizontal Eckhardt gel electrophoresis (see Section I for a detailed description)
APPENDIX III. SOYBEAN ROOT NODULES FORMED BY VARIOUS TRANSCONJUGANTS
Fig. 1. Soybean root nodules formed by the transconjugant IA728 (pPA1) on cultivar "Peking" (described in Section II)
Fig. 2. Soybean roots inoculated with the transconjugant IA728 (pNA6) harvested 5 weeks after inoculation. pNA6 is a pVK102 derivative carrying the 6.8 kb HindIII nod fragment of R. fredii strain USDA193.
Fig. 3. Soybean root nodules formed by the transconjugants IA728 (pPA1::Tn5) on cv. "Peking", harvested 6 weeks after inoculation.

Abbreviations: BS::Tn5, BamHI-SalI fragment with a Tn5 insertion; SEgp::Tn5, SalI-EcoRI fragment with Tn5 insertion in the extreme right region.

As described in Section III, Tn5 insertions in nodD or nodN regions produced only white "bumps" 5 weeks after inoculation.
Fig. 4. Soybean root nodule formed by the transconjugant IAN3 (pNA2D1) on cv. "Peking" (see Section III), harvested 4 weeks after inoculation. IAN3 (pNA2D1) is the transconjugant used for functional complementation of the delayed nodulation mutation (due to Tn5 insertion in the nodN region) in the strain IAN3, using a clone of nodD::omega fragment (pNA2D1).
APPENDIX IV. HYBRIDIZATION PROFILE OF CLONED NODULATION FRAGMENTS OF _R. FREDII_ STRAIN USDA193 DNA
Fig. 1. Hybridization of $^{32}\text{P}$-labeled 3.5 kb BamHI-EcoRI nod probe from *R. meliloti* (see Section II) to different nod clones of *R. fredii* USDA193, used in this dissertation.

Lane 1: HindIII digested pBR322 derivative carrying the 5.2 kb HindIII nod fragment; lanes 2 and 3: pVK101 derivative carrying both 5.2 kb HindIII and 2.8 kb EcoRI nod fragments (pPA3) digested with HindIII and EcoRI restriction enzymes, respectively; lanes 4 and 5: cosmid clone 54 (see Fig. 1 in Section II) carrying 5.2 kb HindIII (11.3 kb EcoRI) and 5.3 kb EcoRI (12.5 kb HindIII) nod fragments of pRjaUSDA193, digested with HindIII and EcoRI restriction enzymes, respectively.
APPENDIX V. IDENTIFICATION OF ADDITIONAL REGIONS OF R. FREDII USDA193 PLASMID DNA INVOLVED IN SOYBEAN NODULATION
Fig. 1. HindIII restriction enzyme digested cosmid DNA isolated from bacteria (by alkaline lysis method as described in Maniatis et al., 1982) that were present in soybean root nodules after inoculation of cv. "Peking" with IA728 transconjugants carrying pVK102 cosmid bank of pRjUSDA193 (see Summary and Discussion).

Lane 9 shows lambda DNA separately digested with HindIII and EcoRI enzymes and run together as a size standard.

Hybridization of Southern blot of this DNA using $^{32}$P-labeled 3.5 kb nod fragment of R. meliloti as probe showed strong hybridization to 5.2 kb HindIII fragment (data not shown). Hybridization data suggested that only lanes 5 and 7 represent complete digestion.
Fig. 2. Hybridization of $^{32}\text{P}$-labeled 3.5 kb $\text{nod}$ probe of $R. \text{meliloti}$ to $\text{HindIII}$ digested cosmid DNA (lanes 2 and 3) isolated from $E. \text{coli}$ (by rapid boiling method of Holmes and Quigley, 1981) transformed with DNA shown in lanes 2 and 5 of Fig. 1. The larger signal seems to be due to partial digestion. Lane 1: $\text{HindIII}$ digested PVK101 derivative carrying only the 5.2 kb $\text{HindIII nod}$ fragment (pPA2) used as positive control.
CONCLUSIONS

Results presented in Fig. 1 and Fig. 2 suggest that sequences flanking the 5.2 kb *HindIII* fragment may be involved in soybean nodulation, since the transconjugant IA728 (pPA2) carrying the 5.2 kb *HindIII* fragment alone was unable to nodulate soybean (as described in Section II). The bacteria isolated from the nodules (carrying the cosmid clones shown in lanes 2 and 5 of Fig. 1) were used to inoculate "Peking". Nodules were formed 3–4 weeks after inoculation.
APPENDIX VI. CHARACTERIZATION OF A SPONTANEOUS

FIX" MUTANT STRAIN OF USDA193
Fig. 1. Hybridization of $^{32}$P-labeled pRmR2 DNA (carrying the nifDH sequences of R. meliloti ) to HindIII and EcoRI digested total DNA from mutant and wild type strains of USDA193.

Lanes 1 and 2 represent HindIII digested DNA from the strains IA13 and USDA193, respectively. Lanes 3 and 4 represent EcoRI digested DNA from the mutant and wild type strains, respectively.

The Fix$^{-}$ mutant, IA13 was isolated after maintaining the wild type strain on TY plates for 27 months (Duteau NM, 1985).
Fig. 2. Hybridization of $^{32}$P-labeled EcoRI 19a fragment (IS66t) (see Section I) of T-DNA to HindIII digested total DNA. Lanes 1 and 2 represent total DNA from IA13 and USDA193 strains, respectively.
CONCLUSIONS

The results presented in Figs. 1 and 2 suggest that the mutant phenotype could be due to a deletion (IS66 homologue is absent) of sequences flanking the nif gene sequences. A genome rearrangement event is also possible to give a different restriction profile for the mutant DNA.