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# Isolation and Characterization of the DNA Region Encoding Nodulation Functions in *Bradyrhizobium japonicum*

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**The DNA region encoding early nodulation functions of *Bradyrhizobium japonicum* 3I1b110 (I110) was isolated by its homology to the functionally similar region from *Rhizobium meliloti*. Isolation of a number of overlapping recombinant clones from this region allowed the construction of a restriction map of the region. The identified nodulation region of *B. japonicum* shows homology exclusively to those regions of *R. meliloti* and *Rhizobium leguminosarum* DNA known to encode early nodulation functions. The region of homology with these two fast-growing *Rhizobium* species was narrowed to an 11.7-kilobase segment. A nodulation-defective mutant of *Rhizobium fredii* USDA 201, strain A05B-2, was isolated and found to be defective in the ability to curl soybean root hairs. Some of the isolated recombinant DNA clones of *B. japonicum* were found to restore wild-type nodulation function to this mutant. Analysis of the complementation results allows the identification of a 1.8-kilobase region as essential for restoration of Hac function.**

Biological nitrogen fixation is a characteristic found solely in some procaryotic organisms. Eucaryotic organisms, however, can benefit from procaryotic nitrogen fixation by establishing a symbiotic association with a procaryotic partner. In many leguminous plants, biological nitrogen fixation occurs through such an association with bacteria of the genera *Rhizobium* and *Bradyrhizobium*. The establishment of this symbiosis is a multistep process involving both bacterial and plant functions. To understand the initial steps in the formation of nitrogen-fixing nodules, which are differentiated outgrowths of the plant root in which the *Rhizobium* spp. reside, we have sought to isolate those bacterial genes that encode nodulation functions.

Recombinant DNA clones of the nodulation genes from a number of *Rhizobium* species have been isolated; the *Rhizobium* species used in most of these studies are all members of the fast-growing group (4, 5, 7, 8, 10, 16, 20, 22, 29, 31). The fast-growing group of *Rhizobium* species are taxonomically divergent from the slow-growing group; in fact, the slow-growing rhizobia have recently been placed in a separate genus, *Bradyrhizobium* (15). Sutton et al. reported the isolation of recombinant DNA clones of *B. japonicum* that expressed soybean root hair curling (Hac) function in a variety of gram-negative bacteria (e.g., *Pseudomonas putida*). However, no evidence was presented that the DNA cloned has any role in nodulation.

Three basic approaches have been used to isolate recombinant DNA clones of the nodulation region from *Rhizobium* spp. The first such clones were isolated from *Rhizobium meliloti* by selecting from a conjugative cosmid library those clones which complemented a nodulation-defective mutant (20). This same approach has been used effectively to isolate the nodulation genes from *Rhizobium* sp. strain ANU 240 (31), *Rhizobium trifolii* (22), and other fast-growing *Rhizobium* species, some of which could nodulate *Glycine max* (4). This approach has also been used to isolate the nodulation genes from slow-growing *Rhizobium parasponiae*, which nodulates the nonlegume parasponia (25). A second approach has been to first isolate nodulation-defective mutants induced by insertion of the transposon Tn*5* (5, 7, 10, 16, 20). The DNA region containing the mutated gene can then be cloned from the mutant by selecting for kanamycin resistance, which is encoded on Tn*5*. The wild-type DNA region is then obtained by using these mutated regions as hybridization probes to a DNA bank from the wild type. This approach has been used successfully to isolate the nodulation genes from *Rhizobium leguminosarum* (7), *R. trifolii* (31), *Rhizobium phaseoli* (10), and *Rhizobium loti* (5). In some of these cases, the DNA regions isolated from different species show homology to each other as exhibited by DNA-DNA hybridization. For example, the nodulation regions of *R. meliloti*, *R. leguminosarum*, and *R. trifolii* show homology (8, 29). Recently, Noti et al. (26) have used the homology between the *nod* genes of *R. meliloti* and *Bradyrhizobium* sp. strain IRC78 to isolate the *nod* genes from this slow-growing rhizobium. We have found that the nodulation regions from *R. meliloti* (encoded on plasmid pRmSL26) (20) and *R. leguminosarum* (encoded on plasmid pIJ1089) (7, 8) hybridize to DNA isolated from cells of *B. japonicum* 3I1B110. Nodulation genes can therefore be isolated from *B. japonicum* by using pRmSL26 or pIJ1089 as hybridization probes. The isolated cloned region can be used to complement a mutant of *Rhizobium fredii* USDA 201 that is deficient in an early step in nodulation. *R. fredii* is a fast-growing *Rhizobium* species that can nodulate some cultivars of soybean (30).

## MATERIALS AND METHODS

**Microbiological techniques.** Bacterial strains and plasmids used in this study are listed in Table 1. *B. japonicum* cultures were grown in yeast extract-mannitol (36) or RDM medium (2). *Escherichia coli* cultures were grown in LB medium (23) or, for plasmid isolation, in M9 medium (24).

**Genomic DNA isolation.** *B. japonicum* DNA was isolated as follows. Cells from a culture in 500 ml of YEM medium were harvested after 4 to 5 days of growth, pelleted, washed in TE buffer (10 mM Tris hydrochloride, 0.5 mM EDTA; pH 7.4), and suspended in 10 ml of 0.1 M Tris hydrochloride (pH

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TABLE 1. Bacterial strains and plasmids

| Bacterial strain or plasmid | Relevant characteristics | Source (reference) |
|---|---|---|
| Bacteria | | |
| *B. japonicum* 3I1B110 | Wild type, colony type I110 | (19) |
| *R. fredii* USDA 201 | Wild type | (16) |
| *R. fredii* A05B-2 | Nod$^-$ mutant of strain USDA 201 | This study |
| *E. coli* DH1 | F$^-$, *recA1 endA1 gyrA96, thi-1 hsdR17 (hsdR hsdM) supE44*, lambda$^-$ | (21) |
| *E. coli* HB101 | F$^-$, *hsdS20* (Rb$^-$ Mb$^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* Str$^r$ *xyl-5 mtl-1 supE44* lambda$^-$ | (3) |
| Plasmids | | |
| pLAFR1 | Tc$^r$, cosmid | (11) |
| pHC79 | Tc$^r$ Ap$^r$, cosmid | (6) |
| pVK102 | Tc$^r$ Kn$^r$, cosmid | (17) |
| pRmSL26 | pLAFR1 clone of *R. meliloti* DNA containing *nod* genes | (20) |
| pIj1089 | pLAFR1 clone of *R. leguminosarum* DNA containing *nod* genes | (7) |
| pRmJ30 | pBR329 clone containing the 8.7-kb *Eco*RI fragment from pRmSL26 that contains *nod* genes | S. Auger |
| pRjUT2, 7, 10, 11, 14, 16, 21, 22 | pHC79 clones of *B. japonicum* DNA | This study |
| pI2, 14, 18 | pLAFR1 clones of *B. japonicum* DNA | This study |
| pR26, 29, 31, 32 | pVK102 clones of *B. japonicum* DNA | This study |

7.4)–0.1 M EDTA–0.2 M NaCl. Chicken egg white lysozyme (100 mg; Sigma Chemical Co., St. Louis, Mo.) was added to the cell suspension, which was then incubated for 60 min at 37°C. After this period, 1 ml of 25% sodium dodecyl sulfate (SDS) was added, followed by the addition of 0.5 ml of predigested proteinase K (Beckman Instruments, Inc., Palo Alto, Calif.). Proteinase K was predigested for 15 min at 37°C in 0.01 M Tris hydrochloride (pH 8.0)–5 mM CaCl. The cell suspension containing SDS and proteinase K was incubated at 65°C for 1 h. The cell lysate obtained by this method was then mixed directly with $CsCl_2$ to give a refractive index of 1.4. This mixture was centrifuged in a Ti60 fixed-angle rotor (Beckman) at 40,000 rpm for 48 h at 20°C. The high-molecular-weight DNA was obtained by puncturing the side of the tube with a 16-gauge needle and collecting those fractions showing high viscosity.

**Recombinant DNA techniques.** Four clone banks of *B. japonicum* DNA were constructed. To generate DNA to clone into the *Eco*RI sites of pHC79 (6) or pLAFR1 (11), the genomic DNA was partially digested with *Eco*RI, and DNA fragments greater than 20 kilobases (kb) were collected after centrifugation on a 10 to 20% NaCl gradient. To clone into the *Hin*dIII site of pVK102 (17), genomic DNA was partially digested with *Hin*dIII and otherwise treated as described above. In the case of pHC79, the vector DNA was further treated with calf intestinal alkaline phosphatase (Boehringer GmbH, Mannheim, Federal Republic of Germany) as described in the specifications of the manufacturer. *B. japonicum* DNA (2 μg) was mixed with vector DNA (1 μg) and allowed to ligate overnight at 12°C in a total volume of 10 μl with T4 DNA ligase (New England Bio-Labs, Beverly, Mass.) as described in the specifications of the manufacturer. The DNA was then packaged into bacteriophage lambda as described by Hohn (14). This mixture was used to transfect *E. coli* DH1 or HB101. Approximately 2,000 clones were obtained from each library and stored as individual clones in microtiter dishes or as mixtures of clones in the presence of 10% dimethyl sulfoxide at −70°C. Random screening of clones in the pLAFR1 library indicated that approximately 90% of the presumptive clones contained inserted DNA; these inserts were of an average size of approximately 21 kb. In the case of the pHC79 library, greater than 95% of the clones contain inserts, with an average insert size of 40 kb. In the pVK102 library (a generous gift from R. Haugland, Allied Corp., Syracuse Research Laboratories, Solvay, N.Y.) greater than 95% of the clones contain inserts, with an average insert size of 26 kb. A second pLAFR1 library prepared by *Eco*RI partial digestion of *B. japonicum* I110 DNA was a gift of J. M. Ligon and R. J. Helfrich, Allied Corp. This library contains greater than 90% inserts, with an average insert size of 19 kb.

Large scale isolation of plasmid DNA was performed by the SDS lysis procedure as described by Maniatis et al. (24). Plasmid isolations from cell culture volumes of 5 or 30 ml were performed by the alkaline lysis method as described by Maniatis et al. (24). In the case of cosmids, the lysozyme concentration was increased to 10 mg/ml, and the volumes of all the solutions added were doubled.

**DNA hybridization.** DNA was electrophoresed in agarose gels and transferred to nitrocellulose paper as described by Southern (33). Radioactively labeled DNA was prepared by nick translation (28). Restriction fragments from various clones to be used as hybridization probes were obtained after separation by electrophoresis in agarose gels. The fragments were extracted from the gel by a freeze-thaw procedure (32). Hybridization to nitrocellulose paper was done in heat-sealed pouches at 63°C as previously described (9). Hybridizations were performed for 16 h, and the nitrocellulose was then washed four times at 63°C in 2× SSC buffer (1× SSC contains 15 mM sodium citrate and 150 mM NaCl [pH 7.0]) containing 0.1% SDS for low-stringency washing and in 0.1× SSC containing 0.1% SDS for high-stringency washing.

**Colony screening.** Procedures were as described previously (13, 23), except that colonies were transferred to Whatman 541 paper (Whatman Inc., Clifton, N.J.). Hybridizations with $^{32}P$-labeled probe were performed by the method of Haugland and Verma (13). Alternatively, colonies from frozen microtiter dishes were replica plated to nitrocellulose filters (Millipore Corp., Bedford, Mass.), grown overnight, and prepared for hybridization (12, 24). After washing, filters were exposed to X-ray film (Kodak X-Omat R) for 24 h at −70°C. Colonies showing positive hybridization signals were restreaked for isolation before analysis.

**Isolation of Nod$^-$ mutant A05B-2.** *R. fredii* USDA 201 was grown for 2 weeks at 37°C in TY medium (1) containing 5 μg of acridine orange per ml. Survivors were picked from TY plates grown at 28°C and inoculated (1 ml per plant) onto 3-day-old seedlings of *G. max* cv. Peking. After 30 days, plants were harvested and tested for $C_2H_2$ reduction. Those lacking $C_2H_2$-reducing ability were analyzed for the presence of nodules. One mutant, designated A05B-2, repeatedly

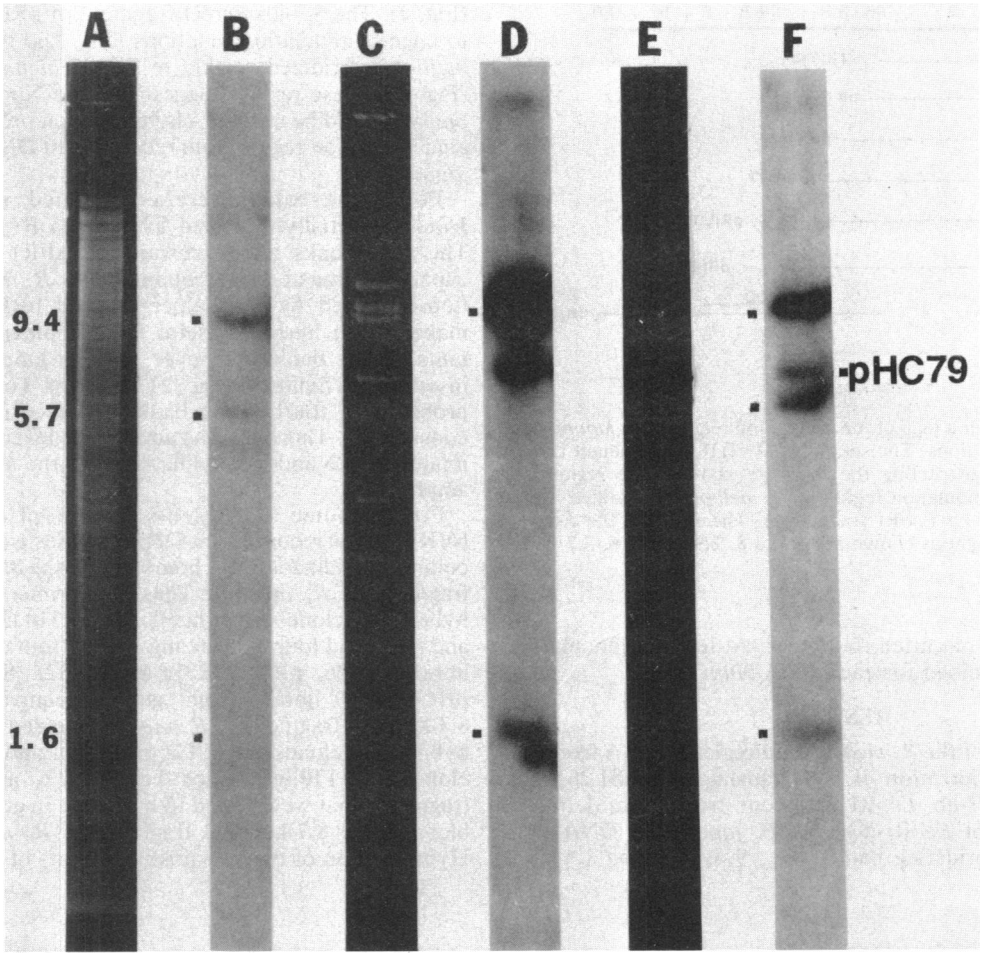


FIG. 1. Hybridization of the 8.7-kb *Eco*RI Nod fragment of *R. meliloti* to *B. japonicum* I110 DNA. Lanes A and B, I110 chromosomal DNA digested with *Eco*RI; lanes C and D, *B. japonicum* DNA clone pRjUT2 digested with *Eco*RI; lanes E and F, *B. japonicum* DNA clone pRjUT10 digested with *Eco*RI. Lanes B, D, and F are Southern blots of DNA probed with the nick-translated 8.7-kb *Eco*RI Nod fragment of *R. meliloti*. The 1.6-kb *Eco*RI fragment, although not visible in lane B, is faintly visible after prolonged exposure (approximately 14 days). The vector pHC79 hybridizes in lanes D and F to contaminating pBR329 DNA present in the probe (the 8.7-kb Nod fragment was obtained from pRmJ30).

failed to produce nodules. Analysis of the plasmid profile of mutant strain A05B-2 showed no apparent difference from the profile of the wild-type strain.

**Transfer of cosmids from *E. coli* to *R. fredii nod* mutant.** The transfer of cosmids to *R. fredii* A05B-2 was performed by the method described by Long et al. (20).

**Plant assay for functional complementation of Nod$^-$ phenotype.** Transconjugants obtained from matings with the Nod$^-$ mutant A05B-2 were harvested and inoculated onto sterile, pregerminated soybean seeds (*G. max* cv. Peking) at the rate of 200 pooled colonies per plant. After 30 days, the plants were harvested and tested for the ability to reduce $C_2H_2$. Nodules were then picked, surface sterilized, and crushed aseptically into 0.1 ml of RDM broth. This cell suspension was streaked onto RDM plates containing the appropriate antibiotics. The colonies that grew were tested by the miniprep procedure for the presence of the complementing cosmid containing I110 DNA. In all cases of positive complementation, the original cosmid clone was recovered from the nodulating bacteria.

**Examination for root hair curling.** Seeds of *Glycine soja* (originally obtained from T. Hymowitz, University of Illinois, Urbana) were surface sterilized in 20% chlorox for 10 min, rinsed twice with sterile distilled water, soaked in concentrated sulfuric acid for 1 min and rinsed with sterile distilled water five times. *G. soja* is a relative of the commercially used *G. max* and exhibits the same nodulation characteristics (34); it has been shown previously to be very useful for microscopic observation of the infection process of *Rhizobium* spp. (34). Sterilized seeds of *G. soja* were germinated on sterile, moist filter paper for 2 days in the dark at room temperature. Seedlings were transferred aseptically to sterile plastic growth pouches (Northrup-King Seed Co., Minneapolis, Minn.) moistened with 10 ml of sterile plant nutrient solution (36). Seedlings were inoculated with approximately $10^8$ cells of either the wild-type *R. fredii* USDA 201 or the mutant strain A05B-2. Plants were watered as needed with half-strength plant nutrient solution.

After inoculation and subsequent growth for 1 to 4 days, seedlings were immersed in phosphate-buffered saline (per liter of water: 0.43 g of $KH_2PO_4$, 1.48 g of $Na_2HPO_4$, and 7.2 g of NaCl) containing 0.05% (wt/vol) toluidine blue for 15 s and then rinsed with phosphate-buffered saline (28). After staining, roots were excised, sectioned longitudinally, and mounted in phosphate-buffered saline under glass cover slips. Roots were examined visually with bright-field optics

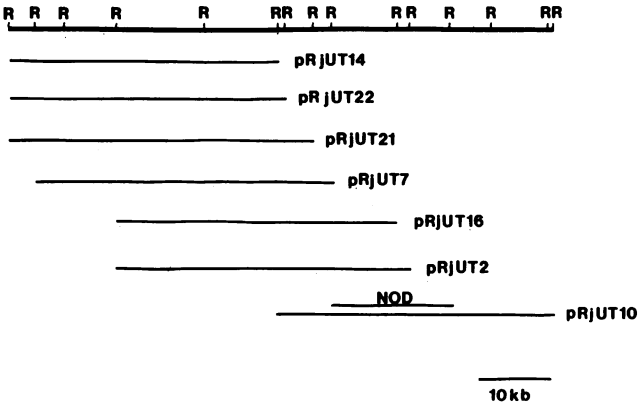


FIG. 2. Restriction map of the nodulation region of *B. japonicum* and surrounding regions. The set of pHC79:I110 recombinant DNA clones used in constructing the map are shown. The region of homology to the nodulation region of *R. meliloti* is identified (i.e., 9.4-, 1.6-, and 5.7-kb *Eco*RI fragments). The sizes of the *Eco*RI fragments left to right as shown are 0.8, 3.8, 2.9, 9.4, 1.6, 5.7, 5.3, 8.8, and 1.0 kb.

for the presence of curled root hairs. At least 10 inoculated plants were examined for each *Rhizobium* strain.

## RESULTS

**Hybridization of the *R. meliloti* Nod region to DNA from *B. japonicum*.** Hybridization of nick-translated pRmSL26 (20) DNA or the 8.7-kb *Eco*RI fragment from pRmSL26 to Southern blots of *Eco*RI-digested *B. japonicum* DNA detected three hybridizing bands (i.e., 9.4, 5.7, and 1.6 kb) (Fig. 1). The 8.7-kb *Eco*RI fragment of pRmSL26 is known to encode nodulation functions (20). The Nod region of *R. meliloti* hybridized weakly to *B. japonicum* genomic DNA (Fig. 1). These results suggest that the Nod region from *R. meliloti* could be used as a hybridization probe to isolate the analogous Nod region from recombinant DNA libraries of *B. japonicum*.

Four gene banks were constructed with *Eco*RI- or *Hin*dIII-(partially) digested DNA from *B. japonicum* I110. The gene banks in the cosmids pLAFR1 or pVK102 are capable of transfer to *B. japonicum* or *R. fredii* by conjugation mediated by the helper plasmid pRK2013 (11); this makes these libraries useful for complementation of mutants. These banks, however, are not amplifiable, and the insert size is limited (range, 21 to 26 kb). To overcome these problems, a fourth gene bank in the cosmid pHC79 was constructed. This bank has an average insert size of approximately 40 kb and is amplifiable upon the addition of chloramphenicol.

Four genome equivalents of the pLAFR1:I110 and pVK102:I110 recombinant DNA libraries were screened, via colony hybridization, for homology to the 8.7-kb *Eco*RI Nod fragment of *R. meliloti*. These experiments yielded three hybridizing clones from the pLAFR1:I110 library (pI2, pI14, and pI18) and four hybridizing clones from the pVK102:I110 library (pR26, pR29, pR31, and pR32). Screening of the pHC79::I110 library (eight genome equivalents) with the 8.7-kb Nod fragment of *R. meliloti* yielded three additional hybridizing clones (pRjUT2, pRjUT10, and pRjUT16). One clone, pRjUT10, contained the 9.4-, 5.7-, and 1.6-kb *Eco*RI fragments that were found to hybridize in genomic Southern blots to the 8.7-kb Nod fragment of *R. meliloti* (Fig. 1). Hybridization of the left-most fragments of pRjUT2 (Fig. 2)

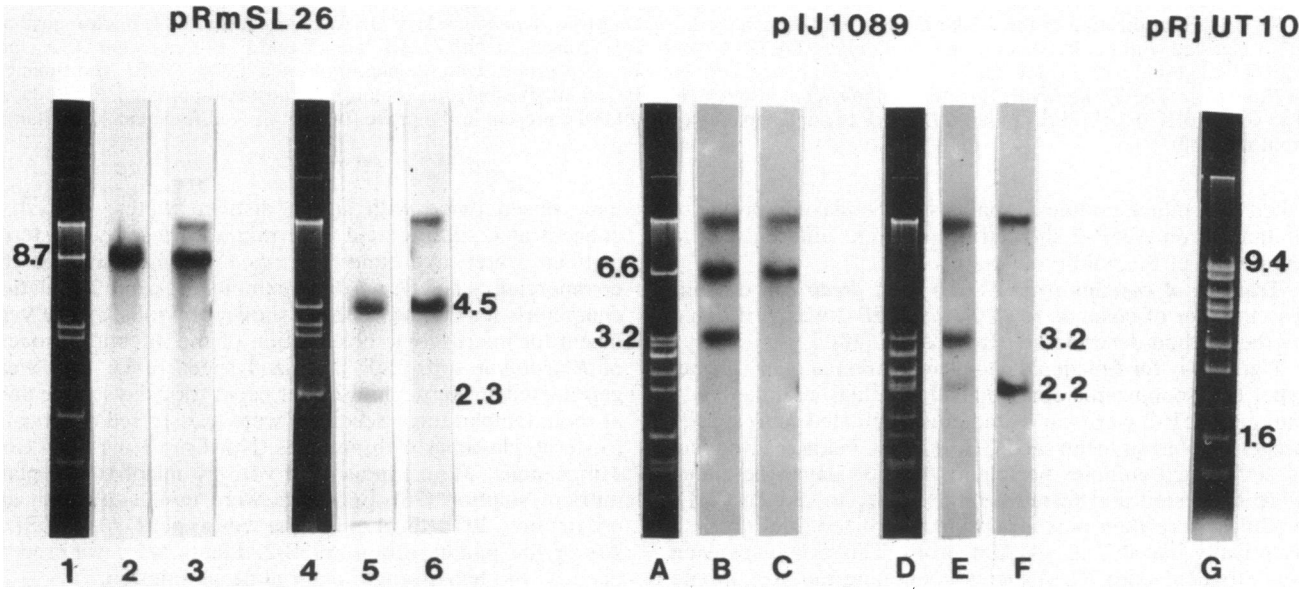


FIG. 3. Homology of the 9.4- and 1.6-kb fragments of pRjUT10 to plasmids pRmSL26 and pIJ1089 containing nodulation genes of *R. meliloti* and *R. leguminosarum*, respectively. Lane 1, pRmSL26 digested with *Eco*RI; lanes 2 and 3, pRmSL26 digested with *Eco*RI and probed against nick-translated 9.4- and 1.6-kb fragments, respectively; lane 4, pRmSL26 digested with *Eco*RI and *Hin*dIII; lanes 5 and 6, pRmSL26 digested with *Eco*RI and *Hin*dIII probed against nick-translated 9.4- and 1.6-kb fragments, respectively; lane A, pIJ1089 digested with *Eco*RI; lanes B and C, pIJ1089 digested with *Eco*RI and probed against nick-translated 9.4- and 1.6-kb fragments, respectively; lane D, pIJ1089 digested with *Eco*RI and *Bam*HI; lanes E and F, pIJ1089 digested with *Eco*RI and *Bam*HI probed against nick-translated 9.4- and 1.6-kb fragments, respectively; lane G, pRjUT10 digested with *Eco*RI. The uppermost hybridizing band of approximately 23 kb seen in some lanes is the vector pLAFR1.

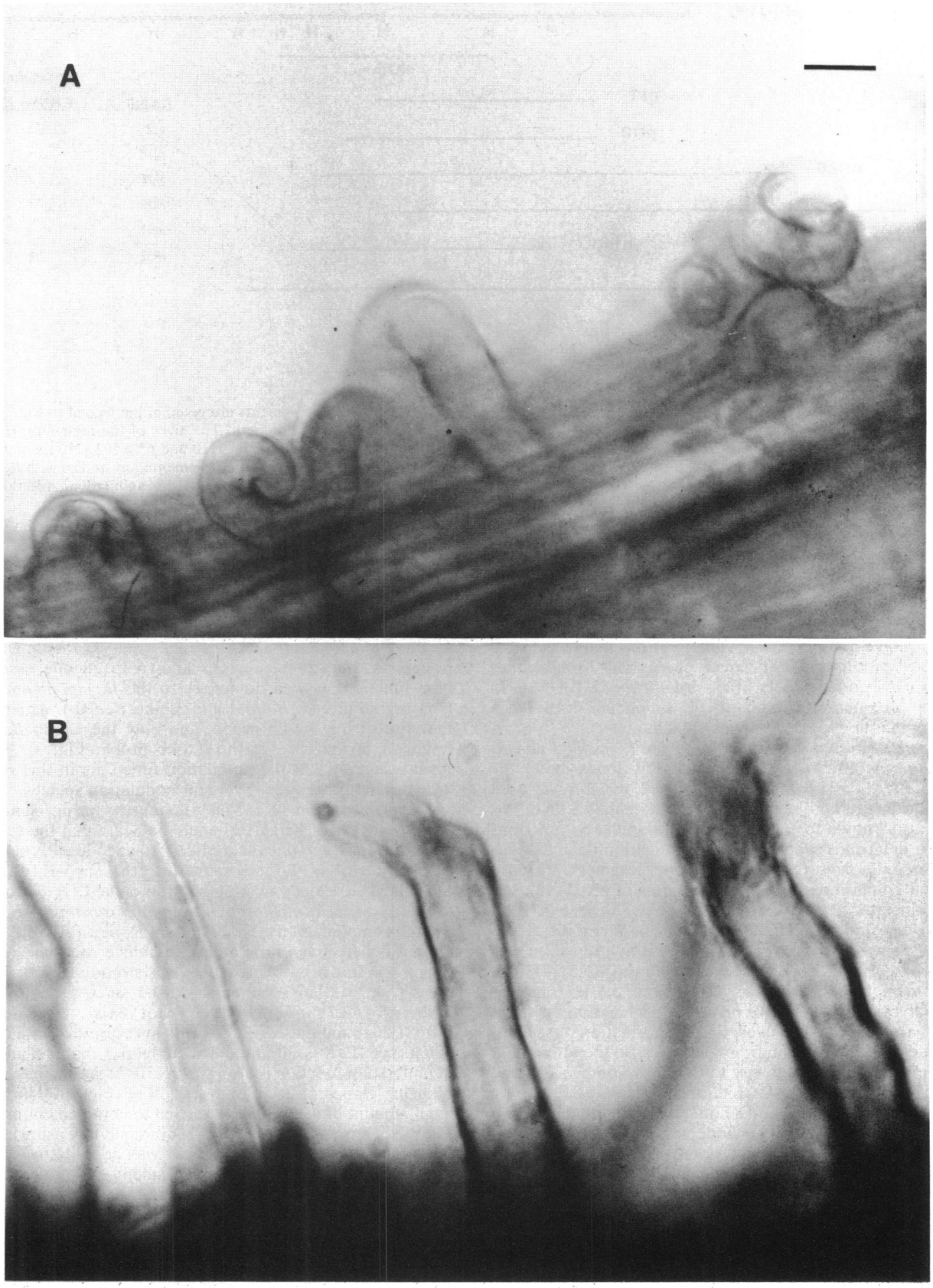


FIG. 4. Light micrograph of the roots of *G. soja* subsequent to inoculation with *R. japonicum* USDA 201 (A) or the mutant strain A05B-2 (B). Prominent corkscrew-like curling of the root hairs is evident in panel A, but only slight deformation of the root hairs is shown in panel B. Bar represents 10 μm.

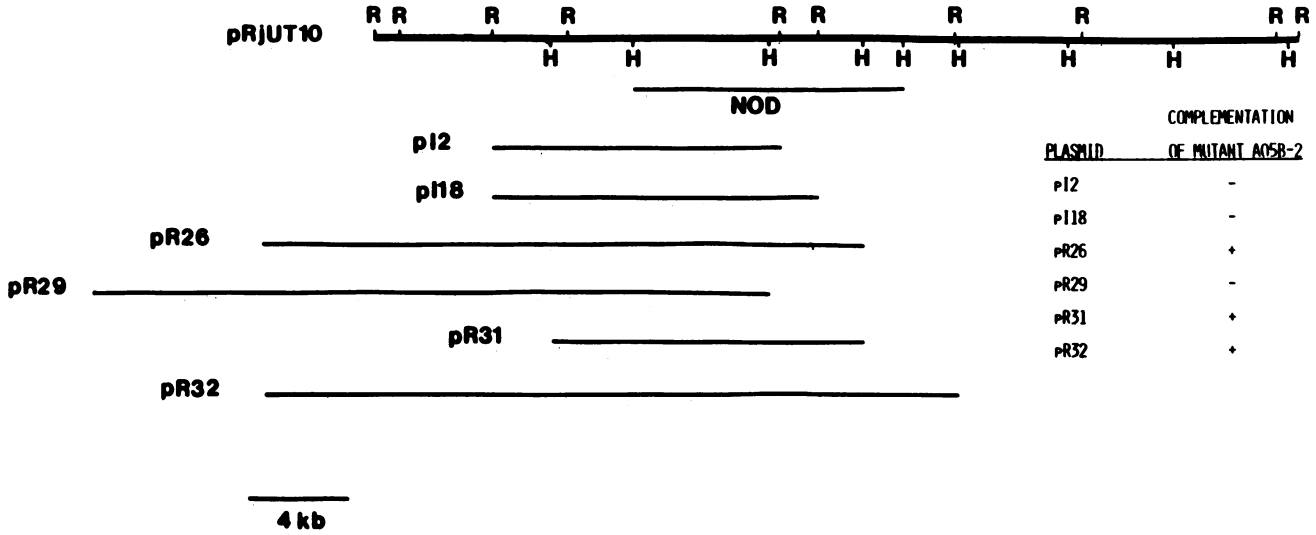


FIG. 5. *Eco*RI and *Hin*dIII restriction enzyme map of pRjUT10. The sizes of the *Eco*RI fragments are given in the legend to Fig. 2. The left-most *Hin*dIII fragment contains vector sequences (not shown) and in pRjUT10 is 10 kb in length. The sizes of the remaining *Hin*dIII fragments from left to right as shown are 3.3, 5.6, 3.9, 1.7, 2.3, 4.5, 4.6, and 4.3 kb. Various pLAFR1:I110 and pVK102:I110 recombinant DNA clones are shown with respect to the map. As shown by the inset table, these clones were used in complementation studies with *R. fredii* mutant strain A05B-2. The overlap between the clones identifies a 1.8-kb *Eco*RI-*Hin*dIII fragment as essential for nodulation. Additionally, the 11.7-kb region containing homology to the 8.7-kb *Eco*RI Nod fragment from *R. meliloti* is indicated.

to the I110 library yielded four additional clones (pRjUT7, pRjUT14, pRjUT21, and pRjUT22). The overlapping clones as well as single and double restriction enzyme digests were used to construct a restriction map of pRjUT10 and adjacent regions (Fig. 2 and 5) (4). The validity of this map was tested by hybridization studies between clones and to subclones and also to genomic blots of *B. japonicum* I110 DNA. In total, approximately 80 kb of *B. japonicum* I110 DNA surrounding the Nod region was cloned.

**Hybridization of pRjUT10 DNA to the *R. meliloti* and *R. leguminosarum* Nod region.** In isolating the clones above, the entire 8.7-kb *Eco*RI Nod fragment from *R. meliloti* was used as a hybridization probe, yet only approximately 3 kb of this fragment is known to encode essential nodulation functions (18). To determine whether the hybridization detected was to the essential Nod region of the 8.7-kb fragment, the 9.4-, 5.7-, and 1.6-kb internal *Eco*RI fragments of pRjUT10 were nick translated and hybridized to *Eco*RI- and *Eco*RI-*Hin*dIII-digested pRmSL26 DNA (Fig. 3). When the 5.7-kb *Eco*RI fragment was used as a probe, no hybridization was detected to pRmSL26 or pIJ1089. When pRmSL26, pIJ1089, or subfragments were used as a probe, the 5.7-kb fragment always hybridized. We have no ready explanation for these results. We suspect, however, that the formation of secondary structure in the 8.7-kb fragment when on the filter could explain the results. Homology to the *B. japonicum* 9.4- and 1.6-kb *Eco*RI fragments was detected only to the 8.7-kb Nod fragment of pRmSL26. This homology lies in the 4.5-kb *Hin*dIII fragment known to encode Nod function (18, 20). In the case of the 9.4-kb fragment, homology was also detected to a 2.3-kb *Hin*dIII fragment that is not known to encode nodulation function. This fragment does, however, encode essential nitrogen fixation (Fix) functions in *R. meliloti* (20). The homology detected to the 2.3-kb fragment may be to these Fix sequences.

To further investigate the homology between the *B. japonicum* I110 Nod region and other *Rhizobium* Nod regions, the homologous 9.4- and 1.6-kb internal *Eco*RI fragments of pRjUT10 were hybridized to pIJ1089 DNA. Clone pIJ1089 contains 6.6- and 3.2-kb *Eco*RI fragments encoding essential early nodulation functions of *R. leguminosarum* (7, 8). Only the 6.6- and 3.2-kb fragments of pIJ1089 hybridized to the *B. japonicum* DNA probes (Fig. 3). Within these fragments, only those regions known to encode essential Nod function showed homology to the *B. japonicum* I110 Nod region (2.2-kb *Bam*HI and 3.2-kb *Eco*RI fragments in the case of the 9.4-kb probe, and only the 2.2-kb *Bam*HI fragment in the case of the 1.6-kb probe; Fig. 3). These results suggest that the nodulation functions in this region are conserved between divergent nodulation species.

**Complementation of a Nod$^-$ mutant by using cloned *B. japonicum* DNA.** The above results indicate that the regions cloned from *B. japonicum* are likely to encode early nodulation functions. To substantiate this, pVK102 and pLAFR1:I110 clones overlapping the cloned region known to hybridize to *R. meliloti* or *R. leguminosarum* DNA were used to complement the Nod$^-$ mutant *R. fredii* A05B-2. This mutant is defective in the ability to induce root hair curling (Hac), as shown by microscopic observation of inoculated roots (Fig. 4). This mutant was used since a comparable mutant of *B. japonicum* does not exist. Transfer of pRmSL26 to mutant A05B-2 results in restoration of a Nod$^+$ phenotype (P. Russell, unpublished data). Complementation of nodulation function in *R. fredii* A05B-2 was accomplished by using clones pR26, pR31, and pR32 (Fig. 5). The minimum amount of cloned DNA needed to complement mutant A05B-2 is represented in pR31. Additionally, by using clones that terminate at different points, it was possible to identify a single 1.8-kb *Eco*RI-*Hin*dIII fragment as containing an essential region for complementation (Fig. 5). The data do not allow us to conclude whether this 1.8-kb fragment encodes an entire gene for Hac function or only part of a gene.

**Homology of the *R. meliloti* Nod region to pRjUT10.** As originally analyzed, the 8.7-kb *Eco*RI Nod fragment of *R. meliloti* hybridized to a 16.7-kb region defined by 9.4-, 5.7-,

and 1.6-kb *Eco*RI fragments. Further hybridization of the 8.7-kb *R. meliloti* DNA to *Hin*dIII digests of pR26, pR29, pR31, and pR32 narrowed the region of homology to 11.7 kb as defined by 5.3-, 4.0-, and 1.7-kb *Hin*dIII fragments (Fig. 5 and 6), which includes the identified Hac region.

## DISCUSSION

The DNA regions encoding early nodulation function such as root hair curling (Hac) are widely conserved among fast-growing *Rhizobium* species and a few slow-growing species. This conservation also appears to apply to species of *Bradyrhizobium* (25, 26; this study); therefore, the isolation of this region from recombinant DNA libraries of diverse *Rhizobium* and *Bradyrhizobium* species should be possible.

Previously, Sutton et al. (35) reported the isolation of a clone from *B. japonicum* that expressed root hair curling function in a diversity of gram-negative organisms (e.g., *Pseudomonas* sp.). This region was not shown to be involved in nodulation since there were no data presented to indicate that a mutation in this region affected nodulating ability (35). In contrast, the cloned region described in this report shows strong homology to analogous Nod regions of *R. meliloti* and *R. leguminosarum* and is capable of restoring nodulation function to a $Nod^-$ $Hac^-$ mutant of *R. fredii* USDA 201. Further, recent work shows that Tn*5* insertions into this region results in a $Nod^-$ phenotype (manuscript in preparation). Comparison of the restriction maps of the regions cloned by Sutton et al. (35) to that presented here shows little apparent similarity.

Interestingly, *R. fredii* USDA 201 is a fast-growing *Rhizobium* species taxonomically divergent from *B. japonicum*. *R. fredii* USDA 201 nodulates *G. max* but has a limited host range (16). The fact that *B. japonicum* DNA will complement mutations in *R. fredii* USDA 201 suggests that nodulation genes isolated from these two organisms may be expressed in either cell background. This has potential importance to efforts to genetically engineer superior *Rhizobium* (or *Bradyrhizobium*) inoculum strains.

Approximately 80 kb of DNA surrounding the Nod region has been cloned. Thus far, no homology to *B. japonicum nifH* or *nifDK* genes (kindly provided by B. Chelm and H. Hennecke, respectively) has been detected in the DNA cloned. This suggests that, unlike fast-growing *Rhizobium* species, the Nif and Nod regions may not be closely linked. Most of the DNA cloned, however, lies on only one side of Nod region (Fig. 2). Our efforts to obtain additional clones on the other side of the Nod region by hybridization to the genomic libraries has been unsuccessful thus far. This suggests that this region is underrepresented in our genomic DNA libraries or, perhaps, that a gene lethal to *E. coli* lies in this region.

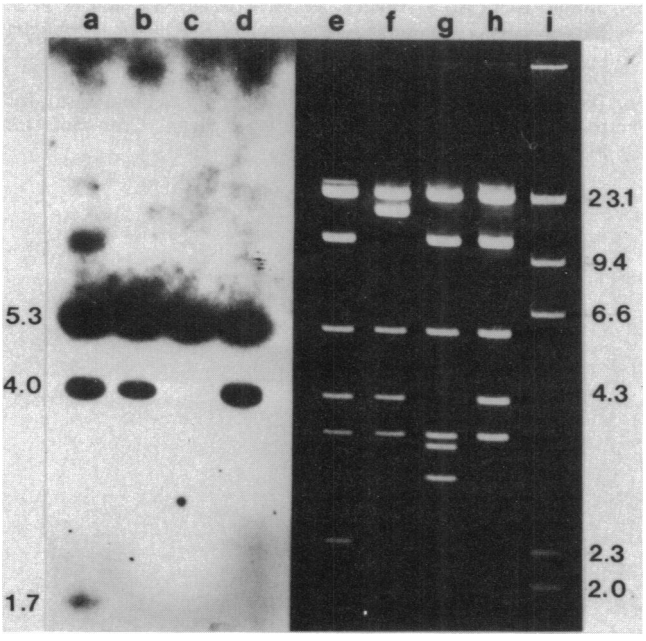


FIG. 6. Hybridization of the 8.7-kb *Eco*RI Nod fragment from pRmSL26 to pVK102:I110 recombinant DNA clones. Lanes a through d show hybridization to the 8.7-kb Nod fragment. Lanes e through h represent *Hin*dIII digests of the respective clones. Lane i contains lambda bacteriophage digested with *Hin*dIII as size markers. Lanes a and e, pR32; lanes b and f, pR31; lanes c and g, pR29; lanes d and h, pR26. The hybridizing band at approximately 9 kb in lane a is due to partial digestion.

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