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A Locus Encoding Host Range Is Linked to the Common Nodulation Genes of *Bradyrhizobium japonicum*

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By using cloned *Rhizobium meliloti*, *Rhizobium leguminosarum*, and *Rhizobium* sp. strain MPIK3030 nodulation (*nod*) genes as hybridization probes, homologous regions were detected in the slow-growing soybean symbiont *Bradyrhizobium japonicum* USDA 110. These regions were found to cluster within a 25-kilobase (kb) region. Specific *nod* probes from *R. meliloti* were used to identify *nodA*-, *nodB*-, *nodC*-, and *nodD*-like sequences clustered on two adjacent *Hin*dIII restriction fragments of 3.9 and 5.6 kb. A 785-base-pair sequence was identified between *nodD* and *nodABC*. This sequence contained an open reading frame of 420 base pairs and was oriented in the same direction as *nodABC*. A specific *nod* probe from *R. leguminosarum* was used to identify *nodIJ*-like sequences which were also contained within the 5.6-kb *Hin*dIII fragment. A *nod* probe from *Rhizobium* sp. strain MPIK3030 was used to identify *hsn* (host specificity)-like sequences essential for the nodulation of siratro (*Macroptilium atropurpureum*) on a 3.3-kb *Hin*dIII fragment downstream of *nodIJ*. A transposon Tn*5* insertion within this region prevented the nodulation of siratro, but caused little or no delay in the nodulation of soybean (*Glycine max*).

Soybean, an important agricultural plant, is infected (or nodulated) by and establishes a symbiosis with the nitrogen-fixing soil bacterium *Bradyrhizobium japonicum*. Nodulation is a complex developmental process requiring several plant and bacterial functions. *B. japonicum* is a member of the so-called slow-growing rhizobia (24), as opposed to the taxonomically divergent fast-growing species of *Rhizobium* that infect such plants as alfalfa (symbiont: *Rhizobium meliloti*), peas (symbiont: *Rhizobium leguminosarum*), and clovers (symbiont: *Rhizobium trifolii*). Although studies of the physiology and molecular biology of nodulation have often used soybeans (19, 50), knowledge of the genetics of nodulation in *B. japonicum* has lagged behind that of the fast-growing rhizobia.

The initial interactions of plant and symbiont that lead to establishment of the symbiosis require at least two sets of genes. One set (*nodABCDIJ*), the so-called common nodulation genes due to their ability to functionally complement nodulation-defective mutants in other rhizobial species, encodes functions important for the early steps of nodulation (20, 23, 40, 48). A second set of genes (*hsn*; *hsnABCD* = *nodEFGH*) (21, 29) imposes on the plant-bacterial interaction a degree of specificity; certain plant-rhizobia combinations are favored, other combinations are excluded. Induction of these nodulation genes requires low-molecular-weight compounds produced by the plant and also a functional *nodD* gene (22, 27). Additionally, Rostas, et al. (42) have identified a 47-base-pair (bp) sequence upstream of many of the known nodulation operons in *R. meliloti* that are essential for *nod* gene induction. This sequence has been termed the Nod box.

Recently, four groups have cloned the common nodulation genes from three different species of *Bradyrhizobium* (31, 35, 38, 43). In each case, the common nodulation genes were found to be distant from the nitrogenase genes, *nifKDH*, and in fact it is generally thought that the symbiotic genes in *Bradyrhizobium* spp. are not as tightly clustered as in *Rhizobium* species. None of the host specificity genes (*hsn*) of any *Bradyrhizobium* species have been isolated. Host specificity functions in *Bradyrhizobium* spp. may be more complex than in *Rhizobium* species in that generally a wider range of plants can be nodulated.

In this study, the DNA in and around the common nodulation genes of *B. japonicum* USDA 110 was further analyzed and a number of symbiotic genes were located. In addition to the *nodDABC* genes found in *Rhizobium* spp., nucleotide sequence analysis of the region upstream of *nodABC* revealed the existence of a 420-bp open reading frame (ORF). This ORF was in a similar position to one identified in another slow-growing rhizobium, *B. parasponia*, designated *nodK* (46). By using site-directed transposon Tn*5* mutagenesis, a region essential for the nodulation of siratro (*Macroptilium atropurpureum*) was identified and found to be linked to the common nodulation genes. Mutations in this region prevented nodulation of siratro but only slightly affected the nodulation of soybean. This region was found to contain homology to the *hsn* genes from *Rhizobium* sp. strain MPIK3030, which nodulates siratro. This region appears to encode *hsn* functions which extend the host range of *B. japonicum*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. *B. japonicum* cultures were grown in YS medium, which contains 1 g of yeast extract per liter added to the previously described minimal salts-vitamin base of Bishop et al. (4), or in RDY medium, containing (per liter) 5 g of gluconic acid, 1 g of glutamic acid, and 1 g of yeast extract, in mineral salts-vitamin base. *Escherichia coli* cultures were grown in LB medium (34), except for JM101, which was grown in TYE medium (45). *R.*

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TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Relevant characteristics	Source or reference
Bacteria		
B. japonicum		
USDA 110	Wild type; colony type I110	30
NAD138	USDA 110(pAN86::Tn*5*)	This work
AN240	USDA 110(pAN146::Tn*5*)	This work
E. coli		
DH1	F$^-$ *recA1 endA1 gyrA96 thi-1 hsdR17 supE44* λ^-	33
HB101	F$^-$ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^-	5
DBCS31-1	*recA trpE* λ^+	D. Berg
JM101	*lac proAB supE thi*/F' *traD36 proAB lacI^{q}ZM15*	36
EG47	*hsdR lac gal rpsL594*	15
R. meliloti		
AK631	*R. meliloti* 41	A. Kondorosi
MG107	AK631 *nodD*::Tn*5*	16
Plasmids		
pRjUT10	pHC79 clone of *B. japonicum*	43
pRK2073	Trimethoprim-resistant, Tra$^+$	32
pZB14	pNM481 with insertion of 2.2-kb *R. meliloti nodAB* fragment	A. Kondorosi
pZB15	pBR329 with insertion of 3.2-kb *Eco*RI fragment of *nodIJ* genes of *R. leguminosarum*	This work
pCB507	pLAFR1 derivative carrying *hsn* region of *Rhizobium* sp. strain MPIK3030	2
pDG39	pVK102 (25) derivative carrying the 3.9-kb *Hin*dIII fragment of *B. japonicum* USDA 110 homologous to *R. meliloti nodD*	This work
pIJ1089	*R. leguminosarum nod* and *fix* genes cloned as a 30-kb fragment in pLAFR1	11
pBR329	Ap^r Tc^r Cm^r	6
Phages		
λ Tn*5*	Km^r	D. Berg
P1 Tn*5*	Km^r	40
P1 Tn*5 lac*	Km^r	D. Kaiser
P1 clr100 cm	Cm^r	15
fFR *nodC*	M13mp8 with insertion of 0.7-kb *nodC* fragment of *R. meliloti*	F. R. Quinones
fMG *nodD*	M13mp18 with insertion of 275-bp *nodD* fragment of *R. meliloti*	F. R. Quinones
fBH *hsnA*	M13mp18 with insertion of 0.5-kb *hsnA* fragment of *R. meliloti*	B. Horvath
fBH *hsnB*	M13mp18 with insertion of 0.5-kb *hsnB* fragment of *R. meliloti*	F. R. Quinones
fBH *hsnC*	M13mp18 with insertion of 1.6-kb *hsnC* fragment of *R. meliloti*	B. Horvath
fFR *hsnD*	M13mp18 with insertion of 320-bp *hsnD* fragment of *R. meliloti*	F. R. Quinones

meliloti and *Rhizobium* sp. strain MPIK3030 were grown in TYA (39) or TY (3) medium as described previously.

DNA isolation. Plasmid DNA was isolated on a large scale as described by Davis et al. (7) or on a small scale essentially as described by Maniatis et al. (34).

Cloning procedures. Cosmid pIJ1089, carrying the nodulation genes of *R. leguminosarum* (11), was digested with *Eco*RI, and a fragment of 3.3 kilobases (kb) containing the *nodIJ* genes (10) was isolated from an agarose gel by electroelution (34). The vector plasmid pBR329 (6) was linearized by *Eco*RI, ligated to the isolated fragment,and transformed into *E. coli* DH1, selecting for tetracycline-resistant colonies on LB medium with tetracycline (20 μg/ml). Tet^r Cam^s colonies were isolated by replica plating onto LB medium containing tetracycline or tetracycline plus chloramphenicol (20 and 30 μg/ml, respectively). The resulting plasmid from one such colony was designated pZB15 and used as the source of *nodIJ* DNA hybridization probe in further experiments. Likewise, the cosmid pRjUT10 containing the common nodulation genes of *B. japonicum* (43) was digested with *Hin*dIII, and a fragment of 3.9 kb containing homology to the *R. meliloti nodD* gene (see Fig. 3) was isolated from an agarose gel by electroelution (34). The vector plasmid pVK102 (25) was linearized by *Hin*dIII, ligated to the isolated fragment, and subsequently transformed into *E. coli* DH1, selecting for tetracycline-resistant colonies on LB medium with tetracycline (20 μg/ml). Tet^r Kan^s colonies were isolated by replica plating onto LB plates containing tetracycline (20 μg/ml) or kanamycin (25 μg/ml). The resulting plasmid from one such colony was designated pDG39.

Southern blotting and hybridization. DNA restriction fragments were transferred to nitrocellulose filters as described previously (48). Internal fragments from the intra-*nod* and *hsn* genes of *R. meliloti* cloned into the phage M13 were labeled as described by Sanger et al. (45) with Klenow enzyme, [^{32}P]dCTP, and a 17-mer primer DNA (Bethesda Research Laboratories, Inc.), except *hsnD*, for which a reverse primer (BioLabs Inc.) was used. The DNA fragments containing the *nodIJ* genes of *R. leguminosarum* and the *hsn* genes of *Rhizobium* sp. strain MPIK3030 were reisolated from agarose gels (34) and labeled by the random primer method of Feinberg and Vogelstein (13). With both methods, specific activities of 10^8 cpm/μg were obtained. Hybridizations were carried out in 50% formamide buffer at 37°C by the method of Kondorosi et al. (26). Filters were washed at 37°C twice for 1 h each in 2× SSC (1× SSC contains 15 mM sodium citrate and 150 mM NaCl, pH 7.0)

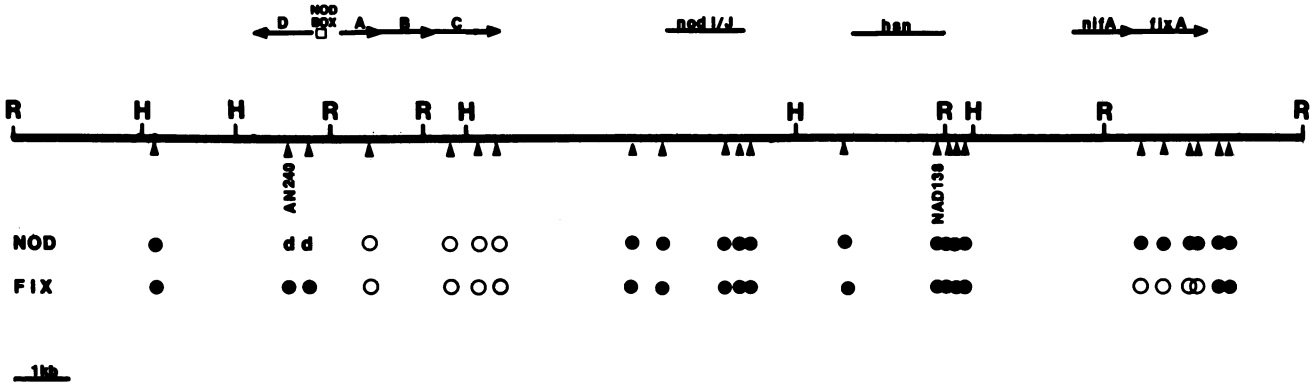


FIG. 1. Nodulation characteristics of *B. japonicum* mutants with the Tn*5* insertion mapping within pRjUT10. Only a portion of pRjUT10 is shown. The sites of insertion are indicated by triangles, and the symbols underneath correspond to different Nod and Fix phenotypes: wild type (●), negative (○), and delayed nodulation (d). Abbreviations: R, *Eco*RI; H, *Hin*dIII. The locations of *fixA* and *nifA* are taken from Lamb and Hennecke (31).

and 0.1% sodium dodecyl sulfate (SDS) and twice for 1 h each in 2× SSC. Exposure of X-ray film was done as described by Maniatis et al. (34).

The Nod box DNA, a 25-mer oligodeoxyribonucleotide [d(ATAAAAACAATCGATTTTACCAATC)], was a gift from A. Kondorosi (Biological Research Center, Szeged, Hungary) and used for hybridization essentially as described previously by Rostas et al. (42).

Enzymes and isotopes. DNA restriction endonucleases and modifying enzymes were purchased from New England Biolabs, Boehringer Mannheim, or Bethesda Research Laboratories and used as specified by the manufacturers. Radioactive nucleotides were purchased from New England Nuclear Corp.

DNA sequencing. DNA sequences were determined by the dideoxy chain termination method (M13 Cloning and Sequencing Handbook, Amersham Corp.). A nested set of deletions of the cloned fragments in mp18 and mp19 was generated by the method of Henikoff et al. (18).

Site-directed Tn*5* mutagenesis. Tn*5* mutagenesis was performed in *E. coli* HMS174 by the method of de Bruijn and Lupski (8) or in *E. coli* EG47 by the method of Quinto and Bender (40). Mutated fragments were conjugated from *E. coli* to *B. japonicum* USDA 110 by the triparental mating system described by Ditta et al. (9). The Tn*5*-mutated fragments were marker exchanged for the corresponding wild-type DNA by the double reciprocal crossover technique of Ruvkun and Ausubel (44). The occurrence of the reciprocal recombination event was confirmed in most cases by hybridizing *Eco*RI digests of total DNA isolated from the mutants with Tn*5* probe pSUP1011 (47). The hybridization data confirmed the location of the Tn*5* insertions mapped on pRjUT10 (data not shown).

Plant tests. Seeds (*Glycine max* cv. Essex) were surface sterilized and germinated as described previously (51). Seedlings (2 days old) were planted in 35-ml serum vials containing sterile vermiculite saturated with sterile plant nutrient solution supplemented with 1% sucrose (51) and covered with sterile 18-oz (ca. 510-g) Whirlpak bags (Nasco). Plants were maintained for 21 days at 26°C in a growth room at 320 $Em^{-2}\ s^{-1}$ with a 14-h photoperiod. Nitrogen fixation activity was detected by the acetylene reduction assay (51) with a Shimadzu GC-8A gas chromatograph equipped with a 6-foot (ca. 2-m) Poropak R column. The detector was maintained at 100°C and the column at 75°C. Plant roots were examined for nodules. For delayed-nodulation assays, seedlings (*G. max*) were sprouted as before and then grown three to a pack in clear plastic pouches (Dispo Seed Pack; Northrup King Seed Co.) as described previously (17). Alfalfa (*Medicago sativa*) and siratro (*M. atropurpureum*) plant assays were carried out in test tubes on nitrogen-free medium as described previously (26, 27).

RESULTS

Identification of symbiotic regions. Previous work in this laboratory identified a 40-kb region of *B. japonicum* USDA 110 DNA which encoded early nodulation functions (e.g., root hair curling, Hac) (43). To identify and delimit symbiotic genes within this region, a pHC79 cosmid clone (pRjUT10) (43) was subjected to site-directed Tn*5* mutagenesis (Fig. 1). The positions of the Tn*5* insertion points were checked and mapped by restriction endonuclease digestions (data not shown). For insertions in the *nodABCD* region, location of the insertions was aided by restriction enzyme sites obtained from the DNA sequence. The mutated DNA was subsequently homogenotized into *B. japonicum* USDA 110, and nodulation and nitrogen fixation assays were performed as described in Materials and Methods.

The following three mutant classes were obtained on soybean (*G. max* cv. Essex) seedlings. (i) Delayed Nod^+: 2 to 4 days delayed in nodulation and reduced efficiency of nodulation (Fig. 2), normal nodule morphology and nitrogen fixation. (ii) Nod^-, with less than 2% of the plants assayed showing some abnormal nodulelike structures after 21 to 28 days. (iii) Nod^+ Fix^-: no significant delay in nodulation compared with the wild-type strain, but these mutants could not fix atmospheric nitrogen. This Fix^- region has been shown previously to have homology to the *fixA* and *nifA* genes of *R. meliloti* (31).

Hybridization of *nod* regions. To further characterize the nodulation regions identified and to locate other nodulation regions previously identified in other *Rhizobium* strains, specific *nod* DNA probes from *R. meliloti*, *R. leguminosarum*, and *Rhizobium* sp. strain MPIK3030 were hybridized to pRjUT10. The *nodABC* and *nodD* genes of *R. meliloti* hybridized to two *Hin*dIII fragments of 5.6 and 3.9 kb (Fig. 3, lanes A, C, and D). The *nodAB* region was localized to the 3.9-kb *Hin*dIII fragment and was wholly contained within the 1.7-kb *Eco*RI fragment (Fig. 3, lane C). The *nodC*-

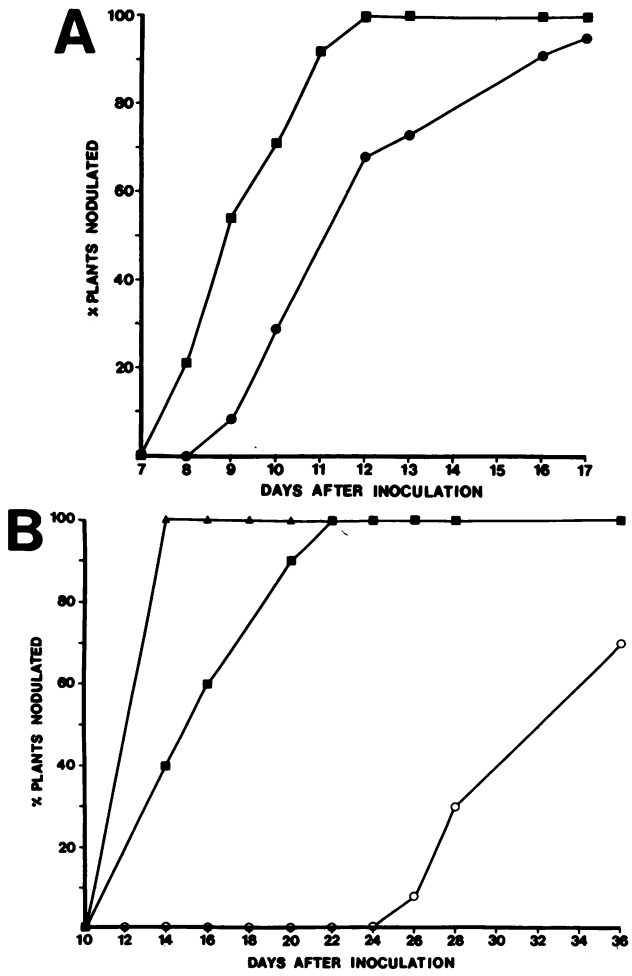


FIG. 2. (A). Nodulation kinetics of wild-type *B. japonicum* USDA 110 (■) and a mutant derivative, AN240 (●), on *G. max* cv. Essex. Nodulation kinetics are shown as percentage of plants nodulated (20 plants). (The Tn*5* insertion in AN240 is in the *nodD*-homologous region.) The average number of nodules per nodulated plant on day 17 was three and four for strains USDA 110 and AN240, respectively. (B). Nodulation kinetics of MPIK3030 (▲), USDA 110 (■), and mutant NAD138 (○) on siratro. Nodulation kinetics are shown as percentage of plants nodulated (15 plants). The average number of nodules per nodulated plant on day 36 was seven, eight, and three for strains MPIK3030, USDA 110 and NAD 138, respectively.

specific homology was predominantly to the left end of the 9.5-kb *Eco*RI fragment, since it also hybridized to the 5.6-kb *Hin*dIII fragment (Fig. 3, lane D).

The *nodD*-specific homology was located on a 5.8-kb *Eco*RI fragment and a 3.9-kb *Hin*dIII fragment (Fig. 3, lane A) and was found to be completely within a 1.8-kb *Eco*RI-*Hin*dIII fragment at the right end of the 5.8-kb *Eco*RI fragment (data not shown).

Within the 9.5-kb *Eco*RI fragment and the 5.6-kb *Hin*dIII fragment, a region hybridized to the *R. leguminosarum nodIJ* probe (Fig. 3, lane E). To localize the *nodIJ*-homologous region, nine pRjUT10 cosmid clones containing Tn*5* insertions within the 5.6-kb *Hin*dIII fragment were restricted with *Hin*dIII and probed with the *nodIJ* fragment (data not shown). The *nodIJ*-homologous region was localized to a 1.5-kb region approximately 0.5 kb from the right

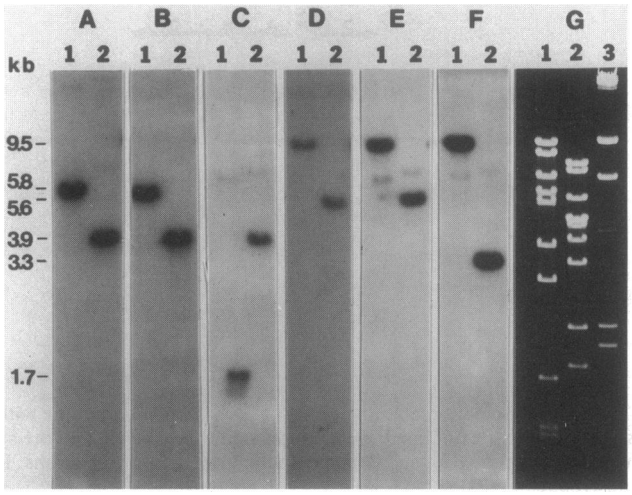


FIG. 3. Hybridization of *nod*-specific probes to restriction digests of pRjUT10. Lanes: 1, pRjUT10 DNA digested with *Eco*RI; 2, pRjUT10 DNA digested with *Hin*dIII; 3, bacteriophage lambda DNA digested with *Hin*dIII. The *nod*-specific probes used were: (lanes A) *R. meliloti nodD*, (lanes B) 25-bp Nod box, (lanes C) *R. leguminosarum nodAB*, (lanes D) *R. meliloti nodC*, (lanes E) *R. leguminosarum nodIJ*, (lanes F) *Rhizobium* sp. strain MPIK3030 *hsn*,and (lanes G) ethidium bromide-stained agarose gels. Hybridization conditions and the *nod*-specific probes are described in Materials and Methods and in Table 1. Numbers in the left margin denote the sizes of fragments that showed interspecies hybridization.

end of the 5.6-kb *Hin*dIII fragment or about 2.5 kb from the end of the *nodC* gene (Fig. 1). The mutants carrying Tn*5* in this region showed wild-type nodulation and nitrogen fixation phenotypes, with at most a 1-day delay in nodulation.

Within the 9.5-kb *Eco*RI and 3.3-kb *Hin*dIII fragments, a region hybridized to the *Rhizobium* sp. strain MPIK 3030 host specificity (*hsn*) probe (Fig. 3, lane F). Therefore, this region lies at the right end of the 9.5-kb *Eco*RI fragment within a 2.9-kb *Hin*dIII-*Eco*RI fragment (Fig. 1). No region within pRjUT10 could be found which was homologous to the host specificity genes *hsnABCD* of *R. meliloti*.

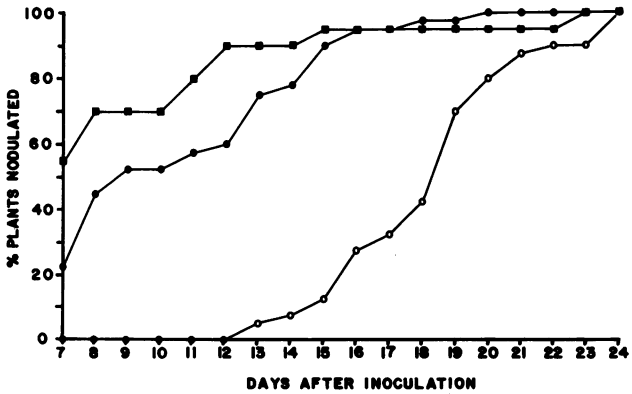


FIG. 4. Nodulation kinetics of wild type *R. meliloti* AK631 (■), an *R. meliloti* Tn*5* insertion mutation in *nodD*, MG107 (○), and strain MG107 containing plasmid pDG39 (●). Nodulation kinetics are shown as the percentage of 40 plants which were nodulated. The average number of nodules per nodulated plant on day 24 was five, three, and six for strains AK631, MG107, and MG107(pDG39), respectively.

Complementation of *nodD*. The *nodD* gene is of special importance since it has been suggested that it encodes a regulatory protein (22, 37). Since hybridization data cannot determine the function of a region, additional experiments were done. The 3.9-kb *Hin*dIII fragment containing the *nodD*-homologous region was cloned into pVK102 (25) and used to complement a *nodD* mutant of *R. meliloti*. The wild-type strain of *R. meliloti*, strain AK631, was able to nodulate alfalfa in 15 days, while the *nodD* mutant strain MG107 was delayed by approximately 9 days (Fig. 4). However, the *nodD* mutant strain containing the *B. japonicum* 3.9-kb *Hin*dIII fragment was able to efficiently nodulate alfalfa with only a slight (2-day) delay (Fig. 4). The data clearly indicate that a functional *nodD* gene was present on the 3.9-kb *Hin*dIII fragment.

Tn*5* insertions into the *B. japonicum nodD* gene resulted in a delayed-nodulation phenotype (Fig. 2A). The delayed phenotype for *nodD* mutations has also been observed in *R. meliloti* (16), in which multiple functional copies of *nodD* have been found. Hybridization of the *R. meliloti nodD* probe to *B. japonicum* genomic DNA revealed multiple bands (data not shown). However, hybridization of *B. japonicum* genomic DNA with the 1.8-kb *Eco*RI-*Hin*dIII fragment (Fig. 1) containing *nodD* revealed only one band. Therefore, it is unclear whether *B. japonicum* contains multiple copies of *nodD*. Work to clarify this is currently in progress.

A region homologous to the Nod box sequence was found within the 5.8-kb *Eco*RI fragment (Fig. 3, lane B). To confirm and precisely locate this sequence, the region 5′ of *nodABC* was subjected to DNA nucleotide sequence analysis. The entire nucleotide sequence from the start of *nodD* to the start of *nodA* was determined (Fig. 5). About 750 bases upstream of the start of *nodA*, the 25-bp Nod box sequence was identified (Fig. 5).

In Fig. 6 a 47-bp conserved region of the 5′-flanking region of the *nodABC* genes of *B. japonicum* is compared with the 5′-flanking region of *nodABC* of *B. parasponiae* (46) and the six conserved 5′-flanking regions of *R. meliloti* identified by Rostas et al. (42). Computer alignment of the *B. japonicum* sequence revealed the existence of the highly conserved sequence, 25 bp in length (i.e., the Nod box).

In addition, within the sequenced region an ORF (ORF1) was identified 5′ to the *nodABC* genes (Fig. 5). This ORF was 420 bases long and in a location analogous to that of an ORF found in *B. parasponiae* and was designated *nodK* (46). The predicted amino acid sequence of ORF1 is also shown in Fig. 5. Although similar in size and location to the *B. parasponiae nodK*, the *B. japonicum* ORF1 had only 30% overall amino acid homology to *B. parasponiae nodK* (46).

Host range locus linked to the *nodABCD* genes. As mentioned previously, homology to the *hsn* genes of *Rhizobium* sp. strain MPIK3030 was detected with a 3.3-kb *Hin*dIII fragment of pRjUT10. Strain MPIK3030 can nodulate siratro, and the *hsn* gene region identified was isolated by its ability to confer the *R. meliloti* the ability to nodulate siratro (2). *B. japonicum* will also nodulate siratro, although its phenotype is Fix$^-$, so the possibility existed that the region detected in pRjUT10 encoded siratro-specific functions. To test this, Tn*5* was inserted into this region, and the nodulation phenotype was tested on soybean and siratro. On soybean, the mutant produced nodules with little or no delay. However, when tested on siratro, this mutant could not elicit nodules until after 20 days (Fig. 2B). After 36 days the average number of nodules per nodulated siratro plant was 7, 8, and 3 for strains MPIK3030, USDA 110, and NAD138, respectively. No differences in nodule color or size could be detected among those induced by these three strains.

A second insertion mutation in the region, generated by Tn*5-lac* mutagenesis (29), was located 0.6 kb to the left of NAD138 and was also found to cause an altered nodulation phenotype on siratro (data not shown). However, Tn*5* insertions located to the right of NAD138 within the 0.5-kb *Eco*RI-*Hin*dIII fragment and about 2.0 kb to the left of that *Eco*RI site showed a wild-type nodulation phenotype on siratro (Fig. 1). Thus, the siratro *hsn*-specific region can be localized to the rightmost end of the large 9.5-kb *Eco*RI fragment of pRjUT10.

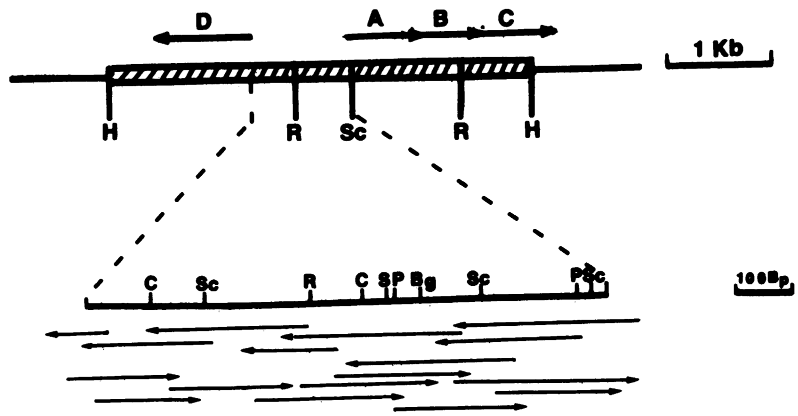
DISCUSSION

The common nodulation genes (*nodABCDIJ*) encode functions important to the early stages of nodule formation. Our data indicate that the arrangement of these genes in *B. japonicum* is similar to that found in other *Rhizobium* species. Regulation of the *nod* genes in *Rhizobium* appears to require a low-molecular-weight host-produced factor (22, 37), a functional *nodD* gene (37), and a conserved Nod box sequence (42). Hybridization and sequence homology to the *R. meliloti nodD* was found in *B. japonicum*. Indeed, the *B. japonicum nodD* could functionally complement a *nodD* mutations in *R. meliloti*. Hybridization of the Nod box oligonucleotide to *B. japonicum* DNA revealed two strongly hybridizing *Eco*RI bands (5.8 and 4.0 kb). The 5.8-kb fragment was adjacent to the *nodABC* genes, in a comparable position to that found in other rhizobia. The 4.0-kb band was unlinked to the common *nod* genes (data not shown). The presence of a functional *nodD* gene and Nod box sequence in *B. japonicum* suggests that regulation of at least the *nodABC* genes will be similar to that found in *Rhizobium* species.

The region 5′ of *nodABC* was sequenced and found to contain 785 bp between the start of *nodD* and *nodA*. Within this region, a 420-bp ORF was identified (ORF1) in the same orientation as *nodABC*. A similar region has been identified in *B. parasponia* and was designated *nodK* (46). The ORF1 of *B. japonicum* was overall only 30% homologous, at the amino acid level, to *B. parasponia nodK*, and therefore these two regions may not encode the same protein, if in fact the regions are translated at all. Thus, at this time, we believe that this region in *B. japonicum* should remain designated ORF1.

The host range of *Bradyrhizobium* species is, in general, much wider than that of *Rhizobium* species. Therefore, the bradyrhizobia are a good model system in which to investigate the genetic determinants of host range. Approximately 8 kb from the common *nod* genes was a region homologous to the siratro *hsn* genes of *Rhizobium* sp. strain MPIK3030 (2). Mutations in this region prevented *B. japonicum* from nodulating siratro but caused little or no delay in nodulation of soybean. This region appeared to encode the siratro *hsn* functions of *B. japonicum*. One or more genes may lie in this region.

A common feature of the symbiotic genes in *Bradyrhizobium* species until now appeared to be the lack of tight clustering of genes, as is found in *Rhizobium* species. This view is largely based on the fact that the *nifKDH* and common *nod* genes appear to be unlinked (31, 43). However, it is now obvious that, excluding this one example, the *sym* genes of *B. japonicum* are clustered. Previous reports have shown a clustering of *nif* and *fix* genes around the *nifKD* and *nifH* loci (1, 14). In this report, we show a clustering of the




```
                                             Nod D ↰
         .       20        .       40         .       60         .       80
5' --GCGTCGAGCGCAACGAGAAGATTTAGATCAAGTCCCTTGAACCGCATGTGATGAGTCTATCCATCGTGTGGATGTGTTCT
      A  D  L  A  V  L  L  N  L  D  L  G  K  F  R  M

         .      100        .      120         .      140         .      160
ATCGAAACAATCGATTTTACCAAACTGGGGGAGGTTGGATAGCAAACTGAAGTTTGGAAAAAGCAATTAGACGCGCCACG
______________________________
     consensus

         .      180        .      200         .      220         .      240
ATGGTTTCCGGTCGTTCACGTGAGCTAAGAGAAGAGCTCCCAGACGTGGCGAGGCACCATGGCGCCGGTTGTTGTTTCTC

                                              ↱ ORF 1
         .      260        .      280         .      300         .      320
CTTAAGCGTGGCCGTTCGAACGCCAAGCGCGTCCCGATAAACGTTGGTATGCAGATATCATCCGTTTCCATATTTCAGGT
                                   ______          M  Q  I  S  S  V  S  I  F  Q  V

         .      340        .      360         .      380         .      400
TTACCCGCGTACAATTGAGGGCGCGTTCAGCCTCATTCTAGTGTCGAAGAATAGAGGCAGACGCAGGTTGCGGATGCCAA
  Y  P  R  T  I  E  G  A  F  S  L  I  L  V  S  K  N  R  G  R  R  R  L  R  M  P

         .      420        .      440         .      460         .      480
GAATTCCAGGCGCCGTTCCAAATGGCTCGCCTGTCCAGTGCATGTGGGGCGTGTCGAAGATGTATAAAAGCCAGTGTGAT
R  I  P  G  A  V  P  N  G  S  P  V  Q  C  M  W  G  V  S  K  M  Y  K  S  Q  C  D

         .      500        .      520         .      540         .      560
CTGATGCCGCTCCGATGCGCCATCGATGAGGCTTGCGCGCCGATGGCCGGCCCTCAGACGTCAAAGCGCCGGAGGCATGC
 L  M  P  L  R  C  A  I  D  E  A  C  A  P  M  A  G  P  Q  T  S  K  R  R  R  H  A

         .      580        .      600         .      620         .      640
GACTGCAGGCACAAAAGCTCGCTGGTGCAGACAGGTTCTGCTAGCCCAACCGCCTCGTCGGCAGCTTCCGAGTTATCGTG
  T  A  G  T  K  A  R  W  C  R  Q  V  L  L  A  Q  P  P  R  R  Q  L  P  S  Y  R

         .      660        .      680         .      700         .      720
AGCGCGGCATCAACCGCACGAGGGTGCAATCAAGCGATCGCTCCGTCGCGCAAAGGCCAGCGCGCGAGTAGGCGAGCAGT
E  R  G  I  N  R  T  R  V  Q  S  S  D  R  S  V  A  Q  R  P  A  R  E

         .      740        .      760         .      780         .      800
GCCGAGCTCCGACAGAGCATGGCGCGCATCTCAGCATTCATCGGCGGTTCGGCGAATAGCGAATTGACGTGACCATCCCC

                               ↱ Nod A
         .      820        .    840           .      860         .      880
GCTCTTCATTCCACCGGCGCAAGGAAGCTCGCCATGAACATTGCCGTCTCCCCGACTTGCGGAAGGATCTTCTGGGCGCG
                    _______        M  N  I  A  V  S  P  T  C  G  R  I  F  W  A  R
                      SD

         .      900        .      920         .      940         .      960
CTCAAGTGCAGTGGAGCCTTTCGTTGGGAAAGTGAACTGCAGCTCGACGATCATGCGAGCTCGCGATTCTTCTAAGAGTT
  S  S  A  V  E  P  F  V  G  K  V  N  C  S  S  T  I  M  R  A  R  D  S  S  K  S

         .
ACGGACGACGGTCTTATCG-- 3'
Y  G  R  R  S  Y
```

FIG. 5. Complete nucleotide sequence of the promoter region of *nodABC*. At the top is a partial restriction map of the region. The arrows denote the sequencing strategy, indicating direction and length of sequence. Predicted amino acid sequences for the start of *nodD*, ORF1, and start of *nodA* are given beneath the nucleotide sequence beginning at bases 47, 289, and 834, respectively. The region designated consensus is the presumptive Nod box sequence. Abbreviations: C, *Cla*I; Sc, *Sac*I; R, *Eco*RI; S, *Sal*I; P, *Pst*I; Bg, *Bgl*II.

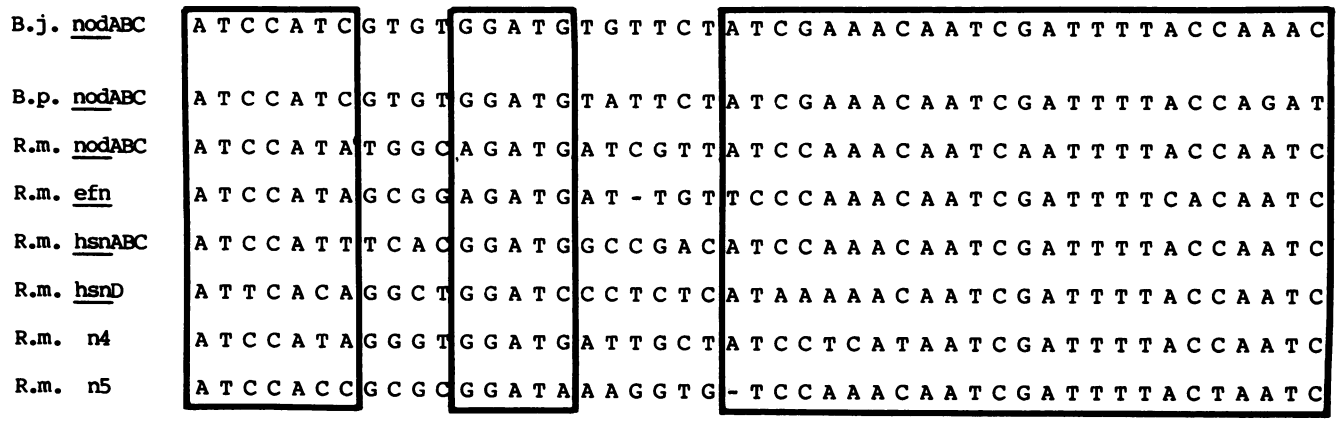


FIG. 6. Comparison of a 47-bp conserved sequence in the 5'-flanking regions for *B. japonicum* (B.j.), *B. parasponia* (B.p.) (46), and *R. meliloti* (*R.m.*) *nodABC*, *efn*, *hsnABC*, *hsnD*, and two additional *R. meliloti* regions, N4 and N5 (27). Sequences read 5' to 3' from left to right.

nifA and *fixA* genes with the common *nod* genes and an *hsn* locus.

Thus far, our examination of the nodulation genetics of *B. japonicum* has revealed many similarities to the better-studied fast-growing *Rhizobium* species. However, apparent differences are also present. For example, both *B. parasponia* and *B. japonicum* contain an ORF between *nodD* and *nodABC*. *B. japonicum* also possesses a host range locus for plants other than its preferred host, soybean. Interestingly, this locus shows no homology to the Nod box sequence. This latter result leaves open the possibility that these genes may be regulated in a unique manner.

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