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Sequence and Analysis of the *nodABC* Region of *Rhizobium fredii* USDA257, A Nitrogen-Fixing Symbiont of Soybean and Other Legumes

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We cloned and analyzed *nodABC* from *Rhizobium fredii* USDA257. These genes are thought to have common functions in initiation of nitrogen-fixing nodules by all rhizobia. In USDA257, they were located in a 9.2-kb *Eco*RI fragment that was not closely linked to either of two copies of the regulatory gene, *nodD*. *nodABC* was present in a 3,094-base pair (bp) sequenced region, which also included a consensus *nod*-box promoter. The three open reading frames contained 654, 642, and 1,239 bp, respectively, and encoded deduced proteins of 21.9, 23.4, and 44.7 kD. The sequence of the *nodABC* region of USDA257 was generally homologous with corresponding regions from other rhizobia, but it diverged significantly in the 5′ nontranslated region and in the 3′ terminus of *nodC*. *nodC* was not translationally coupled to *nodSU*, as in another soybean symbiont, *Bradyrhizobium japonicum*, and the deduced NodC protein was the shortest of any such proteins yet described. Site-directed mutagenesis of the 9.2-kb *Eco*RI fragment confirmed that *nodA*, *nodB*, and *nodC* are essential for nodulation of soybean, but failed to identify other linked *nod* genes. Daidzein, a major isoflavone from soybean roots, was the most potent of nine tested flavonoids in activating a plasmid-borne *nodC::lacZ* fusion. The 9.2-kb fragment complemented *nodA*$^-$, *nodB*$^-$, and *nodC*$^-$ mutants of *R. meliloti* to the Nod$^+$ phenotype on *Medicago sativa*, *M. truncatula*, and *Trigonella foenum-graecum*. Nodule numbers, percentage of nodulated plants, and shoot dry weights, however, were considerably less than in plants inoculated with mutants complemented with *nodABC* from *R. meliloti*.

Additional keywords: broad host range, common *nod* genes, nodulation.

More than 30 nodulation (*nod*) genes have been detected in symbiotic nitrogen-fixing bacteria, a group which includes the genera *Bradyrhizobium*, *Rhizobium*, and *Azorhizobium* (for reviews, see Long 1989; Young and Johnston 1989; Martinez *et al.* 1990). These organisms, which we collectively term rhizobia, nodulate legume species, as well as the ulmaceous genus, *Parasponia*. Their interactions with host plants are known to be complex, variable, and often highly specific. In the beginning, *nod* genes were defined operationally as genes that function in the nodulation process, for the most part, either to define host specificity or to govern infection and nodule development. More recently, genes that are co-regulated with known *nod* genes, but have no known function in nodulation, also have been included in this category (Martinez *et al.* 1990).

The earliest characterized *nod* gene cluster, *nodDABC*, serves a dual function in symbiosis. These genes exist as a contiguous unit in *Rhizobium meliloti* Dangeard, *Rhizobium leguminosarum* Frank bv. *trifolii* Jordan and *viciae* Jordan, and *Bradyrhizobium japonicum* (Buchanan) Jordan, but in some cases (e.g., *R. meliloti*) (Martinez *et al.* 1990), additional copies of *nodD* are present elsewhere in the genome. In others (e.g., *R. fredii* and broad host range *Rhizobium* strain NGR234), *nodD* is not adjacent to *nodABC*, but exists in one or more copies in other portions of the Sym plasmid (Ramakrishnan *et al.* 1986; Appelbaum *et al.* 1988; Perret *et al.* 1991). Although *nodABC* is normally repressed, at least one copy of *nodD* is expressed constitutively. NodD, in conjunction with flavonoids of host origin, is required for activation of *nodABC*, which in turn leads to root hair curling. The activation process involves binding of NodD to a consensus promoter, the *nod*-box, which precedes the *nodABC* operon (Long *et al.* 1989).

nodA, *nodB*, and *nodC* have been collectively termed "common" *nod* genes, because of their high interstrain homology and their interfunctionality among species (Appelbaum 1989). Recent experiments have demonstrated that *nodD* alleles from different rhizobia interact differentially with flavonoids and thus have species-specific functions (Appelbaum 1989; Long 1989). In contrast, considerable evidence supports the contention that *nodABC* indeed is a functionally common unit. All known mutations in this region condition a Nod$^-$ phenotype. Such mutations in *R. meliloti* can be complemented to Nod$^+$ with the corresponding genes from *R. l.* bv. *trifolii* (Fisher *et al.* 1985), *R. l.* bv. *viciae* (Banfalvi *et al.* 1981; Djordjevic *et al.* 1985; Debellé *et al.* 1986), *Bradyrhizobium* sp. (Marvel *et al.* 1985; Noti *et al.* 1985), and *Rhizobium* sp. NGR234 (Bachem *et al.* 1985). Conversely, *nodABC* of *R. meliloti* can complement *nodABC* mutants of *R. l.* bv. *trifolii* (Djordjevic *et al.* 1985; Fisher *et al.* 1985), *R. fredii* USDA201 (Russell *et al.* 1985), and NGR234 (Bachem *et al.* 1985) to Nod$^+$.

Here we describe the cloning and genetic analysis of *nodABC* from *R. fredii* USDA257. This strain is curious in that it exhibits a broad host range for nodulation of

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Nucleotide and/or amino acid sequence data is to be submitted to GenBank, EMBL, and DDBJ as accession number J03702.

legume species, but marked cultivar specificity on soybean (Heron and Pueppke 1984; Scholla and Elkan 1984; Chatterjee *et al.* 1990). Our objective was to provide the first physical characterization of *nodABC* from a broad host range strain and to determine how these genes differ from the corresponding genes of other rhizobia. We show that *nodABC* of USDA257 is unlinked to either of two copies of *nodD*, that portions of the operon diverge significantly from homologous genes from other rhizobia, and that the isoflavone daidzein is a potent inducer of the *nodABC* operon. A plasmid containing *nodABC* from USDA257 only partially complements *nodA*, *nodB*, or *nodC* mutants of other rhizobia.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Table 1 lists *Rhizobium* strains and the plasmids used in this study. Bacterial stocks were maintained in glycerol at −70° C. Rhizobia were cultured in liquid yeast extract-mannitol (YEM) medium (Vincent 1970) and *Escherichia coli* in Luria-Bertani broth (Maniatis *et al.* 1982). When required, antibiotics were supplied in the following concentrations: carbenicillin (100 μg/ml), kanamycin (100 μg/ml), and tetracycline (10 μg/ml).

Table 1. Bacteria and plasmids used in this study

Strain/plasmid	Relevant characteristics	Reference or source
Rhizobium fredii		
USDA257	Wild-type; nodulates Peking soybean	Heron and Pueppke 1987
257B3	Nod⁻ mutant of USDA257, contains Tn*5*	Heron and Pueppke 1987
257B17	257B3 containing pHBK30317; *nodC*::*lacZ* fusion	This study
R. meliloti		
RCR2011	Wild-type	Debellé *et al.* 1986
GMI5382	2011*nodA*::Tn*5*	Debellé *et al.* 1986
GMI5383	2011*nodB*::Tn*5*	Debellé *et al.* 1986
GMI5387	2011*nodC*::Tn*5*	Debellé *et al.* 1986
Plasmids		
pRMSL26	*nod* segment of *R. meliloti*, including *nodD1ABC*, in pLAFR1; Tc^R	Jacobs *et al.* 1985
pRMSL42	*nodAB* of *R. meliloti*, including parts of *nodCD1* in pBR325; Ap^R	Egelhoff *et al.* 1985
pRF1	*nodABC* containing cosmid of USDA257, in pLAFR1; Tc^R	This study
pRK1309	*nodD1* containing cosmid of USDA257, in pLAFR1; Tc^R	This study
pRK1489	*nodD2* containing cosmid of USDA257, in pLAFR1; Tc^R	This study
pHBK301	9.2-kb *nodABC* containing fragment of USDA257 in pGEM7ZF(+); Ap^R	This study
pHBK303	Insert of pHBK301 in pRK415; Tc^R	This study
pHBK30317	pHBK303, *nodC*::*lacZ*-17	This study
pRK415	*IncP* broad host range vector; Tc^R	Keen *et al.* 1988
pGEM7ZF(+)	Cloning vector, Ap^R	Promega-Biotech[a]

[a] Madison, WI.

Molecular methods. Genomic DNA from rhizobia was isolated by the method of Jagadish and Szalay (1984). Preparation of the cosmid library of DNA from USDA257 and its screening by colony hybridization have been described (Heron *et al.* 1989). Plasmid isolation, restriction digestions, cloning, and Southern hybridizations utilized standard procedures (Maniatis *et al.* 1982). DNA probes were labeled with [^{32}P]-dCTP by random priming (USB Biochemicals, Cleveland, OH) and hybridized overnight to filters at 68° C. Hybridization was in 10× Denhardt's solution/6× SSC (1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) containing salmon sperm DNA (100 μg/ml) (Maniatis *et al.* 1982). Before autoradiography, filters were washed in a solution of 0.1% sodium dodecyl sulfate in 0.3× SSC at 68° C. An oligonucleotide for the *nod*-box *Rhizobium* consensus promoter, ATCGAAACAATCGAATTTTACCA (Rostas *et al.* 1986), was kindly provided by W. J. Broughton, University of Geneva. Conditions for labeling and use of the oligonucleotide as a hybridization probe were as described (Lewin *et al.* 1987). Plasmid pHBK303, containing *nodABC* of USDA257, was mutagenized with MudII1734 as described (Castilho *et al.* 1984). After restriction mapping of insertion sites, individual mutated plasmids were transferred triparentally to 257B3, a Nod⁻ deletion mutant that lacks a portion of the Sym plasmid containing *nodABC* (16). Symbiotic phenotypes were assessed as described below.

DNA sequence analysis. The 9.2-kb *Eco*RI fragment from cosmid pRF1 was subcloned into pGEM7ZF(+) as pHBK301. A series of subclones derived from internal restriction fragments then was generated. Sequencing was initiated at these restriction sites by means of the dideoxy-chain termination procedure (Sanger *et al.* 1977). Based on initial sequence analysis, a series of oligonucleotides were synthesized and the sequenced regions extended until the region of interest was completely sequenced. The sequence was verified by complete sequencing of both strands.

Assays for *nod* gene induction by flavonoids. Strain 257B317, which carries a *lacZ* fusion near nucleotide position 2,010 in *nodC*, was grown in YEM medium essentially as described by Maillet *et al.* (1990). Flavonoids known to induce a plasmid-borne *nodC*::*lacZ* fusion from *B. japonicum* in *R. fredii* USDA191 (Kosslak *et al.* 1987) were tested. These included apigenin, biochanin A, chrysin, daidzein, genistein, kaempferol, luteolin, naringenin, and quercetin (all except daidzein, genistein, and luteolin [from ICN Pharmaceuticals, Inc., Covina, CA] were from Sigma Chemical Co., St. Louis, MO). The compounds were added to fresh cultures at concentrations of 5, 10, 50, 100, 200, 400, 600, 1,000, and 10,000 nM. After incubation overnight, β-galactosidase assays were carried out as described by Miller (1972) with the modifications of Mulligan and Long (1985). Cultures were assayed in duplicate, and each experiment was repeated two or three times.

Phenotypic analysis on plants. The ability of pHBK303 to complement mutations in *nodA*, *nodB*, and *nodC* was tested with a series of defined Tn*5* mutants of *R. meliloti*

(Debellé *et al.* 1986). *nodABC*-containing cosmid pRMSL26 served as the positive control in these experiments. Cosmids were transferred triparentally into rhizobia prior to analysis. Seeds of *Medicago truncatula* Gaertn. were from W. J. Broughton, University of Geneva, and those of *M. sativa* L. 'Nitro' were from R. McGraw, University of Missouri. *Trigonella foenum-graecum* L. was from Burpee Seed Co., and soybean (*Glycine max* (L.) Merr. 'Peking') was from C. Morris, University of Missouri.

Seeds were surface-sterilized and germinated on water agar as described (Pueppke 1983). Bacteria were pelleted from log-phase cultures in microcentrifuge tubes, resuspended in sterile water, repelleted, and resuspended in sterile water to 10^8–10^9 cells per milliliter. Seedling roots were dipped into bacterial suspensions and the seedlings transferred into aseptically prepared Leonard jars, which had been fashioned from 6.5 cm square × 10 cm deep Magenta jars (Carolina Biological Supply Co., Burlington, NC). The jars contained vermiculite and Jensen's N-free solution (Vincent 1970). Controls were inoculated with water, and each jar held from two to five seedlings depending on plant size. The jars were incubated in a growth chamber at 400 μmol photons/m^2/sec with a 12-hr photoperiod, and the growth solution was replenished with sterile water as needed. Nodulation was evaluated 25 days after inoculation.

RESULTS

Cloning and sequence analysis of *nodABC*. Cosmids containing *nodABC* were selected by colony hybridization with a library of DNA from *R. fredii* USDA257. The probe was pRMSL42, which contained *nodAB* and portions of *nodC* and *nodD* from *R. meliloti*. The genome of USDA257 contained *Eco*RI fragments of 9.2, 6.0, and 3.0 kb that hybridized to this probe; each was retrieved on a separate cosmid, which appeared not to overlap with the others (Fig. 1). A *nodD*-specific probe, containing the approximately 250-base pair (bp) *Bam*HI/*Bgl*II fragment of pRMSL42, hybridized exclusively to the 3.0- and 6.0-kb

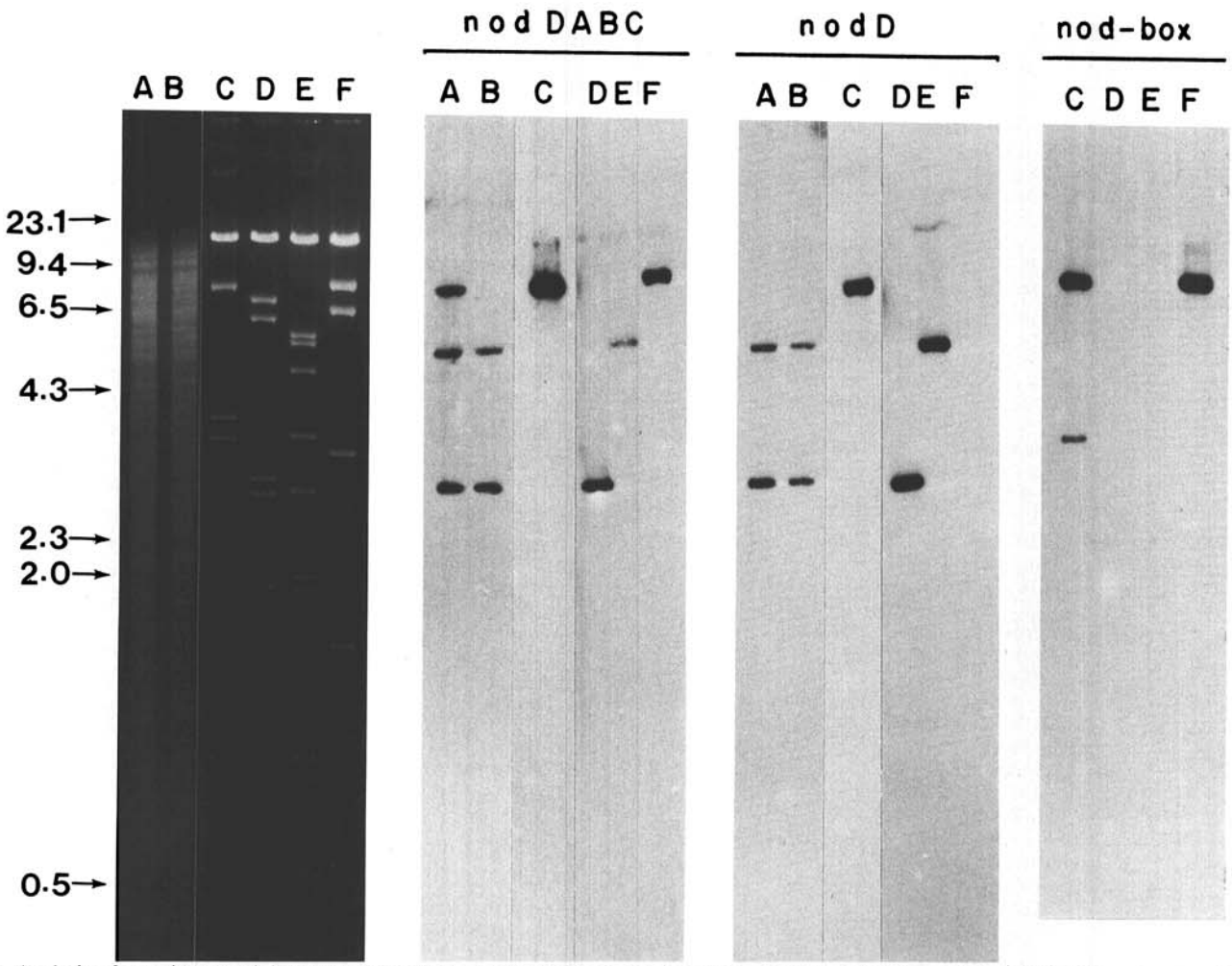

Fig. 1. Analysis of cosmids containing *nod* genes of *Rhizobium fredii*. **A** and **B** contain genomic DNA from strain USDA257 and mutant 257B3, respectively. **C**, pRMSL26 (positive control). **D**, pRK1309. **E**, pRK1489. **F**, pRF1. DNA was digested with *Eco*RI before electrophoresis. The left panel shows ethidium bromide-stained gels. The remaining panels show results of hybridizing replicate Southern blots with the probes identified above individual panels. The *nodDABC* probe was the 2.2-kb *Bam*HI/*Hin*dIII insert of pRMSL42. The *nodD* probe was a 250-bp *Bam*HI/*Hin*dIII subfragment from pRMSL42. The *nod*-box probe was a synthetic oligonucleotide (see Materials and Methods).

*Eco*RI fragments of pRK1309 and pR1489, respectively (Fig. 1). An oligonucleotide probe corresponding to the *nod*-box hybridized to two *Eco*RI fragments of pRMSL26, including the 8.7-kb fragment known to contain *nodABC* (Egelhoff *et al.* 1985). In contrast, this probe hybridized only to the 9.2-kb fragment of pRF1. These data collectively indicated that there are two unlinked copies of *nodD* in the genome of USDA257, and they allowed us to deduce the location of *nodABC* linked to the *nod*-box on the 9.2-kb fragment. This latter fragment consequently was subcloned into broad- and narrow-host range vectors for further analysis.

Figure 2 shows a restriction map of the 9.2-kb *Eco*RI fragment. Preliminary hybridization analysis localized *nodABC* to an internal, 1.7-kb *Bam*HI fragment, and so sequencing was initiated in this area. We ultimately sequenced a 3,094-bp region as indicated on the map in Figure 2. Figure 3 gives this sequence and identifies the three open reading frames that were found within it. These extended from nucleotide position 437 to 1,025 (654 bp; see comments below about the initiation codon), 1,024 to 1,666 (642 bp), and 1,683 to 2,922 (1,239 bp). Based on computerized comparison with known sequences, these reading frames corresponded to *nodA*, *nodB*, and *nodC*, respectively, and encoded proteins of 21.9, 23.4, and 44.7 kD.

Both the nucleotide and deduced amino acid sequences of the three open reading frames were compared with *nodABC* sequences from other rhizobia. This process identified some expected similarities. Thus, there is high, 65–73% overall sequence homology between *nodA*, *nodB*, and *nodC* from USDA257 and homologous genes from *R. loti* (Collins-Emerson *et al.* 1990), *R. meliloti* (Török *et al.* 1984; Egelhoff *et al.* 1985; Jacobs *et al.* 1985), *R. l.* bv. *viciae* (Rossen *et al.* 1984) and *trifolii* (Schofield and Watson 1986), and *Bradyrhizobium* sp. (Scott 1986). This similarity leads to a corresponding 65–74% conservation of amino acid sequence for each of the three genes. In contrast, *nodABC* from *Azorhizobium caulinodans* (Goethals *et al.* 1989) and *R. l.* bv. *phaseoli* (Vázquez *et al.* 1991) are more distantly related, encoding proteins with only about 42–57% amino acid homology to the corresponding gene products from USDA257. An exception to this generality is NodA from *R. l.* bv. *phaseoli*, which was 68% homologous with NodA from strain USDA257. The sequence from USDA257 contained a single *nod*-box (underlined in Fig. 3), which ended approximately 120 bp upstream from the beginning of the *nodA* reading frame. It is highly homologous to the consensus sequence derived from other rhizobia (Spaink *et al.* 1987), differing in only five of 46 conserved nucleotides spanning a 66-bp region.

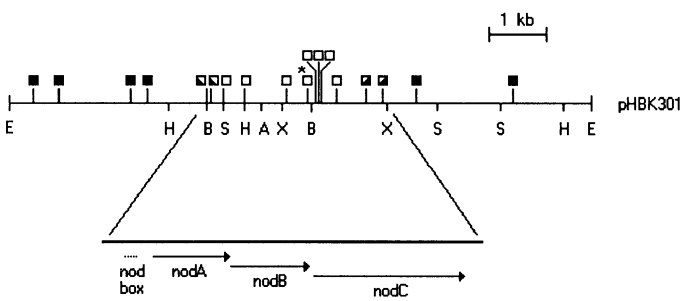

Fig. 2. Coordinated physical and genetic map of the 9.2-kb *nodABC* region of *Rhizobium fredii* USDA257. Restriction sites used for mapping the fragment are indicated. The vertical bars above the fragment represent individual insertion sites for MudII1734. Open and closed boxes indicate insertions that abolish and have no effect on nodulation of Peking soybean, respectively. The half-filled boxes represent insertions with quantitative effects on nodulation as described in the text. The insertion used to monitor induction of *nodC* is indicated with an asterisk. The expanded bar below the dashed lines represents the sequenced region. The *nod*-box and the positions and direction of transcription of *nodA*, *nodB*, and *nodC* are indicated. Abbreviations for restriction enyzmes: A, *Apa*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sac*I; X, *Xho*I.

Computer analysis also identified significant divergence between USDA257 and other rhizobia, especially at the 5′ and 3′ extremes of the *nodABC* region. Strain USDA257 has five potential initiation codons for *nodA* (methionines encircled in Fig. 3). Beginning with the first potential methionine, 70 deduced N-terminal amino acids of NodA of USDA257 were aligned with similar sequences from other rhizobia in Figure 4A. With the exception of the genes from *Bradyrhizobium* sp. and *R. l.* bv. *viciae*, all are believed to initiate from a conserved ATG that aligns with the fourth potential *nodA* initiation codon (position 437) of USDA257. This codon was associated with a well-positioned Shine-Delgarno sequence (GGAG), and there was strong homology in the deduced amino acid sequence 3′ from this site (Fig. 4A). In contrast, the nucleotide sequence upstream from this site was poorly conserved, ranging from 54% homology to the *R. meliloti* sequence to essentially no homology to the *Bradyrhizobium* sequence. These observations made it likely that *nodA* of USDA257, like that from *R. meliloti*, *R. l.* bv. *phaseoli* and *trifolii*, and *A. caulinodans*, initiated from the fourth ATG.

The C-terminus of NodC from USDA257 likewise appeared to be unique. The *R. fredii* protein retained a characteristic cysteine cluster and the hydrophobic domains (Török *et al.* 1984) of NodC from other rhizobia. But the deduced protein was truncated relative to NodC of other organisms, and the last seven amino acids had very low homology to corresponding amino acids of NodC from other rhizobia (Fig. 4B). The divergence with *B. japonicum*, which shares a large number of plant hosts with *R. fredii*, was particularly striking. NodC of *B. japonicum* was 72 amino acids longer than that of *R. fredii*, and the final 24 residues of the protein were encoded by sequences that simultaneously encode the N-terminus of NodS in another reading frame (Göttfert *et al.* 1990). Such sequences were not present in this area of the *R. fredii* genome.

Computerized comparisons of the 3,094-bp sequenced region with the EMBL data base revealed no consistent patterns of extensive homology with genes other than *nodABC*. Several short segments, however, had significant homology with sequences from *Agrobacterium*. These included an 111-bp stretch beginning at nucleotide 478 with 60% homology to a portion of the T-DNA from the Ri plasmid of *A. rhizogenes* (Slightom *et al.* 1986), and a 47-bp segment beginning at nucleotide 2,581 with 70% homology to octopine synthase (DeGreve *et al.* 1982). More extensive homology to sequences from *A. rhizogenes* also have been detected in USDA257, but these lie outside the

nodABC coding region (Krishnan and Pueppke 1991b).

Functional analysis of the 9.2-kb *Eco*RI fragment. The symbiotic importance of genes carried on the 9.2-kb *Eco*RI fragment was assessed by two broad strategies. One involved random mutagenesis of the fragment with Mud-II1734, followed by transfer into Nod$^-$ mutant 257B3, and evaluation of nodulation phenotypes on Peking soybean. This mutant originally was identified during a screen of USDA257 mutants containing random Tn*5* insertions; it is Nod$^-$ on all tested plant hosts and lacks the 9.2-kb *Eco*RI fragment with homology to pRMSL42 (Heron *et al.* 1989). In contrast, both USDA257 and 257B3(pHBK303) pro-

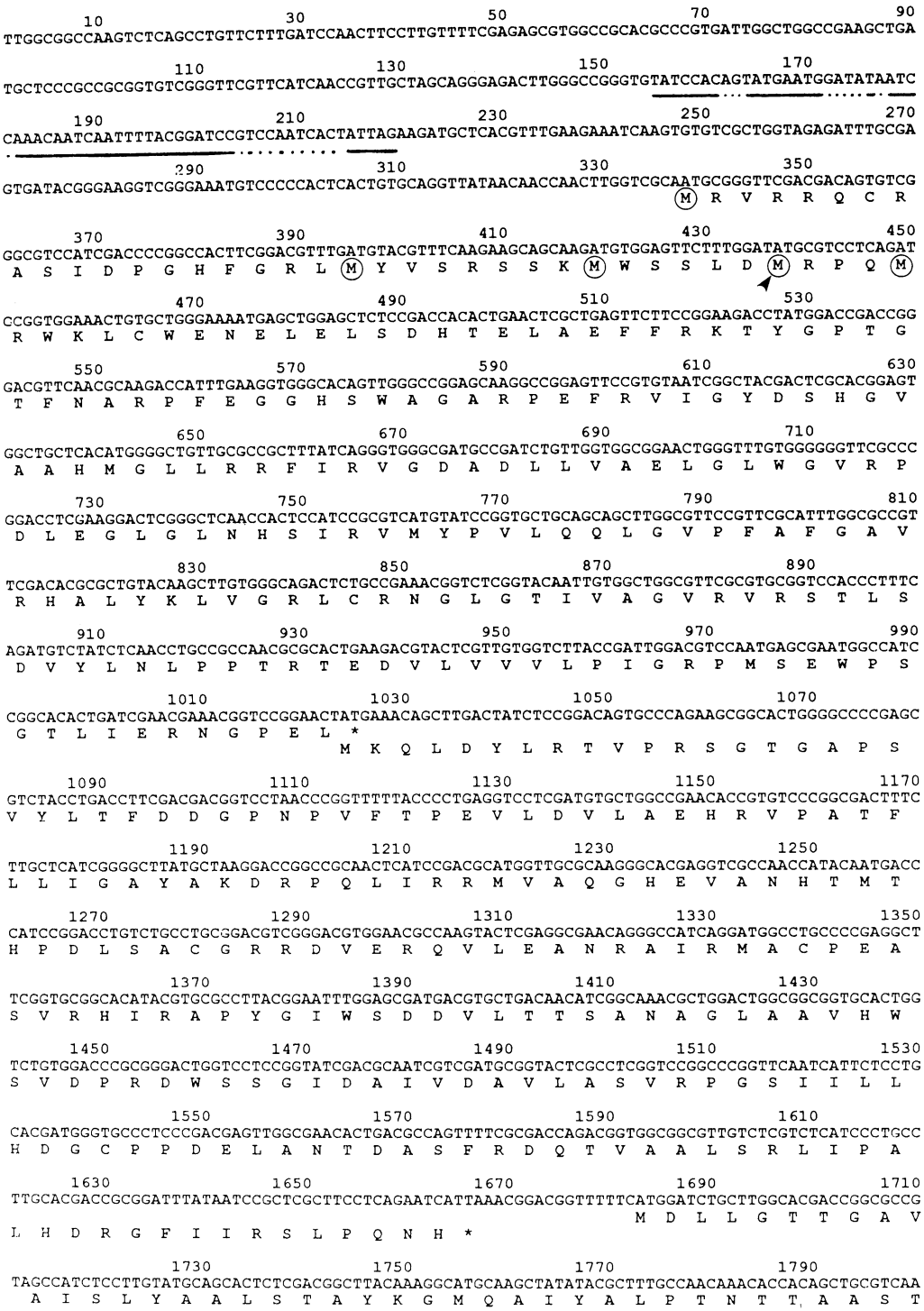

Fig. 3. Sequence of the *nodABC* region of *R. fredii* USDA257. The sequence covers 3,094 nucleotides and includes open reading frames corresponding to *nodA* (begins at position 338), *nodB* (begins at position 1,024), and *nodC* (begins at position 1,683). Deduced amino acid sequences for the three proteins are indicated. Methionines encoded by the five potential initiation codons for NodA are encircled. The consensus *nod*-box is underlined, and the most likely actual initiation site (see text) is identified with an arrow. **(Fig. 3.** *continued on next page.)*

duced normal nitrogen-fixing nodules on Peking soybean. A total of 18 MudII1734 insertions in pHBK303 were mapped and tested in the 257B3 background (Fig. 2). All strains containing insertions within *nodABC* retained a Nod^- phenotype. Conversely, all strains with insertions well outside the borders of this region yielded a Nod^+ phenotype indistinguishable from that of 257B3(pHBK303). Four insertions near the termini of the operon gave intermediate phenotypes, in which nodules formed, but in relatively low numbers.

A MudII1734 insertion within *nodC* (Fig. 2) was chosen to monitor the activation of the operon by nine flavonoids. The inducers were selected based on their known abilities to activate a *nodC*::*lacZ* fusion in another strain of *R. fredii* (Kosslak *et al.* 1987). Three of the compounds were essentially inactive at 1,000 nM (Table 2) and showed minimal inducer activity (less than twofold induction) at the highest concentration tested, 10,000 nM. Naringenin reproducibly increased β-galactosidase activity by three- to fivefold, and the remaining five compounds uniformly induced the fusion by five- to sevenfold. Concentrations required for half-maximal induction, however, differed sharply among the five compounds. By this criterion, daidzein was a 20-fold more potent inducer than genistein or chrysin (Table 2).

We also evaluated the ability of the 9.2-kb fragment to complement nodulation-defective mutants of *R. meliloti*. In one series of experiments, complementation of insertion-mutations in *nodA*, *nodB*, and *nodC* of *R. meliloti* was examined independently in three plant hosts of *R. meliloti* (Table 2). *nodABC* from USDA257 restored nodulation of *M. truncatula*, *M. sativa*, and *T. foenum-graecum* by each of the mutants, but symbiotic phenotypes were far from wild-type. In every case, the number of nodules produced by transconjugants containing *nodABC* of USDA257 was less than that produced by transconjugants containing *nodABC* of *R. meliloti* (Table 3). This attenuation was particularly evident with *T. foenum-graecum*,

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     1810                1830                1850                1870                1890
CGCCCGTGACCGGCTCCGGTGCACCGCCGAGCGTGGATGTTATCGTCCCCTGCTACAATGAGGATCCGCGCGCGCTCTCGGCGTGCCTAG
  P  V  T  G  S  G  A  P  P  S  V  D  V  I  V  P  C  Y  N  E  D  P  R  A  L  S  A  C  L  A

               1910                1930                1950                1970
CTTCCATTGCAAAGCAAGACTACGCTGGAGAGTTGCGGGTCTACGTGGTTGACGACGGTTCTGGCAATCGCAACGCCATCATACCTGTAC
  S  I  A  K  Q  D  Y  A  G  E  L  R  V  Y  V  V  D  D  G  S  G  N  R  N  A  I  I  P  V  H

     1990                2010                2030                2050                2070
ACGATCATTATGCGTGCGACCCGAGGTTCCGCTTTATCCTGATGCCAAAGAACGTCGGAAAGCGCAAGGCCGAGATTGTCGCAATACGGG
  D  H  Y  A  C  D  P  R  F  R  F  I  L  M  P  K  N  V  G  K  R  K  A  E  I  V  A  I  R  E

               2090                2110                2130                2150
AATCATCGGGAGATTTGGTGCTCAACGTTGACTCGGACACGACCATTGCGCCGGACGTAGTCACGAAACTTGCCCTGAAGATGTACAGTC
  S  S  G  D  L  V  L  N  V  D  S  D  T  T  I  A  P  D  V  V  T  K  L  A  L  K  M  Y  S  P

     2170                2190                2210                2230                2250
CCGCGGTCGGCGCCGCGATGGGTCAGTTGACGGCCAGCAACCGCAGCGACACATGGCTGACGCGGTTGATCGACATGGAGTACTGGCTCG
  A  V  G  A  A  M  G  Q  L  T  A  S  N  R  S  D  T  W  L  T  R  L  I  D  M  E  Y  W  L  A

               2270                2290                2310                2330
CCTGCAACGAGGAACGAGCAGCACAGGCTCGCTTTGGAGCCGTTATGTGTTGCTGCGGCCCGTGTGCCATGTACCGGCGGTCCGCACTCC
  C  N  E  E  R  A  A  Q  A  R  F  G  A  V  M  C  C  C  G  P  C  A  M  Y  R  R  S  A  L  L

     2350                2370                2390                2410                2430
TATTGCTGCTCGATAAATACGAGACGCAACTGTTTCGAGGCAGGCCAAGCGACTTCGGGGAAGACCGCCACCTCACAATCCTCATGCTGA
  L  L  L  D  K  Y  E  T  Q  L  F  R  G  R  P  S  D  F  G  E  D  R  H  L  T  I  L  M  L  N

               2450                2470                2490                2510
ATGCAGGCTTTCGAACCGAGTACGAACCGGACGCCATCGCGGCGACGGTCGTTCCAAACTCGATGGGGGCCTATCTGCGCCAACAACTGC
  A  G  F  R  T  E  Y  E  P  D  A  I  A  A  T  V  V  P  N  S  M  G  A  Y  L  R  Q  Q  L  R

     2530                2550                2570                2590                2610
GCTGGGCACGCAGCACGTTTCGCGACACATTGCTCGCGCTCCGCCTACTGCCGGGCCTTGATCGCTATCTTACGCTGGACGTGATCGGAC
  W  A  R  S  T  F  R  D  T  L  L  A  L  R  L  L  P  G  L  D  R  Y  L  T  L  D  V  I  G  Q

               2630                2650                2670                2690
AGAATCTTGGTCCGCTGCTCCTAGCCCTCTCGGTCCTGACGGGGCTAGCACAGCTCGCTCTGACGGCCACAGTGCCTTGGTCGACGATCC
  N  L  G  P  L  L  L  A  L  S  V  L  T  G  L  A  Q  L  A  L  T  A  T  V  P  W  S  T  I  L

     2710                2730                2750                2770                2790
TGATGATTGCATCTATGACAATGGTCCGCTGCGGCCTGGCGGCGTTTCGAGCGCGAGAGCTGCGATTCCTTGGGTTTTCGCTGCACACCC
  M  I  A  S  M  T  M  V  R  C  G  L  A  A  F  R  A  R  E  L  R  F  L  G  F  S  L  H  T  L

               2810                2830                2850                2870
TCCTCAACGTCGCTCTCCTGCTCCCCCTCAAAGCATATGCGTTGTGCACGTTGAGCAACAGCGACTGGCTGTCGCGTGGTTCCCCGGCTG
  L  N  V  A  L  L  L  P  L  K  A  Y  A  L  C  T  L  S  N  S  D  W  L  S  R  G  S  P  A  A

     2890                2910                2930                2950                2970
CCGCACCCAACGGCGTAAAGGATTCTCCTGAACCCCATTGCTAAAACAAGCGCTGCGGAGCCGTCTGAAACTGCGGCACCCGCGTCGCTG
  A  P  N  G  V  K  D  S  P  E  P  H  C  *

               2990                3010                3030                3050
GCTCAGCCTTGCGCAGCTTGCGAGGCTGCTGACATCAGTCAGTCACTGCGGTGGCGGTAGCCGGCTTTGCCAAACAGGCAAGCGAATTTA

     3070                3090
AGATAGGACGAGAAATTGCAGCTATTGACTCGAG
```

Fig. 3. *continued from previous page.*

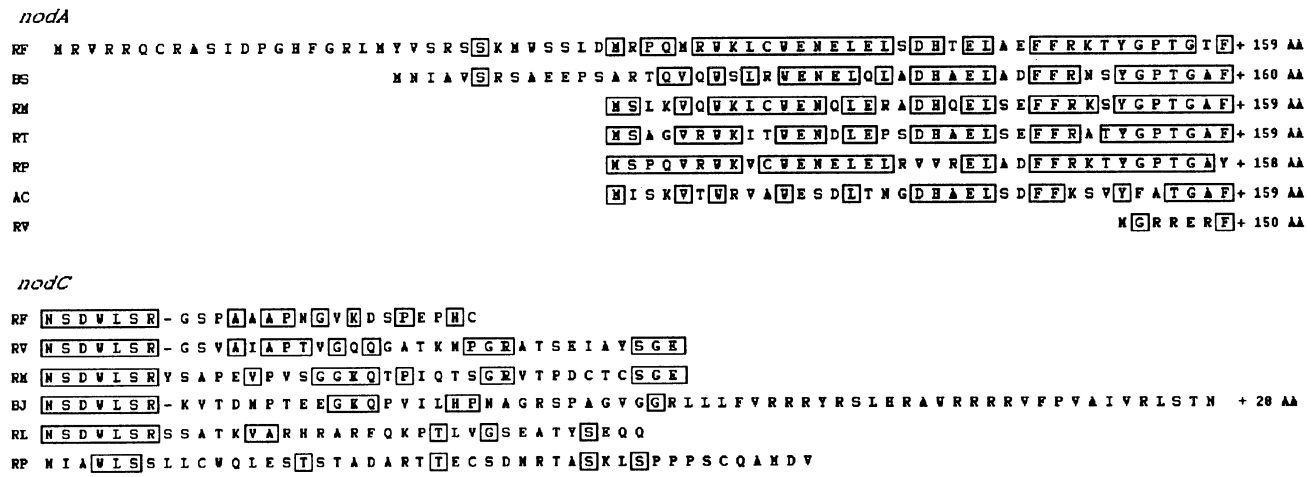

Fig. 4. Comparison of the deduced N-terminus of NodA and the deduced C-terminus of NodC from *Rhizobium fredii* USDA257 with deduced homologous protein sequences from other rhizobia. RF, BS, BJ, RM, RT, RP, AC, RL, and RV refer, respectively, to sequences from *R. fredii*, *Bradyrhizobium* sp. from *Parasponia* (Scott 1986), *B. japonicum* (Göttfert *et al.* 1990), *R. meliloti* (Egelhoff *et al.* 1985; Jacobs *et al.* 1985), *R. leguminosarum* bv. *trifolii* (Schofield and Watson 1986), *R. l.* bv. *phaseoli* (Vázquez *et al.* 1991), *A. caulinodans* (Goethals *et al.* 1989), *R. loti* (Collins-Emerson *et al.* 1990), and *R. l.* bv. *viciae* (Rossen *et al.* 1984).

Table 2. Induction of a *nodC*::*lacZ* fusion by flavonoids[a]

Compound	Class	Fold activity (at 1 μM)	Half-maximal induction (nM)
Daidzein	Isoflavone	5.5–6.3	10
Luteolin	Flavone	5.9–6.1	50
Apigenin	Flavone	4.8–6.3	50
Genistein	Isoflavone	5.6–6.9	200
Chrysin	Flavone	5.4–5.6	200
Biochanin A	Isoflavone	0.9–1.4	...
Naringenin	Flavone	2.7–4.2	400
Quercetin	Flavonol	1.7	...
Umbelliferone	Flavonol	1.2	...

[a] Compounds were tested at concentrations ranging from 5 to 10,000 nM in replicate experiments as described in Materials and Methods. Uninduced cultures expressed 70–100 Miller units of β-galactosidase activity, depending on the experiment.

in which the percentage of nodulated plants was reduced by nearly 50% relative to controls.

Plants inoculated with transconjugants containing *nodABC* from *R. meliloti* were uniformly green and healthy. Plants inoculated with transconjugants containing *nodABC* of *R. fredii* were less uniform; most were severely stunted, with yellow or purple (in the case of *M. truncatula*) leaves. These differences were clearly reflected in reduced shoot dry weights. In *M. sativa*, for example, dry weights of controls inoculated with the transposon-mutants averaged about 7 mg per plant. Whereas, values for mutants complemented with pRMSL26 ranged from 32 to 45 mg per plant, corresponding weights for plants inoculated with mutants of *nodA*, *nodB*, and *nodC* complemented with pHBK303 were only 40, 37, and 40% as great, respectively. Similar relationships were evident for *M. truncatula* and *T. foenum-graecum* (data not shown).

DISCUSSION

In comparison to many other rhizobia, relatively little is known about *R. fredii* and the *nod* genes that it deploys in symbiosis. Only recently discovered, the species appears to be both evolutionarily distinct from its siblings and internally heterogeneous (Chen *et al.* 1988; Young and Johnston 1989). Strain-dependent variability in host specificity also is apparent (Heron and Pueppke 1984; Chatterjee *et al.* 1990). These unique biological traits prompted us to begin characterizing strain USDA257 as a model representative of the group. We recently described *nolC*, a negatively acting host-specificity gene in this strain (Krishnan and Pueppke 1991a). Here we provide evidence that USDA257 has two copies of *nodD*, neither of which is closely linked to *nodABC*. This arrangement appears to be roughly similar to that in two other strains of *R. fredii*, USDA191 and USDA193 (Ramakrishnan *et al.* 1986; Appelbaum *et al.* 1988).

Little is known about the host inducers that naturally interact with the *nodD* gene product to activate transcription of *nodABC* in *R. fredii*. There is evidence that soybean roots contain daidzein, naringenin, and genistein (Kosslak *et al.* 1987; Bassam *et al.* 1988; Sadowsky *et al.* 1988; Cho and Harper 1991; Graham 1991), and high concentrations (5,000 nM) of all three compounds can induce a heterologous *nodC*::*lacZ* fusion in *R. fredii* USDA191 (Kosslak *et al.* 1987). High concentrations of many other structurally diverse flavonoids, however, exhibit comparable inducer activities in this system. We found that at considerably lower concentrations (<100 nM), daidzein was by far the most potent inducer of a *nodC*::*lacZ* fusion from *R. fredii* USDA257 in a *nodC*-mutant of strain USDA257. At high enough concentrations, both daidzein and genistein can enhance β-galactosidase activity five- to sevenfold, but half-maximal activity requires 20 times more genistein than daidzein. Although it is conceivable that minor or unidentified flavonoids such as luteolin (Cho and Harper 1991; Graham 1991) may play a role in induction, our observations suggest that daidzein is by far the most likely native inducer of the *nodABC* operon in strain USDA257.

We have for the first time sequenced *nodABC* from *R. fredii* and compared its structure to *nodABC* from other rhizobia. *nodA*, *nodB*, and *nodC* of USDA257 share

Table 3. Complementation analysis of the *nodABC* region of *Rhizobium fredii*

Recipient	Plasmid	Mean nodule no. (±SE) per plant[a]			*M. sativa* dry weight (mg/plant)
		Trigonella foenum-graecum	*Medicago truncatula*	*M. sativa*	
RCR2011 (wild-type)	None	18.5 ± 2.4 (95)	14.6 ± 1.7 (100)	9.4 ± 1.2 (100)	32.0
GMI5382 (nodA⁻)	None	0	0	0	7.8
GMI5383 (nodB⁻)	None	0	0	0	6.7
GMI5387 (nodC⁻)	None	0	0	0	7.1
GMI5382 (nodA⁻)	pHBK303	10.0 ± 2.0 (63)	6.6 ± 1.4 (93)	4.4 ± 1.1 (84)	18.1
GMI5383 (nodB⁻)	pHBK303	8.7 ± 1.9 (63)	5.7 ± 0.9 (89)	7.8 ± 1.1 (100)	15.1
GMI5387 (nodC⁻)	pHBK303	10.5 ± 1.8 (79)	2.9 ± 1.0 (50)	5.2 ± 1.1 (85)	13.0
GMI5382 (nodA⁻)	pRMSL26	15.4 ± 1.9 (100)	11.1 ± 1.1 (100)	15.1 ± 1.8 (100)	44.6
GMI5383 (nodB⁻)	pRMSL26	20.1 ± 1.4 (100)	11.7 ± 1.1 (100)	11.3 ± 1.2 (100)	40.5
GMI5387 (nodC⁻)	pRMSL26	20.8 ± 2.0 (100)	11.3 ± 1.0 (100)	13.8 ± 1.3 (100)	32.7

[a] Uninoculated control plants remained nodule-free. All data are means from two separate experiments ($N = 20$). Numbers in parentheses are the percentages of plants that produced nodules.

nucleotide and amino acid sequence homology with these previously described genes. This includes the 1-bp translational couple between the termination codon of *nodA* and the initiation codon of *nodB*, as well as conservation of the signature hydrophobic domains and a characteristic cysteine cluster of *nodC* (Török *et al.* 1984). Moreover, a consensus *nod*-box promoter was present and in the expected position to mediate NodD-dependent activation of the *nodABC* operon. The region of greatest divergence among the rhizobia corresponded to the 3′ end of *nodC*. All deduced NodC proteins except those from *R. l.* bv. *phaseoli*, were highly homologous within a perfectly conserved block of seven amino acids ending with arginine, as shown in Figure 3. Thereafter, the proteins diverged. The tail of the *R. fredii* protein contained 18 amino acids, 11 of which were hydrophilic and only four of which were conserved with the slightly longer termini of the *R. meliloti* and *R. l.* bv. *viciae* proteins. The *B. japonicum* protein extends for 90 amino acids beyond the last conserved arginine (Göttfert *et al.* 1990).

The composition and organization of genes downstream from *nodC* is variable, depending on the *Rhizobium* species. In *R. meliloti* and biovars of *R. leguminosarum*, the operon includes genes termed *nodI* and *nodJ* (Young and Johnston 1989). In *B. japonicum*, *nodSU* is inserted between *nodC* and *nodIJ* so that the open reading frames for *nodC* and *nodS* overlap (Göttfert *et al.* 1990). Interestingly, none of these genes seems to be indispensible for normal symbiosis (Göttfert *et al.* 1990; Martinez *et al.* 1990). We have been unable to detect *nodIJ* or *nodSU* adjacent to *nodC* in *R. fredii*. Strain USDA257 does in fact contain *nodSU*, but this region is elsewhere in the genome (Krishnan and Pueppke, in preparation). These observations make it likely that the organization of symbiosis loci of USDA257 is unique.

In spite of the structural similarities between *nodABC* of USDA257 and *nodABC* of other rhizobia, cloned copies of the *R. fredii* genes did not fully complement mutants of *R. meliloti* to wild-type. Transconjugants nodulated three plant hosts of *R. meliloti*, but nodule number, percentage of plants that nodulate, and plant dry weight were substantially and consistently reduced relative to controls. pRK415 and pLAFR1 are known to be unstable under certain conditions (Long *et al.* 1982; Keen *et al.* 1988), and thus failure to complement to wild-type may simply reflect instability of the reintroduced DNA. In the absence of antibiotic selection, we have found pRMSL26 to be stable in *R. meliloti* and pHBK303 to be lost at about 2% per generation. Irrespective of whether the complementing plasmid was pRMSL26 or pHBK303, however, plasmid-encoded resistance to tetracycline was uniformly present in about 1% of the *Rhizobium* colonies recovered from alfalfa nodules. This makes it unlikely that plasmid instability accounts for the observed differences in complementation. A second possibility is that heterologous *R. fredii* genes may not be fully expressed in *R. meliloti*. Cosmids containing not only *nodABC*, but also *nodD* of *R. fredii*, can in fact complement *nodA*, *nodB*, *nodC*, and *nodD* mutants of *R. meliloti* to wild-type (Ramakrishnan *et al.* 1986). Although this suggests that *nodD* of *R. fredii* may be required for full expression of *R. fredii* common *nod* genes in *R. meliloti* backgrounds, *nodD* from another broad host range strain, NGR234, is not required for the expression of its common *nod* genes in *R. meliloti* (Bachem *et al.* 1985).

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