CURRENT MICROBIOLOGY Vol. 45 (2002), pp. 378–382
DOI: 10.1007/s00284-002-3772-z

Current Microbiology
An International Journal

Molecular Cloning and Characterization of *nodD* Genes from *Rhizobium* sp. SIN-1, a Nitrogen-Fixing Symbiont of *Sesbania* and Other Tropical Legumes

Debashis Rana,[1] Hari B. Krishnan[2]

[1]Department of Plant Microbiology and Pathology, University of Missouri, Columbia, MO 65211, USA
[2]Plant Genetics Research Unit, USDA-ARS and Department of Agronomy, 108W Curtis Hall, University of Missouri, Columbia, MO 65211, USA

Received: 17 January 2002 / Accepted: 18 February 2002

Abstract. *Rhizobium* sp. SIN-1, a nitrogen-fixing symbiont of *Sesbania aculeata* and other tropical legumes, carries two copies of *nodD*, both on a *sym* plasmid. We have isolated these two *nodD* genes by screening a genomic library of *Rhizobium* sp. SIN-1 with a *nodD* probe from *Sinorhizobium meliloti*. Nucleotide sequence and the deduced amino acid sequence analysis indicated that the *nodD* genes of *Rhizobium* sp. SIN-1 are most closely related to those of *R. tropici* and *Azorhziobium caulinodans*. *Rhizobium* sp. SIN-1 *nodD*1 complemented a *S. meliloti nodD*1*D*2*D*3 negative mutant for nodulation on alfalfa, but failed to complement a *nodD*1 mutant of *S. fredii* USDA191 for soybean nodulation. A hybrid *nodD* gene, containing the N-terminus of *S. fredii* USDA191 *nodD*1 and the C-terminus of *Rhizobium* sp. SIN-1 *nodD*1, complemented the *nodD*1 negative mutant of USDA191 for nodulation on soybean.

Members of the genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Azorhizobium*, collectively termed rhizobia, can enter into symbiotic association with a large number of leguminous plants. Successful symbiosis depends on signal exchange between plant and rhizobia. Legume roots release secondary metabolites, mostly flavonoids, which are perceived by the rhizobia. In response, the symbiont releases a return signal, the Nod factors. These compounds are oligomers of β-1,4-linked N-acetyl-D-glucosamine that carry various substitution groups and fatty acid side chains [4, 11]. Nod factors at very low concentrations are able to induce a variety of responses including root hair deformation, cortical cell division, induction of plant genes, and the elicitation of nodule-like structures [11].

The nodulation (*nod*) regulon comprises a set of bacterial genes that direct the communication circuit between the plant and rhizobia. Among them, *nodD*, a member of the *LysR* family of transcriptional regulators, plays a crucial role in the early recognition events between rhizobia and legumes [7, 14]. The *nodD* product functions mainly as a regulator of other nodulation genes. This gene is usually expressed constitutively at low levels and, in the presence of plant flavonoids, its product activates the transcription of the common *nod* genes and other host-specific *nod* genes. Usually a highly conserved *nod* box region, present in the promoter region of inducible *nod* genes, is required for *nodD*-dependent regulation. In addition, the *nodD* product contributes to host specificity by its selective ability to recognize plant signals from different hosts [3, 7].

Many *Sesbania* species are fast growers and fix nitrogen under flooded and dry conditions. A few *Sesbania* species form stem nodules. *S. rostrata* has been shown to incorporate as much as 200 kg of nitrogen/hectare/season [2]. Its symbiont, *Azorhizobium caulinodans*, which nodulates both the stem and roots, has attracted considerable attention [2]. We are examining the symbiotic association between *Sesbania aculeata* and its symbiont, *Rhizobium* sp. SIN-1. The fatty acid profile, 16S rRNA gene sequence and LPS structure of this strain indicate it is phylogenetically related to *R. galegae* [12], a species that nodulates temperate legumes. In contrast to *A. caulinodans*, where the *nod* genes are located on the chromosome, the *nod* genes of *Rhizobium* sp. SIN-1 are

Correspondence to: H.B. Krishnan; *email*: KrishnanH@missouri.edu

located on the *sym* plasmid [12]. Here we report the molecular cloning and partial characterization of the *nodD* genes of *Rhizobium* sp. SIN-1.

Materials and Methods

Bacterial strains and growth conditions. *Rhizobium* sp. SIN-1, *Sinorhizobium fredii* USDA191, and *S. meliloti* were routinely grown in yeast extract-mannitol (YEM) medium at 30°C. *S. fredii* USDA191 *nodD*1$^-$ and *nodD*1*D*2$^-$ negative mutants [1] were from M.J. Sadowsky (University of Minnesota, St. Paul). The *nodD*1*D*2*D*3$^-$ negative mutant of *S. meliloti* [9] was obtained from F.M. Ausbel (Harvard Medical School, Boston, MA). *E. coli* was cultivated in Luria-Bertani medium at 37° C. When required, the media were supplemented with antibiotics (in $\mu g\ ml^{-1}$) ampicillin, 50; tetracycline, 10; kanamycin, 50, spectinomycin, 25; chloramphenicol, 25.

Cloning of *Rhizobium* sp. SIN-1 *nodD* genes. A genomic library of *Rhizobium* sp. SIN-1 was constructed in pLAFR1 as described by Sambrook et al. [13]. Twelve hundred tetracycline-resistant colonies were screened by colony hybridization. A 0.2-kb *Bg*/II-*Bam*HI fragment of pRMSL26 [6] containing the N-terminus portion of the *nodD*1 of *S. meliloti* was labeled with [α-^{32}P]dCTP and used as a probe. Cosmid DNA from the positive colonies (pDBR100 and pDBR200) was digested with *Eco*RI and subjected to Southern blot analysis [13]. DNA sequence of *Rhizobium* sp. SIN-1 *nodD*1 and *nodD*2 was determined with Taq Dye Deoxy TM Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Construction of *nodD*1 hybrids. The coding region of *nodD*1 of *S. fredii* USDA191 [1] and *Rhizobium* sp. SIN-1 contains a conserved *Bam*HI site. This site was used to create hybrid *nodD*1 genes. To create a hybrid between the N-terminus region of *nodD*1 of USDA191 and C-terminus of *nodD*1 of SIN-1, the following steps were performed. First, the 1.55-kb *Eco*RI-*Bam*HI fragment containing the N-terminus of *nodD*1 of USDA191 was cloned into pRK415. The resulting plasmid was digested with *Bam*HI-*Xba*I and ligated with a 1.6-kb *Bam*HI-*Xba*I fragment containing the C-terminus of *Rhizobium* sp. SIN-1. Similarly, a hybrid nodD composed of the N-terminus of *nodD*1 of SIN-1 and the C-terminus of USDA191 was also created by utilizing appropriate restriction sites. The *nodD*1, *nodD*2, and the hybrid *nodD* constructs were transferred by triparental mating into appropriate *Rhizobium* backgrounds with pRK2013 as the helper plasmid. Nodulation phenotypes were tested in Magenta-type Leonard jars as described earlier [10]. Plants were harvested 5 weeks after inoculation, and nodulation responses were assessed visually.

Results

***Rhizobium* sp. SIN-1 has two *nodD* genes.** To identify *nodD* homologous sequences in *Rhizobium* sp. SIN-1, the *Eco*RI-digested genomic DNA of this species was probed with *S. meliloti nodD*1. Southern blot analysis revealed two hybridizing bands of 5.7 kb and 3.4 kb (Fig. 1). We designated the 5.7 kb as the *nodD*1 and the 3.4 kb fragment as the *nodD*2. In order to isolate the *nodD* homologs of *Rhizobium* sp. SIN-1, a size-fractionated genomic library in the cosmid vector pLAFR1 was screened by colony hybridization with *S. meliloti nodD*1 as the probe. Five positive cosmids were isolated, and

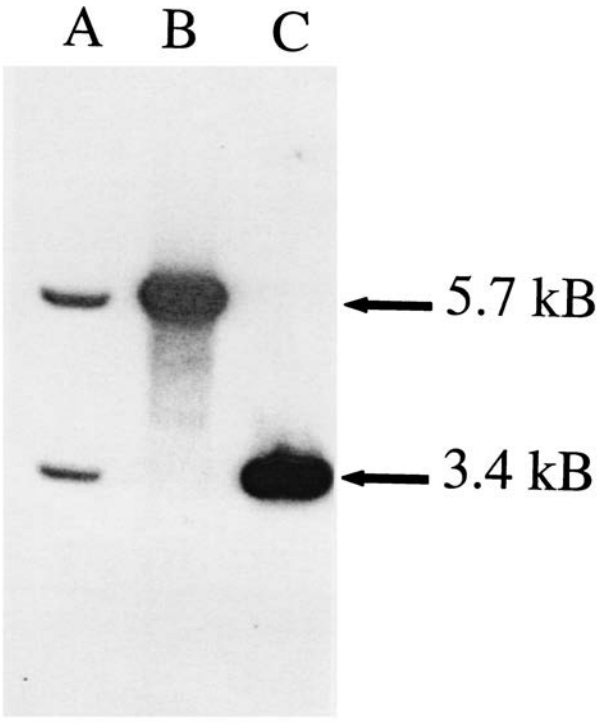

Fig. 1. Analysis of cosmids containing *nodD* genes of *Rhizobium* sp. SIN-1. Genomic DNA from *Rhizobium* sp. SIN-1 (lane A), cosmid DNA from pDBR100 (lane B), and pDBR200 (lane C) were digested with *Eco*RI and hybridized with a *S. meliloti nodD*1 probe (0.2 kb *Bg*/II-*Bam*HI fragment of pRMSL26). Sizes of the hybridizing fragments are indicated on the right side of the figure.

Southern blot analysis confirmed the presence of a 5.7-kb fragment in three of the cosmids, while a 3.4-kb fragment was found in the other two cosmids. We selected two cosmids (*nodD*1, pDBR100 and *nodD*2, pDBR200) for further characterization. The 5.7-kb insert from pDBR100 was cloned into pGEM7Zf(+) as pDBR101 and into the mobilizable vector pRK415 as pDBR102. Similarly, the 3.4-kb fragment from pDBR200 was cloned into pGEM7Zf(+) and pRK415, resulting in plasmids pDBR201 and pDBR202, respectively.

Sequence analysis of *nodD* genes of *Rhizobium* sp. SIN-1. The physical maps of the *nodD*1-and *nodD*2-containing fragments are shown in Fig. 2. A 1496-bp region of the plasmid pDBR101 was sequenced. The nucleotide sequence analysis revealed a single open reading frame (ORF) coding for a protein of 308 amino acids. The deduced molecular weight of this protein is 34,366 with an isoelectric point of 8.5. Similarly, 1232 bp of the plasmid pDBR201 was also sequenced. This sequence includes one ORF that codes for a protein with 310 amino acids. The molecular weight of the deduced protein is 35,201 with a theoretical isoelectric point of

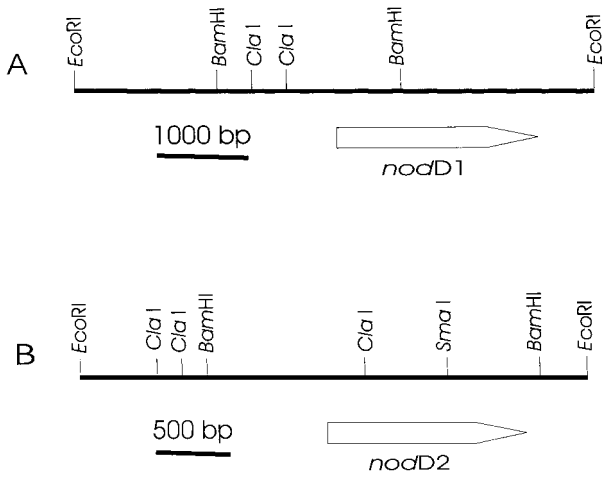

Fig. 2. Restriction maps of the *nodD*1 (2A) and *nodD*2 (2B) containing fragments of *Rhizobium* sp. SIN-1. Restriction sites used for mapping the fragments are indicated. The arrows below the maps indicate the position of the ORFs and their direction of transcription.

8.3. The nucleotide and the deduced amino acid sequences of *nodD*1 and *nodD*2 of *Rhizobium* sp. SIN-1 have been deposited in GenBank nucleotide sequence database under accession numbers AF254355 and AF254356. A computer search of the Swiss-prot database revealed that the NodD1 and NodD2 are similar to other known NodD proteins from different *Rhizobium* spp. In both cases, the homology is higher at the N-terminal region than the C-terminal region of the protein. The NodD1 and NodD2 have 60–68% identity with other known NodDs. However, NodD1 and NodD2 of *Rhizobium* sp. SIN-1 have only 48% identity with each other. The relationship between known NodDs was examined by creating a dendrogram with sequences from several different rhizobia, with the help of the MegAlign computer program. The dendrogram revealed that the NodD amino acid sequences from *Rhizobium* sp. SIN-1 clustered with the NodDs of *R. tropici* and *A. caulinodans* (data not shown).

Complementation analysis. *Rhizobium* sp. SIN-1 *nodD*1 and *nodD*2, cloned into the broad host range vector pRK415, were mobilized into *nodD*1$^-$ negative and *nodD*2$^-$ negative mutants of *S. fredii* USDA191, and a *nodD*1*D*2*D*3$^-$ negative mutant of *S. meliloti* by triparental mating. The transconjugants were tested for complementation of the *nodD* mutations by screening for nodulation on their appropriate hosts. *Rhizobium* sp. SIN-1 *nodD*1 was able to complement the *nodD*1*D*2*D*3$^-$ negative mutant of *S. meliloti*, but SIN-1 *nodD*2 could not. Wild-type *S. meliloti* produced an average of 14 ± 4 nodules/plant, while the *nodD* triple mutant of *S. meliloti* carrying the *Rhizobium* sp. SIN-1 *nodD*1 produced 12 ± 5 nodules/plant on *Medicago sativa*. Interestingly, neither *Rhizobium* sp. SIN-1 *nodD*1 nor *nodD*2 was able to complement the *S. fredii* USDA191 *nodD*1$^-$ negative mutant, even though the *nodD*1 of *Rhizobium* sp. SIN-1 and *S. fredii* USDA191 share extensive amino acid homology (Fig. 3). The N-terminal regions share 79% similarity, while the C-terminal regions are less conserved and show 61% similarity (Fig. 3). We created two hybrid *nodD*s by exchanging the DNA region encoding the N and C terminus of NodD1 of SIN-1 and USDA191. One of the constructs, pDBR301, carried the N-terminus of USDA191 NodD1 and the C-terminus of SIN-1 NodD1. Another construct, pDBR302, carried the N-terminus of SIN-1 NodD1 and the C-terminus of USDA191 NodD1. The USDA191 *nodD*1$^-$ negative mutant carrying pDBR301 induced 21 ± 5 nodules/plant on the soybean cultivar Peking, more than the control strain carrying its own *nodD*1 (12 ± 3 nodules/plant). However, pDBR302, the other hybrid NodD, did not complement the USDA191 *nodD*1-negative mutant for nodulation of the soybean cultivar Peking.

Discussion

Our study indicates that *Rhizobium* sp. SIN-1, like *S. fredii* USDA191, *Rhizobium* sp. NGR234, and *B. japonicum*, has two copies of *nodD* [8]. Occurrence of multiple copies of *nodD* has been reported for several different *Rhizobium* spp. *S. meliloti* has three *nodD* genes [9], while *R. tropici* has five [17]. On the other hand, *R. leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *trifolii*, and *Azorhizobium caulinodans* have only one *nodD* gene. Even though *nodD* is involved in responding to plant signals, there is no correlation between the number of copies of *nodD* and host range. For example, *Rhizobium* sp. NGR234, which nodulates 110 genera of legumes, has only two copies of *nodD*, while *R. meliloti*, which nodulates only a few legumes, has three copies of *nodD* [8]. The importance of more than one copy of *nodD* in rhizobia is not fully understood. It is proposed that different *nodD* genes may be involved in fine-tuning the response of rhizobia during nodulation of different host plants [8].

Our complementation studies have shown that *Rhizobium* sp. SIN-1 is a functional gene. The *nodD*1 of SIN-1 was able to complement a *nodD*1*D*2*D*3$^-$ negative mutant of *S. meliloti* for nodulation of alfalfa. This observation is consistent with earlier studies that have shown complementation of *nodD* mutations with heterologous *nodD* genes. For example, the NGR234 *nodD*1$^-$ negative mutant strain ANU1255 can be complemented by the *R. leguminosarum* bv. *viciae nodD* for root hair curling and for nodulation [5]. In contrast, the *nodD*1 of

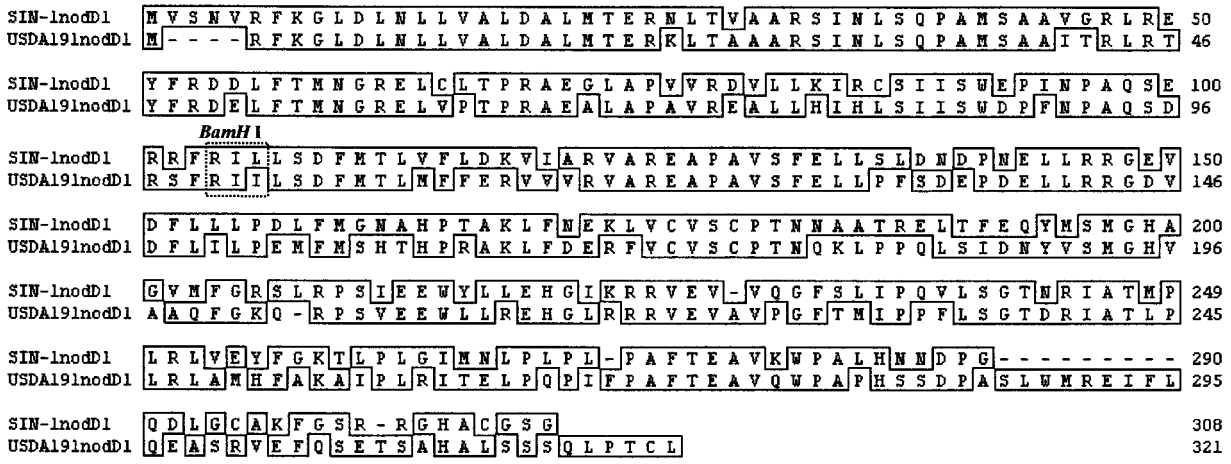

Fig. 3. Amino acid comparison of *nodD*1 between *Rhizobium* sp. SIN-1 and *Sinorhizobium fredii* USDA191. Alignment was created with the PILEUP program (Genetics Computer Group, Madison, WI). Identical amino acids are boxed. The *Bam*HI site used for the construction of hybrid *nodD*1 is also shown.

SIN-1 was unable to complement a *nodD*1$^-$ negative mutant of *S. fredii* USDA191 for nodulation on soybean. A similar situation has also been reported, in which *nodD*1 of *S. meliloti* was unable to complement a *nodD* mutant of *R. leguminosarum* bv. *trifolii* [15]. The *nodD*2 of *Rhizobium* sp. SIN-1 was unable to complement the triple *nodD* mutant of *S. meliloti* or the *nodD*1 mutant of *S. fredii* USDA191. This result suggests that *nodD*2 has structural features, perhaps associated with some specialized function within *Rhizobium* sp. SIN-1, that distinguish it from other *nodD*s.

Complementation studies with hybrid *nodD*s demonstrate that the N-terminus of *nodD*1 from *S. fredii* USDA191 cannot be substituted with the N-terminus of *nodD*1 from SIN-1 for soybean nodulation. On the other hand, the introduction of a hybrid *nodD* carrying the N-terminus of USDA191 *nodD*1 and the C-terminus of *nodD*1 of SIN-1 enabled the *nodD*1$^-$ negative mutant of USDA191 to form even more nodules than the wild type. These results demonstrate that the N-terminus of USDA191 *nodD*1 contains sequences essential for nodulation of soybean. Why the hybrid *nodD* caused USDA191 to initiate more than the normal number of nodules on soybean is not clear. A different hybrid *nodD*, combining the N-terminus of *R. meliloti nodD*1 with the C-terminus of *R. trifolii nodD*, activated several inducible *nod* promoters in a flavonoid-independent manner [16]. We have not tested whether a similar situation occurs with the *S. fredii–Rhizobium* sp. SIN-1 *nodD* hybrid. We speculate that the hybrid *NodD* may be responding to a wider spectrum of inducing compounds, resulting in enhanced expression of *nod* genes. The *nodD* genes are also known to alter the *nod* factor profile when supplied in *trans* [3]. Thus, it is possible that the hybrid *NodD* alter the composition of the Nod factors produced by *S. fredii* USDA191, and thus the nodulation phenotype. Clearly further investigation is required to explain how the hybrid *NodD* causes the observed increase in nodule numbers on soybean plants.

ACKNOWLEDGMENTS

We thank Drs. Laura Green and Larry Darrah for critical reading of this manuscript, and Dave Pinkerton for photographic assistance.

Literature Cited

1. Appelbaum ER, Thompson DV, Idler K, Chartrain N (1988) *Rhizobium japonicum* USDA191 has two *nodD* genes that differ in primary structure and function. J Bacteriol 170:12–20
2. Boivin C, Ndoye I, Molouba F, Lajudie P, Dupuy N, Dreyfus B (1997) Stem nodulation in legumes: diversity, mechanisms, and unusual characteristics. Crit Rev Plant Sci 16:1– 30.
3. Demont N, Ardourel M, Maillet F, Prome D, Ferro M, Prome JC, Denarie J (1994) The *Rhizobium meliloti* regulatory *nodD*3 and *syrM* genes control the synthesis of a particular class of nodulation factors N-acylated by (ω-1)-hydroxylated fatty acids. EMBO J 13:2139–2149
4. Denarie J, Debelle F, Rosenberg C (1992) Signaling and host range variation in nodulation. Annu Rev Microbiol 46:497–531
5. Djordjevic MA, Schofield PR, Ridge RW, Morrison NA, Bassam BJ, Watson JW, Rolfe BG (1985) *Rhizobium* nodulation genes involved in root hair curling (hac) are functionally conserved. Plant Mol Biol 4:147–160
6. Egelhoff TT, Fisher RF, Jacobs TW, Mulligan JT, Long SR (1985) Nucelotide sequence of *Rhizobium meliloti* 1021 nodulation genes: *nodD* is read divergently from *nodABC*. DNA 4:241–248
7. Györgypal Z, Kiss GB, Kondorosi A (1991) Transduction of plant signal molecules by the *Rhizobium* NodD proteins. BioEssays 13:575–581
8. Hanin M, Jabbouri S, Broughton WJ, Fellay R, Quesada-Vincens

D (1999) Molecular aspects of host-specific nodulation In: Stacey G, Keen NT (eds) Plant-microbe interactions, vol. 4. St. Paul, MN: American Phytopathological Society, pp 1– 37
9. Honma MA, Asubel FM (1987) *Rhizobium meliloti* has three functional copies of the *nodD* symbiotic regulatory gene. Proc Natl Acad Sci USA 84:8558–8562
10. Krishnan HB, Pueppke SG (1991) Sequence and analysis of the *nodABC* region of *Rhizobium fredii* USDA257, a nitrogen-fixing symbiont of soybean and other legumes. Mol Plant-Microbe Interact 5:512–520
11. Long SR (1996) Rhizobium symbiosis: Nod factors in perspective. Plant Cell 8:1885– 1898
12. Rana D, Krishnan HB (1995) A new root-nodulating symbiont of the tropical legume *Sesbania*, *Rhizobium* sp. SIN-1, is closely related to *R. galegae*, a species that nodulates temperate legumes. FEMS Microbiol Lett 134:19–25
13. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press
14. Schlaman HRM, Okker RJH, Lugtenberg BJJ (1992) Regulation of nodulation gene expression by NodD in rhizobia. J Bacteriol 174:5177–5184
15. Spaink HP, Wijffelman CA, Pees E, Okker RJH, Lugtenberg, BJJ (1987) *Rhizobium* nodulation gene *nodD* as a determinant of host specificity. Nature 328:337–340
16. Spaink HP, Okker RJH, Wijffelman CA, Tak T, Goosen-de Roo L, Pees E, van Brussel AAN, Lugtenberg BJJ (1989) Symbiotic properties of rhizobia containing a flavonoid-independent hybrid *nodD* product. J Bacteriol 171:4045–4053
17. Van Rhijn PJ, Feys B, Verreth C Vanderleyden J (1993) Multiple copies of *nodD* in *Rhizobium tropici* CIAT899 and BR816. J Bacteriol 175:438–447