JOURNAL OF BACTERIOLOGY, Jan. 1993, p. 438–447
0021-9193/93/020438-10$02.00/0

Vol. 175, No. 2

Multiple Copies of *nodD* in *Rhizobium tropici* CIAT899 and BR816

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Received 31 August 1992/Accepted 6 November 1992

***Rhizobium tropici* strains are able to nodulate a wide range of host plants: *Phaseolus vulgaris*, *Leucaena* spp., and *Macroptilium atropurpureum*. We studied the *nodD* regulatory gene for nodulation of two *R. tropici* strains: CIAT899, the reference *R. tropici* type IIb strain, and BR816, a heat-tolerant strain isolated from *Leucaena leucocephala*. A survey revealed several *nodD*-hybridizing DNA regions in both strains: five distinct regions in CIAT899 and four distinct regions in BR816. Induction experiments of a *nodABC-uidA* fusion in combination with different *nodD*-hybridizing fragments in the presence of root exudates of the different hosts indicate that one particular *nodD* copy contributes to nodulation gene induction far more than any other *nodD* copy present. The nucleotide sequences of both *nodD* genes are reported here and show significant homology to those of the *nodD* genes of other rhizobia and a *Bradyrhizobium* strain. A dendrogram based on the protein sequences of 15 different NodD proteins shows that the *R. tropici* NodD proteins are linked most closely to each other and then to the NodD of *Rhizobium phaseoli* 8002.**

Soil bacteria of the genus *Rhizobium* are characterized by their ability to establish nitrogen-fixing nodules on the roots of specific plants, mainly legumes. This symbiotic relationship is a complex interaction between each *Rhizobium* species and its limited group of host plants.

The host range is already determined at early stages of the plant-bacterium interaction, which is governed by the nodulation (*nod*) genes. Some of these genes (*hsn* genes) affect host specificity, whereas others (common *nod* genes) perform general functions necessary for nodulation of any host (32). The induction of both the common *nod* and *hsn* genes requires the product of the regulatory gene *nodD* in conjunction with a plant signal, identified as a flavonoid (13, 41, 43, 56). NodD binds to a 50-bp conserved DNA region, called the *nod* box (44), upstream of the inducible *nod* genes; in the presence of plant signals, NodD acts as a positive transcription activator (14). The regulation of nodulation gene expression by NodD in rhizobia was recently reviewed by Schlaman et al. (46). NodD shows a certain flavonoid specificity that restricts *nod* gene induction in plants that secrete flavonoids that activate NodD. Therefore, NodD takes part in determining host specificity (24, 51).

Rhizobia that nodulate *Phaseolus vulgaris* comprise two species: *Rhizobium leguminosarum* bv. phaseoli type I and *Rhizobium tropici*, previously called *R. leguminosarum* bv. phaseoli type II (34–36). *R. leguminosarum* bv. phaseoli type I strains have multiple copies of *nifH* genes (35, 42) and a narrow nodulation host range and hybridize with the *psi* (polysaccharide inhibition) gene (5, 42). *R. tropici* strains have a single copy of the *nifH* gene, have a broad-host-range spectrum, and do not hybridize with the *psi* gene (8, 33, 35). In addition, the pSym plasmids of *R. tropici* strains, exemplified by CIAT899, promote an effective and fully differentiated symbiotic process in *Agrobacterium tumefaciens* transconjugants inoculated on beans, in contrast to pSym plasmids of *R. leguminosarum* bv. phaseoli strains (6, 34).

In this report we describe the cloning and characterization of the multiple *nodD*-hybridizing DNA regions of CIAT899, the reference strain of *R. tropici* type IIb (36), and BR816, a heat-tolerant isolate from *Leucaena leucocephala* that also nodulates *P. vulgaris* effectively, even at high temperatures, and that belongs to the species *R. tropici* according to all phenotypic criteria.

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MATERIALS AND METHODS

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

Media and growth conditions. *Escherichia coli* strains were maintained on LB agar (38) and grown in LB broth supplemented with the appropriate antibiotics. The concentrations of antibiotics used for *E. coli* were 10 μg of tetracycline per ml, 50 μg of spectinomycin per ml, and 100 μg of ampicillin per ml. *Rhizobium* strains were maintained on yeast extract-mannitol (YM) medium (23) or on tryptone-yeast (TY) medium (4). The concentrations of antibiotics used for *Rhizobium* strains were 10 μg of tetracycline per ml, 150 μg of spectinomycin per ml, and 30 μg of nalidixic acid per ml.

Bacterial matings. *Rhizobium* strains were grown overnight at 30°C in TY broth. *E. coli* donor and helper cells were grown overnight at 37°C, diluted 100-fold, and grown for another 5 h. Samples of donor, helper, and acceptor cells (1:1:2 ratio) were pooled, washed, and suspended in 10 mM $MgSO_4$. Mating mixtures were spread on TY agar and incubated overnight at 30°C in a humid atmosphere. Mating patches were taken up with a sterile loop, washed twice in 10 mM $MgSO_4$, and spread on selective plates in appropriate dilutions.

DNA isolation, manipulation, and sequencing. Genomic and plasmid DNAs were isolated from *E. coli* and the *Rhizobium* species as described by Ausubel et al. (1). Phage DNA was isolated as reported by Sambrook et al. (45). Restriction endonucleases (Boehringer Mannheim Biochemicals) were used as recommended by the manufacturer. Plasmid patterns of *Rhizobium* strains were visualized by the procedure of Eckhardt (11). Double-stranded DNA sequencing of pUC subclones was carried out with an AutoRead Sequencing Kit (Pharmacia-LKB) on an A.L.F. automated sequencer (Pharmacia-LKB). Sequence data were processed by using the Assemgel program (PCgene; Intelligenetics). The PCgene software was also used for sequence compari-

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristic(s)	Source or reference
Rhizobium strains		
CIAT899	Wild-type isolate from *P. vulgaris*	EMBRAPA, Brazil
BR816	Wild-type isolate from *L. leucocephala*	EMBRAPA, Brazil
AD822	CIAT899 cured of pSym	C. Quinto, Mexico
E. coli NM539	Spi^- host for recombinant lambda EMBL3 phages	15
Phages		
EMBL3	Lambda replacement vector	15
BRD40	Lambda EMBL3 containing *nodD1* region of BR816	This study
BRD2	Lambda EMBL3 containing *nodD1ABC* region of BR816	This study
BRD31	Lambda EMBL3 containing *nodD2* region of BR816	This study
BRD39	Lambda EMBL3 containing *nodD3* region of BR816	This study
BRD3	Lambda EMBL3 containing *nodD4* region of BR816	This study
CD24	Lambda EMBL3 containing *nodD1ABC* region of CIAT899	This study
CD5	Lambda EMBL3 containing *nodD2* region of CIAT899	This study
CD21	Lambda EMBL3 containing *nodD3* region of CIAT899	This study
CD29	Lambda EMBL3 containing *nodD4* region of CIAT899	This study
CD20	Lambda EMBL3 containing *nodD5* region of CIAT899	This study
Plasmids		
pVK100	Km^r Tc^r cosmid derivative of pRK290 (IncP-1)	29
pVK*x*	pVK100 recombinant cosmids containing the different *nodD* genes of CIAT899 and BR816 (Table 2)	This study
pRG960SD	IncP-? (unclassified) cloning vector containing the *uidA* reporter gene	53
pGUS32	pRG960SD containing the *nodABC* promoter (3.2-kb *Bam*HI fragment) of BR816 orientated toward *uidA*	This study
pMP158	pMP190 containing the *nodD* of *R. leguminosarum* bv. viciae	50
pGV910-26	pGV910 containing the *nodD* of NGR234	M. Holsters and G. Van den Eede, Belgium
pRK2073	Mobilizing plasmid, Str^r	12
pEK12	pBR322 containing the *nodABC* region of *R. meliloti*	47
pUC19	Cloning vector	55
pSK	Cloning vector	52

son of deduced proteins by pairwise (Palign) or by multiple (Clustal) alignments.

DNA hybridization. DNA hybridizations were conducted overnight on nylon membranes (HybondN; Amersham Corp.) as described by Silhavy et al. (48). [α-^{32}P]dCTP-labeled probes (specific activity, $>5 \times 10^7$ cpm per μg of DNA) were obtained by using a nick translation kit of Amersham. Blots were autoradiographed at -80°C with Fuji RX films and intensifying screens (Kyokko Special). For the *nodD* probe, the internal fragment of the *nodD* of *R. leguminosarum* bv. viciae (a 1.2-kb *Bgl*II-*Sal*I fragment of pMP158) or an internal fragment of the *nodD* of NGR234 (a 0.8-kb *Sal*I-*Bam*HI fragment of pGV910-26) were used. As a *nodABC* probe, a 2.5-kb *Bgl*II-*Hin*dIII fragment of pEK12 (*R. meliloti*) was used.

Construction of a genomic library with phage EMBL3 as a vector. High-molecular-weight *Rhizobium* DNA was partially digested with *Sau*3A, dephosphorylated, and ligated into the *Bam*HI site of the lambda EMBL3 phage (15). The ligated mix was packaged in bacteriophage lambda heads with the packaging kit of Boehringer and was used to infect host cells of *E. coli* NM539, an Spi^- host for selecting recombinant phages (15). About 1.5×10^4 independent plaques were recovered and amplified.

Cloning of the different *nodD* genes. An overview of the steps used to clone the different *nodD* genes into the broad-host-range vector pVK100 is presented in Table 2.

Preparation of root and seed exudates. Seeds of *P. vulgaris* cv. Carioca 80 or cv. Negro-Argel, *Macroptilium atropurpureum*, and *L. leucocephala* were sterilized by immersion in H_2SO_4 for 10 min, washed three times in water, rinsed in 95% ethanol for 2 min, rinsed in 0.02% $HgCl_2$ for 4 min, and then washed six times in sterile water. For the collection of seed exudates, the seeds were germinated in aerated sterile water for 3 days (1 ml of water per seed). Root exudates were collected by growing sprouted seeds in 5 ml of water for 2 weeks. Both seed and root exudates were filter sterilized after collection.

Assay for *nod* gene induction. Overnight *Rhizobium* cultures grown in YM medium were diluted 10-fold in induction medium (seed or root exudates or 100 nM naringenin) and further incubated for 5 h at 30°C. Then the β-glucuronidase activity was measured spectrophotometrically by using the substrate *p*-nitrophenyl-β-D-glucuronide (27). Units were calculated as defined by Miller (38).

Nucleotide sequence accession numbers. The sequences reported for the *nodD1* gene of CIAT899 and for the *nodD2* gene of BR816 will appear in the GenBank data base under accession numbers L01273 and L01272, respectively.

RESULTS

Hybridization of *R. tropici* DNA with *nodD*-specific probes. Both *R. tropici* CIAT899 and BR816 carry two plasmids of about 120 and 215 MDa. The largest plasmid of CIAT899 was identified previously as the symbiotic plasmid (6). Hybridization experiments with a *nif/fix*- or *nod*-specific probe have shown that this is also the case for BR816 (data not shown). A ^{32}P-labeled fragment containing *nodD* of *R. leguminosarum* bv. viciae was used to probe the Southern blot of

TABLE 2. Cloning strategies for the *nodD*-hybridizing fragments of BR816 and CIAT899

Plasmid	Insert	Method of construction and cloning vector
pVK40	BR816 *nodD1*	4.3-kb *Bam*HI fragment of BRD40 in pUC19 2.6-kb *Hin*dIII fragment into *Hin*dIII-digested pVK100
pVK31	BR816 *nodD2*	4.3-kb *Sal*I fragment of BRD31 in pUC19 3.5-kb *Bgl*II-*Xho*I fragment into *Bgl*II-*Xho*I-digested pVK100
pVK39	BR816 *nodD3*	3.6-kb *Sal*I fragment and the adjacent 0.5-kb *Sal*I fragment of BRD39 in pUC19 3.9-kb *Eco*RI fragment into *Eco*RI-digested pVK100
pVK3	BR816 *nodD4*	3.5-kb *Sal*I fragment of BRD3 in pUC19 3.5-kb *Sal*I into *Xho*I-digested pVK100
pVK24	CIAT899 *nodD1*	5.8-kb *Sal*I fragment of CD24 in pUC19 3.5-kb *Pst*I fragment into *Pst*I-digested pSK 3.5-kb *Bam*HI-*Sal*I fragment into *Bgl*II-*Xho*I-digested pVK100
pVK5	CIAT899 *nodD2*	6-kb *Eco*RI fragment of CD5 in pUC19 6-kb *Eco*RI fragment into *Eco*RI-digested pVK100
pVK21	CIAT899 *nodD3*	7.7-kb *Eco*RI fragment of CD21 in pUC19 7.7-kb *Eco*RI fragment into *Eco*RI-digested pVK100
pVK29	CIAT899 *nodD4*	7.8-kb *Eco*RI fragment of CD29 in pUC19 7.8-kb *Eco*RI fragment into *Eco*RI-digested pVK100
pVK20	CIAT899 *nodD5*	1.8-kb *Eco*RI fragment of CD20 in pUC19 1.8-kb *Eco*RI fragment into *Eco*RI-digested pVK100

*Eco*RI- or *Sal*I-digested genomic DNAs from CIAT899 and BR816. In both strains, multiple hybridization bands were detected (Fig. 1). The same pattern was obtained with the *nodD* gene of *Rhizobium* NGR234 as a probe. Considering the size of the hybridization bands, it can be concluded that there are probably multiple copies of *nodD* in both strains. To determine whether the *nodD*-containing region is located on the pSym plasmid, a *nodD1* probe of *R. leguminosarum* bv. viciae was hybridized to a Southern blot of an Eckhardt gel from CIAT899 and BR816 (Fig. 2). In each strain, the *nodD* probe displayed hybridization only with the largest plasmid, which was previously identified as the pSym plasmid. The same hybridization pattern was obtained with the *nodD* gene of *Rhizobium* strain NGR234 as a probe.

Isolation and physical mapping of *nodD*-hybridizing DNA of *R. tropici*. To isolate the nodulation genes of *Rhizobium* strains CIAT899 and BR816, we constructed a phage genome bank of the total DNA by using the lambda phage EMBL3 as a vector (see Materials and Methods). Phages containing *nodD*-homologous DNA could be isolated by plaque hybridization with the *nodD* of *R. leguminosarum* bv. viciae as a probe. Nineteen (indicated as BRD*x*) and 13 (indicated as CD*x*) hybridizing plaques of the genome libraries of BR816 and CIAT899, respectively, were further analyzed by restriction enzyme analysis and hybridization. Hybridization of *Sal*I-digested DNA from these *nodD*-containing phages (only *Sal*I liberates the entire insert out of the phage) and *Sal*I-digested total DNA from BR816 and CIAT899 with the *nodD* probe shows that all of the hybridizing *nodD* fragments of the *Rhizobium* strains are represented in the selected phages (Fig. 3 and 4). This hybridization pattern shows that the phages CD5, -24, -29, and -20, respectively, contain the 11.0-, 5.8-, 4.3-, and 2.0-kb hybridizing *Sal*I fragments of CIAT899 and that CD21 contains both the 5.4- and 1.0-kb hybridizing *Sal*I fragments (a preliminary mapping of this phage shows that those two fragments are adjacent). For strain BR816, the *nodD*-hybridizing phages BRD31, BRD39, BRD3 contain the 4.3-, 3.7-, and 3.5-kb *Sal*I fragments, respectively. Phage BRD40 contains the *Sal*I fragments of 2.5 and 1.2 kb. The 3.7-kb *Sal*I hybridizing fragment in BRD40 is a result of the partial digestion, which also indicates that the fragments of 1.2 and 2.5 kb are adjacent. The phage BRD2 contains a DNA region overlapping with BRD40. Here, the entire 1.2-kb *Sal*I fragment and only a part of the 2.5-kb fragment are present.

To define the locations of those *nodD*-hybridizing fragments in reference to *nodABC*, the *nodD*-containing phages were hybridized against *nodABC* probes of *R. meliloti*. For

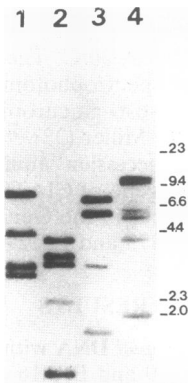

FIG. 1. Southern blot hybridization of a *Rhizobium* total DNA probe with nick-translated *nodD* from *R. leguminosarum* bv. viciae. Lanes: 1, total DNA of BR816 digested with *Eco*RI; 2, total DNA of BR816 digested with *Sal*I; 3, total DNA of CIAT899 digested with *Eco*RI; 4, total DNA of CIAT899 digested with *Sal*I.

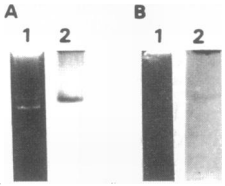

FIG. 2. Southern blot hybridization of the plasmid profile of *Rhizobium* strains BR816 and CIAT899. (A) Plasmid pattern of BR816 (lane 1) and autoradiogram (lane 2) after hybridization with *nodD*; (B) plasmid pattern of CIAT899 (lane 1) and autoradiogram (lane 2) after hybridization with *nodD*.

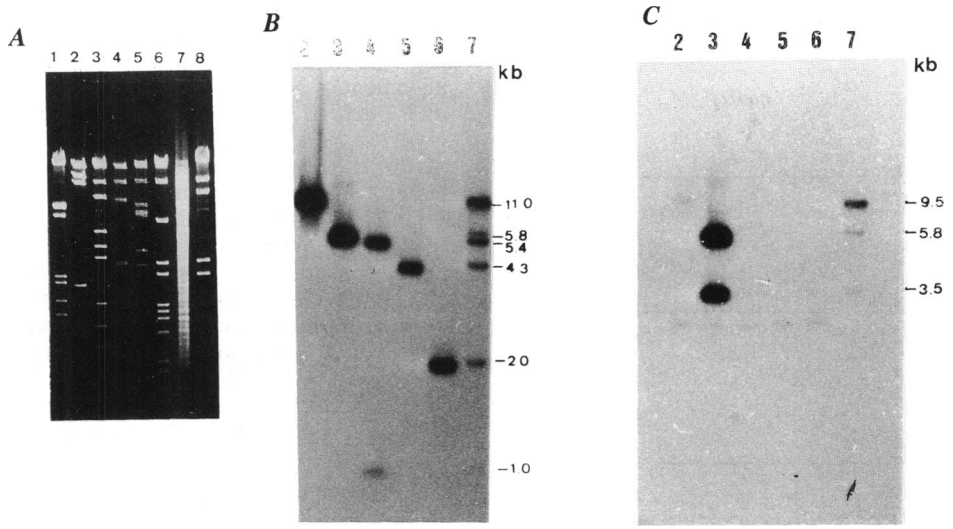

FIG. 3. Analysis of the CD phages containing the *nodD*-hybridizing DNA fragments of CIAT899. (A) *Sal*I restriction digest; (B) Southern hybridization with *nodD* of *R. leguminosarum* bv. viciae; (C) Southern hybridization with *nodABC* of *R. meliloti*. Lanes: 1, lambda digested with *Hin*dIII and *Eco*RI; 2, CD5 digested with *Sal*I; 3, CD24 digested with *Sal*I; 4, CD21 digested with *Sal*I; 5, CD29 digested with *Sal*I; 6, CD20 digested with *Sal*I; 7, total DNA of CIAT899 digested with *Sal*I; 8, lambda digested with *Hin*dIII.

both strains CIAT899 and BR816, a *nodD*-containing phage that showed a strong hybridization signal against the *nodABC* probe, namely, CD24 for CIAT899 (Fig. 3C) and BRD40 and BRD2 for BR816 (data not shown), was detected. This hybridization of total DNA of CIAT899 with *nodABC* gives three hybridization bands corresponding to 3.5-, 5.8-, and 9.5-kb *Sal*I fragments. Only the two smallest *Sal*I fragments could be detected in CD24 (Fig. 3C). Further analysis of CIAT899 DNA with other restriction enzymes indicates that this 9.5-kb hybridization signal results from a partial digest. The total DNA of BR816 digested with *Sal*I gives three fragments corresponding with hybridization signals at 1.4, 1.6, and 2.8 kb. A physical map of this BR816 *nodABC* region is shown in Fig. 5.

The *nodD* copies that are adjacent to *nodABC* were named *nodD1* according to the convention for other *Rhizobium* strains (see below). The others were numbered arbitrarily (Table 2). For further characterization, the *nodD*-containing fragments were subcloned into pUC19 (Table 2) and subjected to a detailed restriction analysis. Physical maps with predicted locations for *nodD* genes are shown in Fig. 6 and 7.

Induction capacities of the different *nodD* copies. Each of the *nodD* copies of *R. tropici* CIAT899 and BR816 was cloned individually in the broad-host-range vector pVK100 (Table 2) to determine its effect on the expression of the *nodABC* operon in the presence of root exudates. For this purpose, a *nodABC-uidA* transcription fusion was constructed. *R. tropici* CIAT899 and BR816 both possess a high endogenous β-galactosidase activity, so that a *nodABC-lacZ* fusion could not be used for induction experiments. For the construction of a *nodABC-uidA* fusion, we took advantage of the physical map of the *nodDABC* region of BR816 shown in Fig. 5. The 3.2-kb *Bam*HI fragment contains no putative *nodD* gene but does contain the part of the *nodABC* region expected to contain the promoter.

To test the induction capacity of the different *nodD* copies, each *nodD* construct, borne on IncP-1 vector

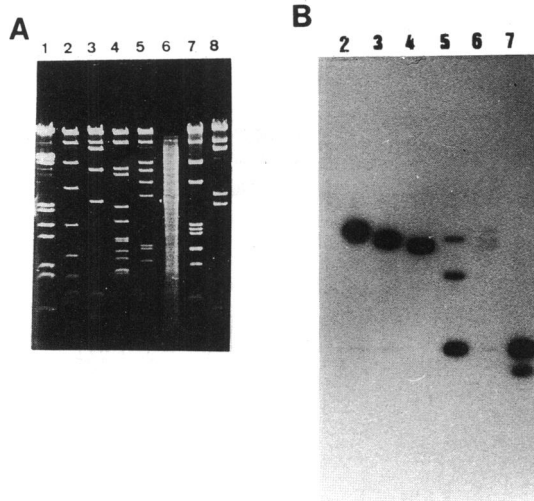

FIG. 4. Analysis of the BRD phages containing the *nodD*-hybridizing fragments of BR816. (A) *Sal*I restriction digest; (B) Southern hybridization with *nodD* of *R. leguminosarum* bv. viciae. Lanes: lambda digested with *Hin*dIII and *Eco*RI; 2, BRD31 digested with *Sal*I; 3, BRD39 digested with *Sal*I; 4, BRD3 digested with *Sal*I; 5, BRD40 digested with *Sal*I; 6, total DNA of BR816 digested with *Sal*I; 7, BRD2 digested with *Sal*I; 8, lambda digested with *Hin*dIII.

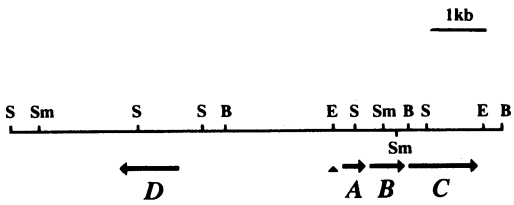

FIG. 5. Physical map of the *nodDABC* region of strain BR816. S, *Sal*I; Sm, *Sma*I; B, *Bam*HI; E, *Eco*RI; D, *nodD*; A, *nodA*; B, *nodB*; C, *nodC*; ▲, *nod* box.

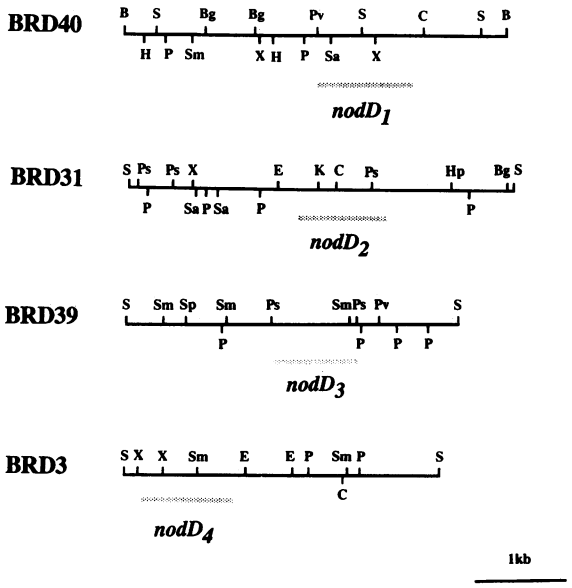

FIG. 6. Physical map of *nodD*-hybridizing regions of BR816. B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; Hp, *Hpa*I; H, *Hin*dIII; Ps, *Pst*I; P, *Pvu*I; Pv, *Pvu*II; K, *Kpn*I; Sa, *Sac*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xho*I.

pVK100 was introduced into AD822(pGUS32), a strain deleted for the pSym plasmid and carrying a *nodABC-uidA* fusion on pRG960SD, an IncP-? (unclassified) derivative that is compatible with IncP-1 (53). The induction capacities of the different *nodD* genes were measured in the presence of seed and root exudates of *P. vulgaris*, *L. leucocephala*, and *M. atropurpureum* as described in Materials and Methods. The values presented in Table 3 are the mean values of three replicates, and variation from each given value is within 10%. Apparently, for both strains under the conditions tested, the highest induction activity is found with one particular *nodD* allele, namely, *nodD1* for CIAT899 and *nodD2* for BR816. However, the following observations suggest that one or more other *nodD* alleles in each strain have a function in nodulation gene regulation. (i) With naringenin as an inducer, all *nodD* alleles cause some induction. (ii) In the case of black bean seed exudates, *nodD3* of BR816 and *nodD5* of CIAT899 cause some induction, although less than that caused by the above-mentioned active alleles. Moreover, the β-glucuronidase activities measured in AD822 transconjugants containing the most active *nodD* allele are different from those observed in the corresponding wild-type strains containing all of the *nodD* alleles. At this stage, the interpretation of these data should be made with caution, since we do not know whether the cloned *nodD* genes are comparably well expressed in the different strains.

Determination of the *nodD1* sequence of CIAT899 and the *nodD2* sequence of BR816. For a detailed molecular analysis of *nodD*, we determined the nucleotide sequences of the *nodD1* gene of CIAT899 and the *nodD2* gene of BR816. The approximate position of the *nodD* gene within the corresponding DNA fragment was established by Southern hybridization. For the regions of interest, several overlapping pUC subclones were isolated and sequenced (Fig. 8). Open reading frames encoding proteins of 304 and 314 amino acids for CIAT899 and BR816, respectively, were evident from analysis of the sequences (Fig. 9 and 10). The predicted molecular masses of the deduced gene products are 35.1 kDa for the *nodD1* gene of CIAT899 and 35.4 kDa for the *nodD2* gene of BR816. The proteins encoded by these open reading frames were found to have strong homology to other NodD proteins already sequenced. Figure 11 shows an amino acid alignment of NodD1 of CIAT899, NodD2 of BR816, NodD1 of *R. meliloti*, NodD of *R. leguminosarum* bv. viciae, and NodD of *R. leguminosarum* bv. trifolii. The NodD proteins of the *R. tropici* strains have 73.7% identical amino acid residues at corresponding positions. When all five NodD proteins are compared, the percentage of identity is reduced to 47.5%. However, at the amino terminus the sequence is highly conserved: of the first 80 residues, 52 are identical and 67 are similar. A putative helix-turn-helix DNA-binding motif near the N-terminal end can be recognized by using the weight matrix method for helix-turn-helix motif detection developed by Dodd and Egan (10). The DNA sequences were also scanned for the presence of a *nod* box, the promoter region of inducible nodulation genes, by using the consensus sequence published by Spaink et al. (50). In CIAT899, a region showing significant homology to the *nod* box consensus sequence was observed in front of the *nodABC* operon, upstream of *nodD1* (Fig. 9). No extensive *nod* box-like sequence could be found in the sequenced region of BR816. For a detailed comparison of the different NodD proteins, a multiple sequence alignment was carried out with 15 protein sequences to construct a NodD-based dendrogram (Fig. 12). This dendrogram shows that the *R. tropici* NodD proteins are linked most closely to each other and then to NodD1 of *R. leguminosarum* bv. phaseoli 8002 (70.1% homology to NodD1 of CIAT899 and 71% homology to NodD2 of BR816). The lowest homology was found with

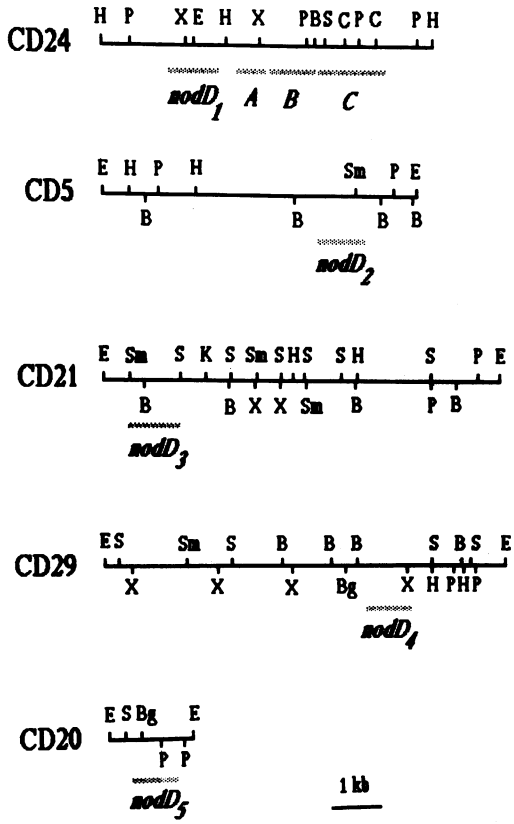

FIG. 7. Physical map of *nodD*-hybridizing regions of CIAT899. B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; E, *Eco*RI; P, *Pst*I; K, *Kpn*I; S, *Sal*I; Sm, *Sma*I; X, *Xho*I.

TABLE 3. Expression of the *nodABC-uidA* fusion in the presence of *Phaseolus*, *Leucaena*, and *Macroptilium* root and seed exudates and in the presence of the commerical flavonoid naringenin (100 nM)

Inducer	Expression of *nodABC-uidA* fusion (Miller units) in:											
	AD822	BR816	AD822 transconjugants containing *nodD* alleles of BR816				CIAT899	AD822 transconjugants containing *nodD* alleles of CIAT899				
			nodD1	*nodD2*	*nodD3*	*nodD4*		*nodD1*	*nodD2*	*nodD3*	*nodD4*	*nodD5*
Water	53	452	103	253	95	101	151	85	95	71	85	99
Naringenin	52	2,647	235	1,228	167	204	728	988	183	216	150	167
Root exudates												
Brown bean	66	1,525	116	649	113	133	240	190	135	118	137	135
Leucaena	69	581	91	703	120	130	229	209	97	70	102	108
Siratro	77	1,238	97	319	115	129	165	127	93	87	110	93
Seed exudates												
Brown bean	75	547	83	630	121	125	452	243	123	93	95	121
Black bean	63	1,580	153	823	241	135	639	551	115	125	129	205
Leucaena	67	611	87	1,257	105	98	172	246	89	94	67	70
Siratro	69	1,009	93	653	95	93	647	258	117	86	95	97

the NodD of *Azorhizobium caulinodans* (50.3% for NodD1 of CIAT899 and 47.8% for NodD2 of BR816).

DISCUSSION

Rhizobia that nodulate *P. vulgaris* form a very heterogenous group. Most of them can be classified in two species, *R. leguminosarum* bv. phaseoli type I and *R. tropici*, but many of isolates cannot be assigned to either of these species (9, 28, 36). In this report we have studied two strains of *R. tropici*: CIAT899, the reference strain (type IIb) originally isolated from *P. vulgaris* (18, 36), and BR816, an isolate from *L. leucocephala* with a strong ability to nodulate and fix nitrogen at higher soil temperatures because of its heat tolerance. To find the broad-host-range determinants of these two strains, we started looking for the *nodD* genes, because the NodD proteins have the potential to play a role in host determination because they can recognize specific signals of the plant (24, 51). A survey for structural homology with *nodD* revealed several copies in both strains: four copies for BR816 and five copies for CIAT899. This was first demonstrated by hybridization experiments (Fig. 1) and

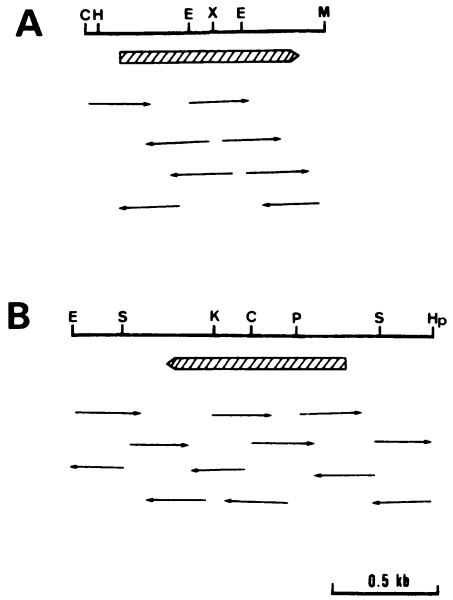

FIG. 8. Sequencing strategies for the 1.1-kb *Cla*I-*Mbo*I fragment containing *nodD1* of CIAT899 (A) and the 1.7-kb *Eco*RI-*Hpa*I fragment containing *nodD2* of BR816 (B). C, *Cla*I; H, *Hin*dIII; Hp, *Hpa*I, P, *Pst*I; K, *Kpn*I; M, *Mbo*I; S, *Sph*I; X, *Xho*I; E, *Eco*RI.

```
1    ATCGATAAGCTTACGCCGATGTACTCGTCTGCTAATCGACATACTTGT
49   CAGGTTATCGACATTTTCCTCATGCACCATCATGCCGAATCGGTAAAA
97   TTGATTGTTTGGATGGCAACCATCCACATCTTGAATGAAGGAAAAGAT
                                                    M
145  GCGCTTCAAAGGACTGGACTTAAATCTTCTCGTCGCGCTCGACGCATT
      R  F  K  G  L  D  L  N  L  L  V  A  L  D  A  L
193  GATGACCGAGCGTAACCTGACGGCCGCGGCACGCAGCATCAATCTCAG
      M  T  E  R  N  L  T  A  A  A  R  S  I  N  L  S
241  CCAGCCTGCGATGAGCGCTGCTGTGGGCCGATTGCGTGTCTATTTCGA
      Q  P  A  M  S  A  A  V  G  R  L  R  V  Y  F  E
289  GGATGAACTGTTTACGATGAATGGTCGCGAACTTGTCCTGACGCCGCG
      D  E  L  F  T  M  N  G  R  E  L  V  L  T  P  R
337  TGCGAAGGGCCTTGTTTCGGCCGTACGTGAAGCCTTACTCCATATCCA
      A  K  G  L  V  S  A  V  R  E  A  L  L  H  I  Q
385  GCTTTCGATCATTTCCTGGGAGCCGTTTGATCCCTTTCAGTCGGATCG
      L  S  I  I  S  W  E  P  F  D  P  F  Q  S  D  R
433  CCGTTTCAGGATCATTCTTTCCGATTTCCTCACACTCGTGTTTATGGA
      R  F  R  I  I  L  S  D  F  L  T  L  V  F  M  E
481  AAAGGTCGTGAAGCGTCGTGCGCGGGAAGCCCCAGGCGTGAGCTTCGA
      K  V  V  K  R  R  A  R  E  A  P  G  V  S  F  E
529  ATTCCTGCCCCTCGCCGATGATTACGACGAGCTTTTGCGCCGTGGCGA
      F  L  P  L  A  D  D  Y  D  E  L  L  R  R  G  E
577  AGTCGATTTTATCATTCTCCCGGACGTGTTCATGCCAACTGGACATCC
      V  D  F  I  I  L  P  D  V  F  M  P  T  G  H  P
625  TCGAGCGAAACTGTTTGAGGAAAGGCTCGTATGCGTGGGCTGTGGCAG
      R  A  K  L  F  E  E  R  L  V  C  V  G  C  G  R
673  GAACCAAGAGCTATCACAGCCGCTTACATTCGACAGATACATGTCCAT
      N  Q  E  L  S  Q  P  L  T  F  D  R  Y  M  S  M
721  GGGGCACGTCGCGGCCAAATTCGGGAATTCACGAAGACCATCAATCGA
      G  H  V  A  A  K  F  G  N  S  R  R  P  S  I  E
769  AGAATGGTATTTGCTCGAACACGGTTTTAAGAGACGTATCGAGGTCGT
      E  W  Y  L  L  E  H  G  F  K  R  R  I  E  V  V
817  CGTGCAGGGCTTCAGCATGATCCTGCCCGTTCTGTCCAATACCAATCG
      V  Q  G  F  S  M  I  L  P  V  L  S  N  T  N  R
875  CATAGCGACCGTGCCGTTGCGACTGGCGCAACATTTCGCAGAAGTTTT
      I  A  T  V  P  L  R  L  A  Q  H  F  A  E  V  L
913  GCCCCTTCGGATCATGGACCTTCCACTGCCGCTTCCCCCATTCACAGA
      P  L  R  I  M  D  L  P  L  P  L  P  P  F  T  E
961  GGCCGTTCAATGGCCTGCGCTTCAAAACAGTGATCCGGCGAGCCTATG
      A  V  Q  W  P  A  L  Q  N  S  D  P  A  S  L  W
1009 GATGCGCGGGATTTTATTACAGGAAGCATCGCGCCTTGCCCTCCCGTC
      M  R  G  I  L  L  Q  E  A  S  R  L  A  L  P  S
1057 GGCAGAGCATTGAGTGGATGCCAGCAATGTTGCTGGAGCCCATCTCAT
      A  E  H  -
1105 GCGGATACTCGCAAGATC
```

FIG. 9. DNA sequences of *nodD1* of CIAT899. The DNA sequence of the 1.1-kb *Cla*I-*Mbo*I fragment and the deduced protein sequence (single-letter amino acid code) are given. *nodD1* extends from position 143 to position 1066. The location of the *nod* box upstream of *nodD1* is underlined.

```
1    GTTAACAGTCAGAAAATCCCTTTTGCTTGGTTGCTGCATGGTGCGGGG
49   GCTGAAACCGCGGATCGCGATCTTTCTCGTTACCCTGTTTCAGGGCGA
97   CATCACCCTTCACTGCGTACGCGAAAATTTTCAAGTGATGCCGTCGAA
145  GGCCCCCATGGCGGCATCGTCCAACGTGAGATAGCGAACGAGGCGCGC
193  GATAGCGCAGTTCAAGCGACGTCTTCTGGACGTGCAGTATGCCCATTG
241  GACATTGGGCGGCTTGTTGGGACCTGCTGCGAGGACGGCATGCGAACC
289  GATAGGAGCGGCCATCCGAGAGCGGTCGGCTGACCGTTGCGTAAATGG
337  TGTGCGGGGGCCTGCCTTGATTTTGAACTAATTGTATTACTAATTAAG
485  AATACTGATGGGTTTGGATGCCATACATTTCACGGTTGGATAGATAAG
433  ACATGCGGTTCAAGGGCCTTGATCTAAATCTTCTGGTTGTGCTCGACG
       M  R  F  K  G  L  D  L  N  L  L  V  V  L  D  A
481  CTCTGATGACCGAGCGTAATCTCACGGCGGCGGCACGCAGCATCAATC
       L  M  T  E  R  N  L  T  A  A  A  R  S  I  N  L
529  TGAGCCAGCCCGCGATGAGCGCGGCCGTCGCGCGGTTACGCACCAATT
       S  Q  P  A  M  S  A  A  V  A  R  L  R  T  N  F
577  TTCGCGATGATCTATTTGCGATGGCCGGCCGCGAATTTATCCCGACAC
       R  D  D  L  F  A  M  A  G  R  E  F  I  P  T  P
625  CGCGTGCGGAAGGGCTCGCCCCCGCGGTGCGCGACGCTCTGCTGCAGA
       R  A  E  G  L  A  P  A  V  R  D  A  L  L  Q  I
673  TTCAGCTCTCCATTGTTTCCTGGGAACCGTTTAACCCGGCCCAGTCGG
       Q  L  S  I  V  S  W  E  P  F  N  P  A  Q  S  D
721  ATCGCCGCTTCAGAATCGTGCTTTCCGATTACGTCACACTCGTCTTTT
       R  R  F  R  I  V  L  S  D  Y  V  T  L  V  F  F
769  TTGAAAAGGTCGTCGCGCGTGCGGCGCAGGAAGCTCCCGGCATCAGCT
       E  K  V  V  A  R  A  A  Q  E  A  P  G  I  S  F
817  TCGATTGTCTGCCTCTTGCCGATGACTTCGAGGAACTTCTGCGCCGCG
       D  C  L  P  L  A  D  D  F  E  E  L  L  R  R  G
875  GCGACATCGATTTTCTGATTATGCCGGAATTGTTCATGTCGATGCATC
       D  I  D  F  L  I  M  P  E  L  F  M  S  M  H  P
913  CTCACGCAGCACTGTTTGAGGATAAATTCGTGTGCGTCGGCTGCCGAA
       H  A  A  L  F  E  D  K  F  V  C  V  G  C  R  T
961  CGAACGAGCAGCTATCAGAGCCATTTACATTCGAGAGATACATGTCGA
       N  E  Q  L  S  E  P  F  T  F  E  R  Y  M  S  M
1009 TGGGGCATGTTGCGGTCAAGTTCGGGAACACTCGGAGACCCACCATCG
       G  H  V  A  V  K  F  G  N  T  R  R  P  T  I  E
1057 AGGAGTGGTACCTGCTTGAGCACGGTCTGAAGAGACGTATCGAGGTCG
       E  W  Y  L  L  E  H  G  L  K  R  R  I  E  V  V
1105 TCGTCCAGGGCTTCAGCATGATTCCGCCCATGCTGTCGGGGACAGAGC
       V  Q  G  F  S  M  I  P  P  M  L  S  G  T  E  R
1153 GTATAGGGACCATGCCTTTGCGGCTGGCGCAGCACTTCGCAAAAACAA
       I  G  T  M  P  L  R  L  A  Q  H  F  A  K  T  I
1201 TTCCTCTGCGGATCGTCGAGCTTCCGCTACCAATCCCCCCACTCGCCG
       P  L  R  I  V  E  L  P  L  P  I  P  P  L  A  E
1249 AGGCCGTTCAATGGCCTGCGCTTCACAATAGTGATCCGGCAAGCCTGT
       A  V  Q  W  P  A  L  H  N  S  D  P  A  S  L  W
1297 GGATGCGCGAGCTGTTACTACAGGAGGCGTCCCTTATGGTCTCGCCGC
       M  R  E  L  L  L  Q  E  A  S  L  M  V  S  P  R
1345 GTGCCCCCGTACGTCTGTCCGCCCCTGGTTTTTGACTGCGTCGTTCAA
       A  P  V  R  L  S  A  P  G  F  -
1393 TAGCTCGGTGTGGTGAGGGGCTGCTCGTAATAAGTGTCTCTCCTTGTC
1441 GGCAGGCTTCCATGATGAGGGTGTTGCCCTCACGTTTTTGAAATCTCT
1489 CGACATGGTTCCCCGGCGCATGCCGGAGGATGCCTCTTGCCCAAATCG
1547 CGGCCAGTACGCTTTGGTCAAGTTGTTGTCGCGATAAAACTGAGATGC
1585 GGTCGAGCGGGTCTTGCTTGTCGATGTCAGCTTGCGCCATCCTTCAGG
1633 CCGCGCTCGCAGATTGACCATTTTTAGCACTCTCCACCGAAGAGTGCT
1681 AAAAACTTGGTCGCCTCCTCTTGAATTC
```

FIG. 10. DNA sequences of *nodD2* of BR816. The DNA sequence of the 1.7-kb *Hpa*I-*Eco*RI fragment and the deduced protein sequences (single-letter amino acid code) are given. *nodD2* extends from position 435 to position 1376.

```
RtroD1    1- MRFKGLDLNLLVALDALMTERNLTAAARSINLSQPAMSAAVGRLRVYFED
RbrD2                  V                                A   TN R
RmelD1       R                 K       R               IA   T  G
RlegD1                     R     K       A               IS W D  R
RtriD1                           K                       I    A  N

RtroD1   51- ELFTMNGRELVLTPRAKGLVSAVREALLHIQLSIISWEPFDPFQSDRRFR
RbrD2        D  A A   FIP    E  AP   D   Q     V     N A
RmelD1         S Q    IP    EA AP   D         V A D LN A
RlegD1       D  IIQR   NP  A EP APV             V A D IN AE
RtriD1         L QQ R  P    EA AP              V A D LV AE

RtroD1   101- IILSDFLTLVFMEKVVKRRAREAPGVSFEFLPLADDYDELLRRGEVDFII
RbrD2          V   YV    F    A A Q    I  DC       FE       DI  L
RmelD1              MI   FARI E V          L   D  PH       D   L
RlegD1              MA   F  IIV L          KL   D  PE       D   L
RtriD1        V     M    F   I  V           L HVN  P  R  S DL  L

RtroD1   151- LPDVFMPTGHPRAKLFEERLVCVGCGRNQELSQPLTFDRYMSMGHVAAKF
RbrD2          M EL  SM-  H A   DKF      RT EQ  E F  E          V
RmelD1         F     SSA  K    D A      PT KK LGNIS ET          Q
RlegD1           L   SGA RK R            ST EQ QGK FLEQ
RtriD1           Q   SAT  S     DK       PS  Q RGK SLK F         M

RtroD1   201- GNSRRPSIEEWYLLEHGFKRRIEVVVQGFSMILPVLSNTNRIATVPLRLA
RbrD2            T   T          L                P M  G E  G M
RmelD1          REMK  V Q L      N    L  P  TL PRL  G       L    V
RlegD1          RGLK  V Q L  QQ L     L  P  NL P L  G       I    V
RtriD1          RTLK    Q L          V I  P  NS PML QG       L  L V

RtroD1   251- QHFAEVLPLRIMDLPLPLPPFTEAVQWPALQNSDPASLWMRGILLQEASR
RbrD2            KTI    VE    I  LA        H           EL      L
RmelD1         KY EQTI    VTS   PLF    I     H T  GNI L E
RlegD1         K YEQTI    IEH    LS          H    GNI   E MI
RtriD1         R  EPTI  Q V H   PLS    L   L H    GNI   N I E

RtroD1   301- LALPSAEH--------------   308
RbrD2          MVS R PVRLSAPGF         314
RmelD1         IDPQSDTC                308
RlegD1         HWN RPKVVRLKRPRSFHSRSS  322
RtriD1         IETSSERCSQEPRATQSW      318
```

FIG. 11. Amino acid sequence alignment (Clustal program) of different NodD proteins. All of these sequences are available in the data bases. NodD sequences from *R. tropici* CIAT899 (RtroD1), *R. tropici* BR816 (RbrD2), *R. meliloti* 1021 (RmelD1), *R. leguminosarum* bv. viciae 1001 (RlegD1), and *R. trifolii* ANU843 (RtriD1) as shown. The complete sequence of NodD1 of *R. tropici* CIAT899 is shown. Residues in other NodD proteins that differ from those in NodD1 are indicated; blank spaces indicate residues that are identical to those in NodD1. A putative helix-turn-helix DNA-binding motif near the N end is underlined.

further confirmed by the cloning data (Fig. 3 and 4). All of the *nodD* genes are present on the pSym plasmid, as shown by the hybridization of the Eckhardt gels of both strains with a *nodD* probe (Fig. 2). From comparison of the restriction patterns of the *nodD*-containing phages and from physical maps of the *nodD*-hybridizing DNA fragments, we can deduce that the different *nodD* copies are not clustered. The fact that we could find five copies of *nodD* in CIAT899 is in contrast to the first results published by Vargas et al. (54), who found only one band that hybridized to the *nodD* probe in CIAT899. However, their latest results, presented by Megias et al. (37), indicate the presence of multiple alleles of *nodD*.

By hybridization of *nodD* to the *nodABC* probe, we could establish the linkage of the *nodD* copies to the *nodABC* genes. For CIAT899, the *nodD*-hybridizing copy represented in phage CD24 is adjacent to the *nodABC* genes, as it is in most other rhizobia, and is therefore referred as *nodD1*. In the case of BR816, the *nodD*-hybridizing copy represented in phage BRD40, referred as *nodD1*, is close to *nodABC* but separated by approximately 3 kb. Nucleotide sequence analysis of a part of this region indicates a DNA region with homology to the *nodE* gene (data not shown). In CIAT899, the *nodE* homolog is 19 kb downstream of *nodABC* (54).

NodD1 of CIAT899 and NodD2 of BR816 have all the structural characteristics known for NodD proteins from other rhizobia: a highly conserved N-terminal part containing the helix-turn-helix motif for DNA binding and a less well- conserved C-terminal part that was previously implicated in host-specific recognition of flavonoid inducer molecules (24).

The presence of multiple copies of *nodD* can offer some advantage for the *Rhizobium* bacterium when it receives signal molecules from the host plants. *R. meliloti*, which nodulates three different hosts, *Melilotus*, *Medicago*, and *Trigonella* species, has three functional copies of the *nodD* gene (17, 22). The two inducer-dependent NodD proteins recognize different plant exudates, and so they play a role in the host range specificity (19, 21, 39). A different case is observed with NGR234, a broad-host-range rhizobium that nodulates at least 35 different genera (31). Hybridization

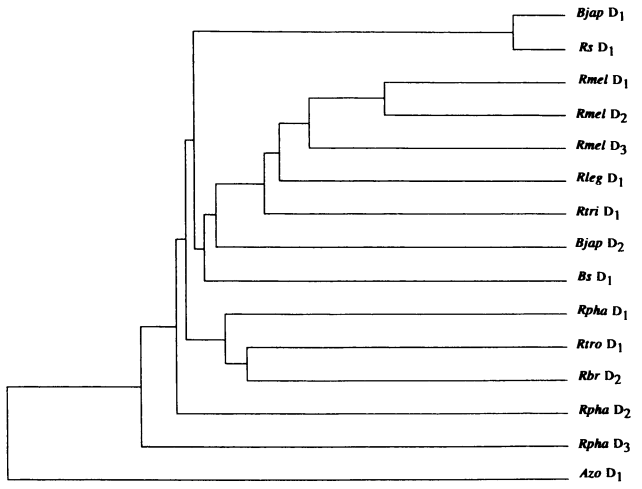

FIG. 12. Dendrogram showing relative distances between NodD proteins of different *Rhizobium* species; the distances are based on multiple sequence alignment (Clustal program). All of these sequences are available in the data bases. NodD proteins from *B. japonicum* USDA110 (*Bjap* D_1 and D_2), *Rhizobium* strain MPIK3030 (*Rs* D_1), *R. meliloti* 1021 (*Rmel* D_1, D_2, and D_3), *R. leguminosarum* bv. viciae 1001 (*Rleg* D_1), *R. trifolii* ANU843 (*Rtri* D_1), *R. leguminosarum* bv. phaseoli 8002 (*Rphas* D_1, D_2, and D_3), *Bradyrhizobium* strain ANU289 (*Bs* D_1), *A. caulinodans* ORS571 (*Azo* D_1), *R. tropici* CIAT899 (*Rtro* D_1), and *R. tropici* BR816 (*Rbr* D_2) are shown.

experiments indicated that NGR234 possesses two *nodD* loci (40). Mutations in *nodD1* result in a Nod^- phenotype on different host plants tested, which leads to the conclusion that the *nodD2* does not play an active role in the control of nodulation by NGR234 (7). A major difference between the narrow-host-range *Rhizobium* species and NGR234 is that the *nodD1* gene of NGR234 responds to a large number of flavonoids (3, 20, 30), so that the nodulation genes of NGR234 are activated by NodD1 in the rhizosphere of many plants. In *Bradyrhizobium japonicum* also, two *nodD* genes are present. *nodD1* contributes to maximal nodulation efficiency, whereas *nodD2* does not play any obvious role in nodulation (2, 16). Interestingly, the *nodD1* gene of *B. japonicum* USDA135 is preceded by a *nod* box sequence (2). The *nodD1* transcription levels are enhanced in the presence of NodD1 protein in combination with certain flavonoids but independently of other *nod* genes (49). To find out the functional role of the *nodD* genes in the *R. tropici* strains, we looked for the regulation of the nodulation genes by the different *nodD* genes in the presence of exudates of their hosts. At this point, it appears that in both strains one particular *nodD* allele contributes most to the induction of an introduced *nodABC-uidA* fusion. Therefore, we postulate that the regulation of nodulation in *R. tropici* follows the model of NGR234, although for some combinations of *nodD* genes and exudates, the activities of the other *nodD* alleles are worth investigating. This hypothesis, based on ex planta experiments, needs to be confirmed with nodulation experiments. First, mutating the active *nodD* allele will give a complete Nod^- phenotype if the other *nodD* genes do not have any role in the nodulation process. Second, if there is still nodulation after mutation of the active *nodD* allele, we can look for possible complementation of NGR234 *nodD1*::Ω by the different *nodD* alleles of *R. tropici* in the nodulation of different hosts.

A recent study by Hungria et al. (25, 26) on the *nod*-inducing compounds present in *P. vulgaris* shows that there are a lot of structurally different *nod*-inducing compounds in the seed and root exudates. Antocyanidins (delphinidin, petunidin, and malvidin) and flavonols (myricetin, quercetin, and kaempferol) present in the seed exudates of black bean are able to induce the *nod* genes in *R. leguminosarum* bv. phaseoli type I strains (25). In the root exudates, eriodicytol, naringenin, and a 7-*O*-glycoside of genistein cause the main induction (26). It will be interesting to determine whether the same compounds are involved in the induction of nodulation genes in *R. tropici*.

ACKNOWLEDGMENTS

This work was supported in part by the STD II program [TS2-0199-C(GDF)] of the Commission of the European Communities. P.J.S.v.R. acknowledges the receipt of a predoctoral fellowship from the Belgian Instituut ter Aanmoediging van het Wetenschappelijk Onderzoek in de Nijverheid en de Landbouw.

We gratefully acknowledge M. Holsters (Laboratory of Genetics, State University of Ghent, Ghent, Belgium) for providing some plasmids. Part of this work was carried out at the Centro de Investigacion sobre Fijacion de Nitrogeno, while P.J.S.v.R. was visiting the research unit of E. Martinez.

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