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Bradyrhizobium (*Arachis*) sp. Strain NC92 Contains Two *nodD* Genes Involved in the Repression of *nodA* and a *nolA* Gene Required for the Efficient Nodulation of Host Plants

WILLIAM K. GILLETTE† AND GERALD H. ELKAN*

Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695-7615

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The common nodulation locus and closely linked nodulation genes of *Bradyrhizobium* (*Arachis*) sp. strain NC92 have been isolated on an 11.0-kb *Eco*RI restriction fragment. The nucleotide sequence of a 7.0-kb *Eco*RV-*Eco*RI subclone was determined and found to contain open reading frames (ORFs) homologous to the *nodA*, *nodB*, *nodD*$_1$, *nodD*$_2$, and *nolA* genes of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii*. Nodulation assays of *nodD*$_1$, *nodD*$_2$, or *nolA* deletion mutants on the host plants *Macroptilium atropurpureum* (siratro) and *Vigna unguiculata* (cowpea) indicate that *nolA* is required for efficient nodulation, as *nolA* mutants exhibit a 6-day nodulation delay and reduced nodule numbers. The *nolA* phenotype was complemented by providing the *nolA* ORF in *trans*, indicating that the phenotype is due to the lack of the *nolA* ORF. *nodD*$_1$ mutants displayed a 2-day nodulation delay, whereas *nodD*$_2$ strains were indistinguishable from the wild type. Translational *nodA-lacZ*, *nodD*$_1$*-lacZ*, *nodD*$_2$*-lacZ*, and *nolA-lacZ* fusions were created. Expression of the *nodA-lacZ* fusion was induced by the addition of peanut, cowpea, and siratro seed exudates and by the addition of the isoflavonoids genistein and daidzein. In a *nodD*$_1$ or *nodD*$_2$ background, basal expression of the *nodA-lacZ* fusion increased two- to threefold. The level of expression of the *nodD*$_2$*-lacZ* and *nolA-lacZ* fusions was low in the wild type but increased in *nodD*$_1$, *nodD*$_2$, and *nodD*$_1$ *nodD*$_2$ backgrounds independently of the addition of the inducer genistein. *nolA* was required for increased expression of the *nodD*$_2$*-lacZ* fusion. These data suggest that a common factor is involved in the regulation of *nodD*$_2$ and *nolA*, and they are also consistent with a model of *nod* gene expression in *Bradyrhizobium* (*Arachis*) sp. strain NC92 in which negative regulation is mediated by the products of the *nodD*$_1$ and *nodD*$_2$ genes.

Bacteria of the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* (collectively termed the rhizobia) invade the roots of plants in the family *Leguminosae* and induce the formation of root nodules to establish a symbiosis with the host plant. The interaction between plant and microbe is specific in that each bacterial species has a host range of legumes with which it can form effective symbioses. This specificity is manifested at several steps, including host recognition, nodule formation, and nitrogen fixation (9, 29, 71). Rhizobia recognize compounds in plant root exudates, flavonoids, and respond with the production of a family of lipooligosaccharide molecules, Nod factors. Purified Nod factors induce several of the plant responses seen in the early stages of nodulation, including root cortical cell division, root hair deformation, and transcription of nodulation-related genes in root hairs (37, 41).

The genes responsible for Nod factor production have been extensively studied (for recent reviews, see references 6, 20, and 67). The common *nod* genes, *nodABC*, are functionally conserved across the rhizobia, are essential for nodulation, and have been implicated in the formation of an N-acylated chitin oligomer core Nod factor (19, 31, 50). Many host-specific nodulation genes which are required for core Nod factor modifications essential for activity on a specific host have been identified (37, 48).

The bacterium's ability to recognize and respond to specific compounds exuded from the plant root is essential for nodulation. This response is primarily mediated by the products of the bacterial *nodD* genes, members of the LysR family of transcriptional activator proteins (25). The NodD proteins activate transcription of *nod* genes in the presence of an appropriate inducer molecule. Typically these molecules are flavonoids, which are secondary metabolites derived from phenylpropanoid metabolism (43, 47), although compounds such as trigonelline and stachydrine are also recognized (44). NodD proteins specifically bind to conserved sequences, called *nod* boxes, in the promoters of regulated *nod* genes (27). The positive regulation of nodulation genes via a NodD-dependent mechanism is common to all rhizobia studied to date, and a functional *nodD* gene is required for nodulation by *Rhizobium* spp. (16, 28, 30). NodD plays a major role in determining the host range of a bacterium through its ability (or inability) to respond to different inducers. Species with a NodD protein(s) which recognizes a wide spectrum of compounds have a corresponding large host range (23). Transfer of *nodD* genes between rhizobial species can result in an extension of host range (29). Recently, it has been reported that specific NodD proteins are required for the production of a family of Nod factors through the differential activation of *nod* genes (10).

Bacteria in the genus *Bradyrhizobium* often have a broader host range (typically tropical legumes) than do those in the genus *Rhizobium*, which, although there are notable exceptions, usually nodulate a small number of temperate legumes (46). Study of *nod* gene function and regulation in *Bradyrhizobium* spp. has led to the discovery of several variations in the strategies employed by *Rhizobium* spp., including the following: atypical arrangement of the common *nod* genes (42), inducibility of the *nodD*$_1$ gene (5), and production of unique Nod factors (57). Much of this research has focused on the well-

* Corresponding author. Phone: (919) 515-3945. Fax: (919) 856-4598.

† Present address: Soybean and Alfalfa Research Laboratories, Bldg. 011, HH 019, Beltsville Agricultural Research Center, USDA-ARS, Beltsville, MD 20705.

characterized soybean symbiont *Bradyrhizobium japonicum* USDA 110, which also nodulates *Vigna unguiculata* (cowpea), *Macroptilium atropurpureum* (siratro), and *Vigna radiata* (mung bean). The regulation of *nod* genes in *B. japonicum* differs significantly from the regulation of *nod* genes in *Rhizobium* spp. A functional *nodD* gene is not required for nodulation of host plants by *B. japonicum* USDA 110 (22, 58). NodD1 activates *nod* gene expression in *B. japonicum* as it does in *Rhizobium* spp., but in the former *nod* gene expression is also activated by the product of the *nodW* gene, the response regulator of the two-component regulatory system *nodVW* (21) required for the nodulation of siratro, mung bean, and cowpea. The $nodD_1$ gene is inducible in *B. japonicum*, and isoflavone-induced expression of this gene and the *nodYABCSUIJ* operon is dependent upon *nodW*. Studies of *nod* gene regulation in *B. japonicum* have also provided examples of negative regulation in the control of nodulation genes. NolA, another transcriptional activator linked to the common *nod* genes and implicated in host specificity (54), negatively regulates both the $nodD_1$ and *nodYABCSUIJ* operons of *B. japonicum* (14). Thus, expression of both the common *nod* operon and of $nodD_1$ are under the complex control of multiple factors in *B. japonicum.*

Other *Bradyrhizobium* species nodulate legumes of significant economic importance (2) other than soybean. Among these legumes are peanuts, which serve as a chief source of high-quality vegetable oil and also play a vital role in less-developed countries, where the seeds are an important food source and the foliage is used for forage and soil improvement (2). *Bradyrhizobium* (*Arachis*) sp. strain NC92 is an effective isolate from peanut, *Arachis hypogaea*, which confers a significant yield increase with certain peanut cultivars (40). *Bradyrhizobium* (*Arachis*) sp. strain NC92 is a member of the cowpea cross-inoculation group (32) and also nodulates cowpea, siratro, mung bean, and *Cajanus cajan* (pigeon pea). Thus, *Bradyrhizobium* (*Arachis*) sp. strain NC92 has a host range which partially overlaps that of *B. japonicum* USDA 110. As a first step in determining the molecular factors involved in establishing this host range, we have isolated and characterized the common *nod* region of *Bradyrhizobium* (*Arachis*) sp. strain NC92. We show that in *Bradyrhizobium* (*Arachis*) sp. strain NC92 *nolA* is required for efficient nodulation of host plants and that $nodD_1$ plays only a minor role in nodulation. Experiments with *nod* gene-*lacZ* translational fusions suggest that NodD1 and NodD2 repress *nodA* expression and that a common factor(s) affects $nodD_2$ and *nolA* expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Table 1 lists bacterial strains and plasmids used in this study. *Escherichia coli* XL1Blue (Stratagene Cloning Systems, La Jolla, Calif.) was used for most applications except when tetracycline was a selective agent, in which case DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was used as the host strain. *E. coli* S17.1 (63) was used as the conjugal donor in matings with *Bradyrhizobium* cells. *E. coli* cells were grown at 37°C in Luria-Bertani media (55) supplemented with appropriate antibiotics at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 25 μg/ml; spectinomycin, 25 μg/ml; streptomycin, 25 μg/ml; and tetracycline, 10 μg/ml. *Bradyrhizobium* cells were grown at 30°C in HM salts (7) plus 1 g each of arabinose, gluconate, and yeast extract per liter (Kuykendall-O'Neill medium [KO] [36]), supplemented with appropriate antibiotics at the following concentrations: kanamycin, 200 μg/ml; spectinomycin, 300 μg/ml; streptomycin, 300 μg/ml; and tetracycline, 300 μg/ml. Tetracycline was added at 100 μg/ml for the maintenance of plasmids in *Bradyrhizobium* in liquid media. Filter-sterilized $HgCl_2$ was added to 10 μg/ml when needed to select for the Hg^r marker (45).

Recombinant DNA techniques. Standard techniques were used for isolation of plasmid and bacterial chromosomal DNA, enzymatic manipulation of nucleic acids, agarose gel electrophoresis, Southern blotting, and autoradiography (55). Transformation of *E. coli* by electroporation was performed with a field strength of 25 kV/cm by using a Bio-Rad (Hercules, Calif.) Gene Pulser apparatus. Restriction fragments were gel purified as described previously (72). Nytran membranes from Schleicher and Schuell (Keene, N.H.) were used for colony hybridizations and Southern transfers. Radioactive DNA probes were generated by using [α-^{32}P]dCTP in random oligonucleotide-primed synthesis (17). Hybridization of radiolabeled probes with Nytran membranes was performed at 65°C in 2× SSPE (1× SSPE is 0.15 M NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, and 50 mM Na_2EDTA)–1% sodium dodecyl sulfate (SDS). Membranes were washed for 5 min at room temperature in 2× SSPE–1% SDS and for 1 h at 65°C in 0.5× SSPE–1% SDS.

Identification and cloning of the common *nod* locus of *Bradyrhizobium* (*Arachis*) sp. strain NC92. Genomic *Bradyrhizobium* (*Arachis*) sp. strain NC92 DNA was digested with a variety of restriction enzymes, separated by electrophoresis in a 0.75% agarose gel, and transferred to nylon membranes. The 3.9-kb *Hin*dIII restriction fragment from pMJS18 (54), containing the $nodD_1YABC$ genes of *B. japonicum* USDA 110, was used to generate radiolabeled probes to detect restriction fragments with homologous sequences in the *Bradyrhizobium* (*Arachis*) sp. strain NC92 genome. The probe hybridized with a single *Eco*RI restriction fragment of approximately 11.0 kb. The fragment of the appropriate size was gel purified from an agarose gel in which was separated 50 μg of *Eco*RI-digested *Bradyrhizobium* (*Arachis*) sp. strain NC92 genomic DNA. Restriction fragments were ligated with *Eco*RI-digested pBluescript SK+ (Stratagene, La Jolla, Calif.), and the entire ligation was used to transform *E. coli* XL1Blue by electroporation. Transformants were selected on media containing ampicillin, 0.004% (wt/vol) 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), and 0.5 mM isopropyl-β-D-thiogalactoside (IPTG). White colonies were screened by colony hybridization for the presence of plasmids containing inserts homologous to the nodulation genes, $nodD_1ABC$, by using the above-mentioned probe. Plasmids from positive clones were purified and confirmed to contain an 11.0-kb *Eco*RI fragment by Southern analysis (data not shown) that was similar in size to that detected by Southern analysis of genomic DNA. A single clone, pCN, was used for further study.

Creation of *Bradyrhizobium* (*Arachis*) sp. strain NC92 deletion and insertion mutants. The Ω Sm^r Sp^r antibiotic resistance cassette from pHP45 (45) was used as a selectable marker in creating insertion and deletion mutations in the *nod* locus of NC92. Plasmids (pCN or pRV, a 7-kb *Eco*RV-*Eco*RI subclone from pCN) containing sequences targeted for insertional disruption or deletion were restricted with appropriate enzymes, and the resulting molecules were made blunt-ended with Klenow fragment and ligated with *Sma*I-digested Ω fragment. Correct construction of insertion and deletion mutations was determined by restriction enzyme analysis, and *Pvu*II fragments from appropriate constructs were cloned into *Sca*I-digested pSUP202 (*Pvu*II does not cleave within the 11-kb *Eco*RI pCN insert or the Ω cassettes). The subsequent pSUP202-based insertion and deletion constructs were used to transform *E. coli* S17.1 (63), which was used as a conjugal donor in matings with *Bradyrhizobium* (*Arachis*) sp. strain NC92 according to the method of Ditta et al. (12). Donor and recipient were mixed in a 1:5 ratio and placed on 0.45-μm-pore-size filter disks on KO agar plates. After 48 h of incubation at 30°C, filters were placed in 5 ml of KO media and vortexed to resuspend the bacteria. Aliquots (10 μl) were spread on KO agar media containing spectinomycin and streptomycin for transconjugant selection and kanamycin (25 μg/ml) to prevent the growth of donor bacteria. Double-crossover recombination events were screened for by analyzing Sm^r Sp^r transconjugants for tetracycline resistance. Sm^r Sp^r Tc^s transconjugants were purified, and the proper genomic organization was verified by Southern analysis (data not shown). The strains used in this study are listed in Table 1, and the extents of the deletions are pictured in Fig. 1. The strains were as follows: BspAABCI, with an insertional inactivation of *nodB*; BspAΔD, containing a deletion between the *Cla*I sites at bp 1387 and 2477 (see Fig. 1 for base pair numbering designations); BspAD1N, harboring a deletion within the $nodD_1$ gene between the *Nhe*I sites at bp 1637 and 2374; BspAD2SK, containing a deletion from the *Sac*II site 356 bp upstream of the $nodD_2$ start codon to the *Kpn*I site located 92 bp downstream of the $nodD_2$ termination codon; BspAD1D2, with a deletion between bp 1445 and 2990, corresponding to a $nodD_1$ $nodD_2$ double mutant; BspA3ΔX, with a deletion spanning bp 1624 to 5442, corresponding to a $nodD_1$ $nodD_2$ *nolA* mutant; and BspABgl, lacking 4,823 bp between the two *Bgl*II sites at bp 1019 and 5842.

Identification of *nodVW* and creation of deletion mutants. A 3.5-kb *Sal*I restriction fragment containing the *nodVW* genes of *Bradyrhizobium elkanii* USDA 83 (66) was used to generate radiolabeled probes to identify homologous sequences in a cosmid-based *Bradyrhizobium* (*Arachis*) sp. strain NC92 genomic library (39). One cosmid clone exhibiting *nodVW* homology contained a single 6-kb *nodVW*-hybridizing *Hin*dIII restriction fragment corresponding to a similar-sized restriction fragment with *nodVW* homology in *Hin*dIII-digested *Bradyrhizobium* (*Arachis*) sp. strain NC92 genomic DNA as identified by Southern analysis (data not shown). This *Hin*dIII fragment was subcloned into the *Hin*dIII site of pBluescript SK+ (p6HVW), and regions of homology to specific *nodV* and *nodW* gene probes were determined by Southern analysis (data not shown). A 2.1-kb *Hin*dIII-*Bam*HI restriction fragment from p6HVW which contained a *Pst*I-*Eco*RV 800-bp restriction fragment responsible for the majority of the *nodW* signal from Southern analysis was subcloned into pBluescript SK+. The resulting clone, p2HBW, was restricted with *Pst*I and *Eco*RV, made blunt-ended with Klenow fragment, and ligated with *Sma*I-digested Ω Hg^r fragment (45). Correctly constructed clones were verified and used for mutagenesis as described above. The *nodVW* deletion was incorporated into the wild type and all of the mutant backgrounds described in this paper.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli		
DH5α	F^- ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17* (r_K^- m_K^+) *thi-1 supE44* λ^- *gyrA96 relA1*	Gibco BRL
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F^+ *proAB*lac^q*Z*Δ*M15* Tn*10* (Tet^r)](Con)	Stratagene
S17.1	Pro^- Res^- Mod^+ *recA*; integrated RP4-Tet::Mu-Kan::Tn*7*; Mob^+	12
Bradyrhizobium (*Arachis*) sp.		
NC92	Wild type	Laboratory collection
BspAABCI	NC92 *nodB*::Ω Sm^r Sp^r	This study
BspAΔD	NC92 Δ(*nodA nod* box $nodD_1$ $nodD_2$)::Ω Sm^r Sp^r	This study
BspAD15	NC92 Δ*nolA*::Ω Sm^r Sp^r	This study
BspAD1N	NC92 Δ$nodD_1$::Ω Sm^r Sp^r	This study
BspAD2SK	NC92 Δ$nodD_2$::Ω Sm^r Sp^r	This study
BspAD1D2	NC92 Δ($nodD_1$ $nodD_2$)::Ω Sm^r Sp^r	This study
BspA3ΔX	NC92 Δ($nodD_1$ $nodD_2$ *nolA*)::Ω Sm^r Sp^r	This study
BspABgl	NC92 Δ(*orf1 orf2 nodA nod* box $nodD_1$ $nodD_2$ *nolA*)::Ω Sm^r Sp^r	This study
Plasmids		
pBSSK+	Cloning vector; Ap^r	Stratagene
pSUP202	Ap^r Tc^r Cm^r; *oriT* from RP4	63
pVK102	RK2 Mob^+ Tc^r Km^r	33
pHP45	Source of Sm^r Sp^r cassette	45
pCN	11.0-kb *Eco*RI fragment containing common *nod* region from *Bradyrhizobium* (*Arachis*) sp. strain NC92 cloned into the *Eco*RI site of pBSSK+	This study
pRV	7.0-kb *Eco*RI-*Eco*RV fragment from pCN cloned into the *Eco*RI-*Eco*RV sites of pBSSK+	This study
pMJS18	Source of *nodDYABC* of *B. japonicum* USDA 110	54
pABLAK1, 3, 5	'*lac*-Km^r cassette vectors in three reading frames	49
pABLAK2, 4, 6	'*lac*-Km^r cassette vectors in three reading frames	49
pAvanodA	1.0-kb *Ava*I fragment[a] from pRV cloned into the *Eco*RV site of pBSSK+	This study
pnodAI	*Bam*HI '*lac*-Km^r cassette[a] from pABLAK2 cloned into the *Sal*I site[a] of pAvanodA	This study
pVKnodAI	*Xho*I-*Bam*HI fragment of pnodAI cloned into the *Xho*I-*Bgl*II sites of pVK102	This study
pnodD1I	1.0-kb *Ava*I fragment[a] from pRV cloned into the *Sal*I site[a] of pABLAK2	This study
pVKnodD1	*Xho*I-*Bam*HI fragment of pnodD1I cloned into the *Xho*I-*Bgl*II sites of pVK102	This study
pnodD2	*Fsp*I-*Sfu*I fragment[a] of pRV cloned into *Sal*I site[a] of pSP72	This study
pnodD2I	*Hin*dIII *lac*-Km^r cassette from pABLAK3 cloned into the *Hin*dIII site of pnodD2	This study
pVKnodD2	*Xho*I-*Bam*HI fragment of pnodD2I cloned into the *Xho*I-*Bgl*II sites of pVK102	This study
pnolAI	*Sac*I fragment[a] of pRV cloned into the *Eco*RI site[a] of pABLAK2	This study
pVKnolAI	*Xho*I-*Bgl*II fragment of pnolAI cloned into the *Xho*I-*Bgl*II sites of pVK102	This study

[a] The 5′ and 3′ overhangs were made blunt with Klenow fragment of DNA polymerase I before cloning.

DNA sequencing. The 7.0-kb *Eco*RI-*Eco*RV restriction fragment was subcloned into pBluescript SK+, and nested deletions were created from both ends of the insert by using the Exo III/Mung Bean Deletion Kit (Stratagene Cloning Systems). The nucleotide sequence was determined for both strands by the dideoxy chain termination method (56) with α-^{35}S-dATP and a Sequenase version 2.0 DNA sequencing kit from United States Biochemical Corporation (Cleveland, Ohio) on single-stranded DNA templates. Sequence alignments were performed with the programs of the Genetics Computer Group, Inc. (Madison, Wis.), and database searches were performed with the BLAST programs of the National Biotechnology Center (Bethesda, Md.) (3). Base pair numbering begins with the *Eco*RV site (bp 1) and ends with the *Eco*RI site (bp 6986) downstream of *nolA*.

Plant testing, nodulation kinetics assays, and acetylene reduction assays. Seeds of *V. unguiculata* cv. Red Caloona (cowpea), kindly provided by W. Broughton, *M. atropurpureum* (siratro), *C. cajan* (pigeon pea), and *V. radiata* (mung bean) were surface sterilized in 10% Clorox for 10 min, rinsed six times in sterile distilled water, germinated overnight at 30°C, planted in sterilized vermiculite, and inoculated with 0.5 ml of inoculum (optical density at 600 nm = 1.0). Seeds of *A. hypogaea* cv. NC7 (peanut) were sterilized and inoculated similarly but planted in modified Leonard jar assemblies (69). For initial screening, nine plants were harvested at each of seven time points for siratro, cowpea, pigeon pea, and mung bean. Peanut nodulation was assayed by harvesting four plants at each of five time points. For kinetic studies, 20 plants (siratro or cowpea) per treatment were harvested at each time point and the roots were placed in 165-ml serum vials and sealed. A 16-cm^3 portion of air was replaced with 16 cm^3 of acetylene, and the bottles were incubated at 30°C. Three 0.5-cm^3 samples were withdrawn and analyzed by gas chromatography as described previously (1). Peak heights were measured and reported as percentages of wild-type values. Roots were subsequently scored for nodules larger than 0.5 mm in diameter. All nodulation kinetics plant tests were repeated in at least two independent experiments.

***nolA* complementation.** The *nolA*-containing 1.9-kb *Kpn*I fragment (bp 4081 to 5944) was subcloned into the *Kpn*I site of pBluescript KS+ and moved as a *Hin*dIII-*Bgl*II fragment (*Hin*dIII site from the vector and *Bgl*II site within the insert) into *Hin*dIII-*Bgl*II-digested pVK102 (33) to create pVKNOLA (verified by restriction enzyme analysis). This plasmid was used to transform *E. coli* S17.1, and a single transformant was used as a conjugal donor in matings with *Bradyrhizobium* (*Arachis*) sp. strain NC92 and BspAD15. pVK102 was also introduced into *Bradyrhizobium* (*Arachis*) sp. strain NC92 and BspAD15 in a similar manner. Purified Tc^r transconjugants were grown in KO media supplemented with 100 μg of tetracycline per ml and used to inoculate (0.5 ml; optical density at 600 nm = 1.0) surface-sterilized, germinated siratro seedlings in sterilized vermiculite. Plants were grown in a growth chamber at 30°C with 14 h of daylight. Five plants from each treatment group were harvested at each time point and assayed as described above for acetylene reduction and nodulation.

Construction of translational β-galactosidase fusions. A series of six plasmids, pABLAK1 to pABLAK6 (49), were the source of the '*lacZ* for translational fusion constructs. The 1.1-kb *Ava*I restriction fragment (Fig. 1) of pRV was used for the *nodA-lacZ* translational fusions by treatment with Klenow fragment and ligation with *Eco*RV-digested pBSSK+ (resulting in pAvanodA). The *nodA-lacZ* fusion was completed by cloning the *Bam*HI-Klenow fragment-treated *lac*-Km^r cassette of pABLAK2 in the Klenow fragment-treated *Sal*I site of pAvanodA

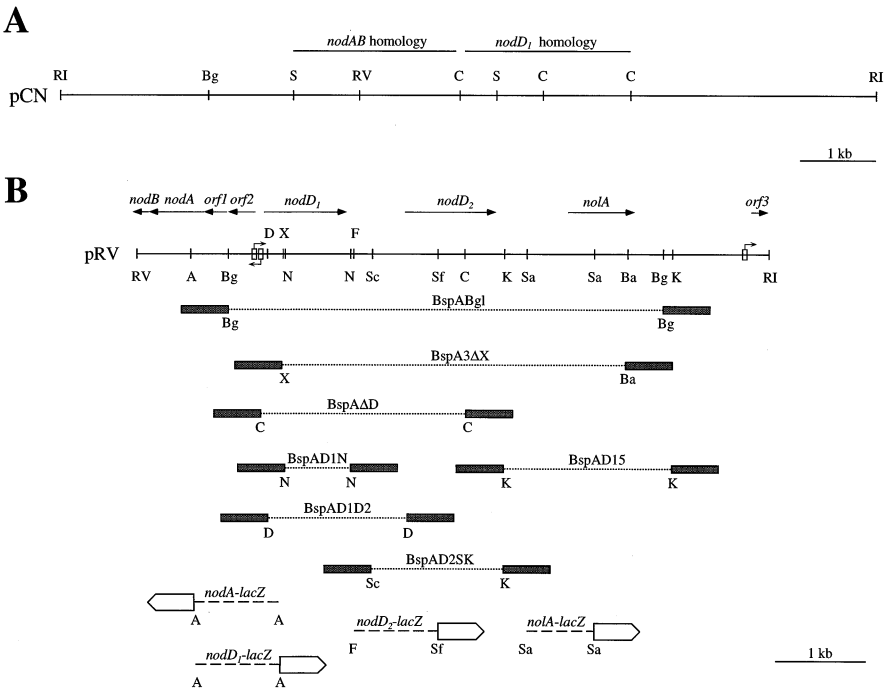

FIG. 1. Physical and genetic maps of the common *nod* locus of *Bradyrhizobium* (*Arachis*) sp. strain NC92. (A) Regions of the 11-kb genomic subclone, pCN, with homology to the indicated probes from *B. japonicum* USDA 110. (B) Restriction map of the sequencing subclone, pRV. Arrows above pRV indicate the positions and orientations of ORFs identified from nucleic acid sequence data. Below the restriction map of pRV are depicted the extents of deletions (dotted lines) in the strains indicated and the regions used in the construction of *lacZ* translational fusions (dashed lines). Stippled rectangles represent wild-type sequence, and open arrows represent *lacZ* coding sequence. Small open rectangles on the pRV restriction map represent *nod* box sequences, with arrows indicating putative directions of control. For clarity, only pertinent restriction sites are shown. Base pair positions referred to in the text are numbered beginning with the first base pair in the *Eco*RV recognition sequence (bp 1) and ending with the last base pair in the *Eco*RI recognition sequence (bp 6986) located 3′ of *nolA*. Restriction sites are abbreviated as follows: A, *Ava*I; Bg, *Bgl*II; Ba, *Bam*HI; C, *Cla*I; D, *Dra*II; F, *Fsp*I; K, *Kpn*I; N, *Nhe*I; RI, *Eco*RI; RV, *Eco*RV; S, *Sal*I; Sa, *Sac*I; Sc, *Sac*II; Sf, *Sfu*I; and X, *Xho*I.

(resulting in pnodAI). The 1.1-kb *Ava*I restriction fragment (Fig. 1) of pRV was also used for the *nodD$_1$-lacZ* translational fusion by treatment with Klenow fragment and ligation with Klenow fragment-treated *Sal*I-digested pABLAK2 (resulting in pnodD1I).

The *nodD$_2$-lacZ* translational fusion was constructed by ligation of the *Fsp*I-*Sfu*I (bp 2344 to 3360) restriction fragment (treated with Klenow fragment) of pRV with *Sal*I-digested, Klenow fragment-treated pSP72 (resulting in pnodD2) followed by cloning of the *Hin*dIII *lac*-Km^r cassette from pABLAK3 into the unique *Hin*dIII site of pnodD2 (resulting in pnodD2I). The *nolA-lacZ* translational fusion was constructed by ligation of the Klenow fragment-treated 817-bp *Sac*I restriction fragment of pRV (bp 4155 to 4972) with *Eco*RI-digested, Klenow fragment-treated pABLAK2. Out-of-frame constructs were created for each of the reporter fusions. Translational fusions were cloned as either *Xho*I-*Bam*HI or *Xho*I-*Bgl*II restriction fragments into *Xho*I-*Bgl*II-digested pVK102. All subclones and constructs were screened by restriction digests for the proper orientation of the subcloned restriction fragments. The correct fusion between the *lacZ* reporter gene and the subcloned restriction fragments was confirmed by DNA sequence analysis (DNA Sequencing Facility, University of Florida, Gainesville). Plasmids with the correct insert were used to transform *E. coli* S17.1, which served as the conjugal donor in matings with *Bradyrhizobium* (*Arachis*) sp. strain NC92 and mutant strains (12). Matings were carried out at 30°C on KO agar plates for 48 h by using 5 μl of logarithmically growing donor cells and 25 μl of logarithmically growing recipient cells. Transconjugants were selected by streaking a loop of cells from the mating mixture on KO plates with tetracycline and kanamycin (*nodA-lacZ*, *nodD$_1$-lacZ*, and *nodD$_2$-lacZ* fusions) or tetracycline (*nolA-lacZ* fusions). All plates contained chloramphenicol (25 μg/ml) to select against the donor.

Flavonoids and seed extracts. Stock solutions of genistein and daidzein (ICN Biomedicals) were 1 mM in methanol. Seed exudates were prepared by surface sterilizing seeds of cowpea (100 seeds), siratro (200 seeds), and peanut (10 seeds) in 95% ethanol for 10 min, rinsing them six times in sterile distilled water, and shaking them overnight in 100 ml of sterile distilled water at 30°C. Exudates were concentrated to 10 ml by rotary evaporation and centrifuged at 5,000 × *g* for 10 min, and the resulting supernatant was filter sterilized and stored at 4°C.

β-Galactosidase assays. Determination of β-galactosidase activity was essentially as described previously (38). Cultures were grown in KO medium at 30°C with tetracycline selection until late log stage and diluted in KO medium to an A_{600} of 0.05 to 0.1, and flavonoids were added to a final concentration of 2 μM or seed extracts were added to 5%. Cultures were incubated for 18 h with shaking at 30°C. Each culture was assayed in triplicate, and cells were permeabilized with SDS-chloroform (38).

Nucleotide sequence accession number. The nucleic acid sequence reported here has been assigned the GenBank accession number U33192.

RESULTS

The common *nod* locus of *Bradyrhizobium* (*Arachis*) sp. strain NC92 is located on an 11-kb *Eco*RI fragment. Southern analysis revealed the presence of an 11.0-kb *Eco*RI restriction fragment with homology to a *nodD$_1$ABC* probe from *B. japonicum* USDA 110 in agreement with the results of Wilson et al. (71) (data not shown). This fragment was cloned, resulting in pCN, and the *nodAB* and *nodD$_1$* loci were localized to the regions of pCN shown in Fig. 1. Southern analysis of *Bradyrhizobium* (*Arachis*) sp. strain NC92 genomic DNA confirmed this organization and the colinearity of pCN with the genomic sequence (data not shown).

Efficient nodulation of host plants by *Bradyrhizobium* (*Arachis*) sp. strain NC92 requires *nolA* but not *nodD$_1$* or *nodD$_2$*. Preliminary results with deletion mutants lacking the *nodD*-hybridizing region (BspAΔD) or the 1.9-kb *Kpn*I fragment (BspD15) of pCN indicated that these regions are important for efficient nodulation of host plants by *Bradyrhizobium* (*Arachis*) sp. strain NC92 (see below). We determined the nucleotide sequence of the 7.0-kb *Eco*RV-*Eco*RI fragment from pCN which contained these regions. Five open reading frames (ORFs) with greater than 70% homology to the nodulation genes *nodA*, *nodB*, *nodD$_1$*, *nodD$_2$*, and *nolA* from other bradyrhizobia, including *B. japonicum* USDA 110 and *B. elkanii* (a

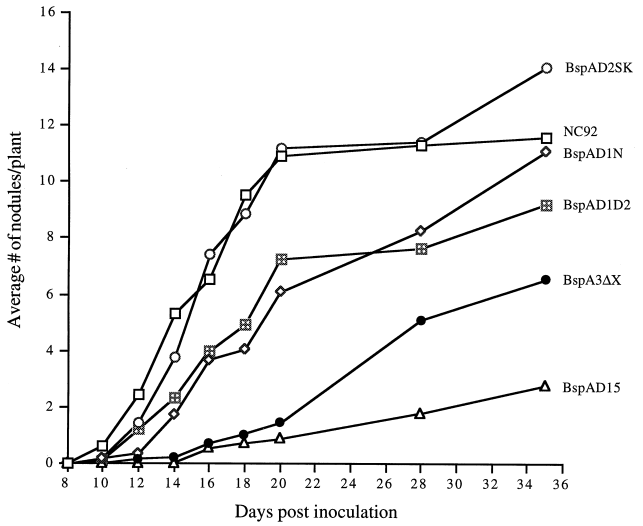

FIG. 2. Nodulation kinetics of *Bradyrhizobium* (*Arachis*) sp. strain NC92 mutants on siratro. Twenty plants were harvested for each treatment at each time point. BspAD2SK was not significantly different from the wild type. BspAD1 and BspAD1D2 were significantly different from the wild type at 12 to 20 dpi ($P = 0.05$). Both BspAD15 and BspA3ΔX were significantly different from the wild type at 12 to 35 dpi ($P = 0.01$).

soybean symbiont previously classified as a *B. japonicum* group II strain and able to elicit the formation of ineffective nodules on peanut [11, 36]), were detected. Strain BspAΔD has a 2,255-bp deletion eliminating 34 nucleotides of the conserved *nod* box proximal to the $nodD_1$ start site, the entire $nodD_1$ gene, and 65% of the 5′ portion of the $nodD_2$ gene. Strain BspAD15 has an 1,863-bp deletion including the entire *nolA* gene but not the *nod* box 3′ of *nolA*, indicating that *nolA* plays an important role in nodulation of host plants by *Bradyrhizobium* (*Arachis*) sp. strain NC92. Both BspAΔD and BspAD15 nodulated 100% of the inoculated host plants (all five host legumes tested) but only after a delay (4 to 9 days as determined by initial screening on siratro and cowpea) compared with the wild type (data not shown).

In order to further delineate the contribution of the identified ORFs to the ability of *Bradyrhizobium* (*Arachis*) sp. strain NC92 to nodulate host legumes and determine if the disruption of the *nod* box sequence in strain BspAΔD was responsible for its nodulation defect, the following mutant strains were constructed: BspAD1N ($nodD_1$), BspAD2SK ($nodD_2$), BspAD1D2 ($nodD_1$ $nodD_2$), and BspA3ΔX ($nodD_1$ $nodD_2$ *nolA*). None of these mutants displayed host-specific defects when assayed on siratro, mung bean, cowpea, pigeon pea, or peanut. The nodulation kinetics of these strains were compared with those of the parent strain on siratro, a promiscuous legume nodulated by members of both *Rhizobium* and *Bradyrhizobium*. The results of a typical nodulation kinetic assay are displayed in Fig. 2.

Strains lacking *nolA*, i.e., BspA3ΔX and BspAD15, exhibited nodulation delays of 6 to 10 days (nodule numbers for both strains were statistically different from those for the wild type at 12 to 35 days postinoculation [dpi] [$P = 0.01$]). The $nodD_2$ deletion strain, BspAD2SK, was indistinguishable from the parent strain. The $nodD_1$ deletion strain, BspAD1N, and the $nodD_1$ $nodD_2$ double mutant, BspAD1D2, both had a slight nodulation delay of 2 days (nodule numbers were significantly reduced from wild-type numbers [$P = 0.05$] at 12 to 20 dpi for both strains). These results indicate that the nodulation defect of strain BspAΔD is due to the deletion of sequences in the $nodA$-$nodD_1$ intergenic region rather than the deletion of $nodD_1$ and $nodD_2$. An additional mutant was constructed to determine the importance of the ORFs located in the $nodA$-$nodD_1$ intergenic region (see below for ORF positions). Bsp ABgl, which carries a 4,823-bp deletion between the *Bgl*II sites of pRV (bp 1019 to 5842), lacks $nodD_1$, $nodD_2$, *nolA*, the *nodA* *nod* box, and the start codons for ORF1 and ORF2. BspABgl was capable of nodulating the five host plants used in these experiments after a delay of greater than 6 days compared with the wild type (data not shown). Strain BspAABCI contains an insertional mutation in its genome at the point corresponding to the unique *Eco*RV site within *nodB*. This strain was unable to nodulate the five host legumes, siratro, cowpea, pigeon pea, mung bean, and peanut (data not shown), confirming the essential nature of the common *nod* genes. Acetylene reduction assays were performed to monitor the progress of an effective symbiosis. In all cases, differences observed in nodulation data were reflected in the acetylene reduction data at the same levels of significance (data not shown).

To determine if the nodulation ability of BspAD1D2 was due to *nodVW*, as is the case for the analogous mutant of *B. japonicum* USDA 110, *Bradyrhizobium* (*Arachis*) sp. strain NC92 was analyzed by Southern analysis and multiple *nodVW*-homologous sequences were discovered (data not shown). One of these regions was isolated and used for the construction of a *nodW* deletion strain. This mutation caused no discernible change in the wild type's nodulation phenotype on the host plants used in the study. Additionally, when incorporated into the mutant strains created in this work, the *nodW* deletion failed to alter the parent strain's nodulation phenotype.

Complementation of the nodulation defect of BspAD15 with *nolA*. To determine if the nodulation defect of BspAD15 is due to the absence of *nolA* or to the disruption of gene expression 3′ to the *nolA* deletion, complementation with the 1.9-kb *Kpn*I *nolA*-containing fragment was attempted (pVKNOLA). This fragment contains *nolA* and sequences 711 bp 5′ and 422 bp 3′ to the *nolA* ORF. At 20 dpi plants inoculated with strain BspAD15(pVK102) were pale green and had fewer nodules ($P = 0.01$) (Fig. 4) and lower values from acetylene reduction assays (16% of wild-type levels with a detection limit of 0.5% of the wild-type levels and a standard deviation of less than 10% of the mean) than those inoculated with NC92(pVK102) or BspAD15(pVKNOLA). The latter two strains were not statistically different from each other. Bacteria isolated from surface-sterilized nodules from all treatment groups displayed the appropriate antibiotic resistance patterns (data not shown). These data indicate that the nodulation defect of strain Bsp AD15 is due to the deletion of *nolA* and not to the disruption of expression from promoters downstream of the deletion.

The organization of the common *nod* locus of *Bradyrhizobium* (*Arachis*) sp. strain NC92 is similar to that of other bradyrhizobia. The ORFs homologous to *nodA*, $nodD_1$, $nodD_2$, and *nolA* encode proteins predicted to be 210, 314, 337, and 237 amino acids in length, respectively. Only the first 117 bp of the ORF homologous to *nodB* is present on the *Eco*RV-*Eco*RI restriction fragment. The predicted amino acid sequence of NodA from *Bradyrhizobium* (*Arachis*) sp. strain NC92 is most homologous (78% identical) to that of NodA from *B. elkanii* (13). The NodD1 proteins from *B. elkanii* and *Bradyrhizobium* (*Parasponia*) sp. (an isolate from *Parasponia regosa*, the only nonlegume known to be nodulated by rhizobia) are both 84% identical to the predicted NodD1 of *Bradyrhizobium* (*Arachis*) sp. strain NC92. The predicted NodD2 protein is most homologous (71% identical) to the sequence predicted for NodD1 of *Bradyrhizobium* (*Arachis*) sp. strain NC92 (this work). The MOTIF program (GCG, Inc.) places the predicted

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  1 MTKATPRRRRWRIGELAGATGVTVRTLHHYEHTGLLAASERTDGGHRMYD 50
    |  ||||||||||||||| |||||||||||||||||||| ||| |||||||
  1 MNRATPRRRRWRIGELAEATGVTVRTLHHYEHTGLLAATERTEGGHRMYD 50
          .         .         .         .         .
 51 RESIQRVHQIRALRELGFSLQEIRRAMDGRTSLTDLLRKHLQRIEVQVAR 100
    ||| ||||||||||||||| ||| || | |||||||||| ||||||||
 51 RESGQRVHQIRALRELGFSLVEIRKAMEGTTSLTDLLRKHLERIEVQVAR 100
          .         .         .         .         .
101 ATQLRDRLRNMTTDGDVRVSVDQLPAALDAMSKVEKRPQPRPCTCALAAD 150
     | ||||||||| |    |||| ||| | |||  | | |   ||| |||
101 TTLLRDRLRNMTIDSEAQVSVDELPATLNAMSRAETRSQTSRCTCNLAAE 150
          .         .         .         .         .
151 REERWRRIRNDLRHCMDRNEHPCSDRTKAVALEARTLISEIAGNDLTGST 200
    || |||||| ||| |||  ||||  | ||||  || |||||||  |   |
151 REDRWRRIRDDLRDCMDGGEHPCGERAKAVAVAARLLISEIAGDDSRVSM 200
          .         .         .
201 ILKVLARLSDPRSLAGWDPHLMQYLDSALVALGDQPH 237
    |||||||| ||||||||| |||||| ||  | |||
201 ILKVLARLSAPRSLAGWDPCLMQYLDLALGGLEDQPY 237
```

FIG. 3. GAP alignment of the predicted amino acid sequences of the NolA proteins from *Bradyrhizobium* (*Arachis*) sp. strain NC92 (upper line) and *B. japonicum* USDA 110 (lower line). Vertical lines connect identical amino acids. The proteins are 78% identical. A helix-turn-helix motif is underlined.

NodD1 and NodD2 proteins in the LysR family of transcriptional activators, which contain a helix-turn-helix motif in the N-terminal portion of the protein. NolA of *Bradyrhizobium* (*Arachis*) sp. strain NC92 is most homologous to NolA of *B. japonicum* USDA 110 (78% identical) (Fig. 3), which is the only other complete NolA sequence in the database. The homology between the predicted NolA proteins of *Bradyrhizobium* (*Arachis*) sp. strain NC92 and *B. japonicum* USDA 110 is most striking in the first 100 amino acids, where the proteins are 90% identical. The N-terminal portion of NolA has a helix-turn-helix motif with homology to the following transcriptional activators: TipA of *Streptomyces lividans* (26) and the MerR proteins of gram-positive and gram-negative bacteria (24).

The organization of the common *nod* region of *Bradyrhizobium* (*Arachis*) sp. strain NC92 (Fig. 1) is similar to that of *B. japonicum* USDA 110 (53) and *B. elkanii* (13) with $nodD_1$ and $nodD_2$ being transcribed divergently from *nodA*. A unique feature of all bradyrhizobial common *nod* loci reported to date is the presence of a single ORF in the $nodD_1$-*nodA* intergenic region. These ORFs are designated *nodY* for *B. japonicum* and *nodK* for *Bradyrhizobium* (*Parasponia*) sp. and *B. elkanii*. No function has been attributed to these ORFs. Two ORFs exist between the $nodD_1$ (bp 1434 to 2378) and *nodA* (bp 749 to 117) genes of *Bradyrhizobium* (*Arachis*) sp. strain NC92. ORF1, at position 1042, encodes a protein of 98 amino acids and ORF2, at position 1320, encodes a protein of 105 amino acids. ORF1 shows no significant homology to sequences in the database and is not preceded by a Shine-Dalgarno sequence. The C-terminal portion (beginning with the methionine at position 63) of the putative ORF2 protein is 40% identical to the N terminus of NodY from *B. japonicum*. No obvious Shine-Dalgarno sequences are present 5′ of the putative ORF2 start codon or the 63rd codon. Two additional ORFs which originate within the $nodD_1$ coding region (bp 2335 to 1673 and bp 1543 to 1085), but which are oriented in the opposite direction, code for proteins of 220 and 152 amino acids. Neither of these predicted proteins is homologous to protein sequences in the databases.

Two sequences homologous to the consensus *nod* box sequence were detected in the *nodA*-$nodD_1$ intergenic region of *Bradyrhizobium* (*Arachis*) sp. strain NC92. The most likely candidate for the *nodA nod* box is 94% identical to the *nodY nod* box of *B. japonicum* and in the same orientation, implicating this sequence in the transcriptional activation of the *nodABC* genes via a NodD protein-mediated mechanism (42, 52). The second *nod* box is located 85 bp upstream of the $nodD_1$ start codon and is oriented so as to control expression of $nodD_1$. This position results in an overlap between the putative *nodA* and $nodD_1$ promoters. This arrangement is similar to that observed in *B. japonicum* and has been noted for its possible role in the coordinated expression of these two operons (70). The *Bradyrhizobium* (*Arachis*) sp. strain NC92 $nodD_1$ *nod* box is divergent from the well-conserved *nodA nod* box (i.e., it is 33% identical to the *Bradyrhizobium* (*Arachis*) sp. strain NC92 *nodA nod* box), as seen in other *Bradyrhizobium* species (5, 13, 70). However, the $nodD_1$ *nod* box of *Bradyrhizobium* (*Arachis*) sp. strain NC92 is only 60% homologous to the $nodD_1$ *nod* box of *B. japonicum*, whereas the *B. japonicum* and *B. elkanii* $nodD_1$ *nod* box sequences are 94% homologous. A third sequence with *nod* box homology is located 950 bp downstream from the end of the *nolA* ORF and is 68% homologous to the *nodA nod* box. A putative ORF (ORF3) beginning 1,243 bp downstream of the end of *nolA* may be under the control of this putative *nod* box. In *B. japonicum*, a recently described isoflavone-inducible gene, *nolY* (15), is located approximately 1.7 kb downstream from *nolA*, in a position similar to that of ORF3 in *Bradyrhizobium* (*Arachis*) sp. strain NC92. A *nod* box sequence is present 5′ of *nolY*, and expression of this gene is dependent on both $nodD_1$ and *nodW*. A mutant with an internal deletion in this gene has a slight nodulation defect on cowpea. The sequence of ORF3 present on pCN codes for the N-terminal 50 amino acids of a protein which has no homology to sequences in the database. Attempts to create *Bradyrhizobium* (*Arachis*) sp. strain NC92 mutants with deletions in ORF3 were unsuccessful, possibly because of a low frequency of double-crossover events owing to the limited amount of sequence available for such events to occur.

Two intergenic sequences homologous to each other (85% over 33 bp) were discovered 322 and 16 bp upstream of the $nodD_2$ and *nolA* translational start sites, respectively, in *Bradyrhizobium* (*Arachis*) sp. strain NC92. These sequences may play a role in the coordinated expression of $nodD_2$ and *nolA* (see below). Homologous sequences (85 to 90%) are present in the analogous positions in *B. japonicum*, *B. elkanii*, and *Bradyrhizobium* (*Parasponia*) sp.

The expression of *nodA* is inducible by isoflavones and seed extracts and does not require $nodD_1$, $nodD_2$, or *nolA*. The level of expression of the *nodA-lacZ* translational fusion in the wild-type strain was determined in the presence and absence of seed extracts and purified inducers. Seed exudates of peanut, cowpea, and siratro and the isoflavones genistein and daidzein all induced expression of the *nodA-lacZ* fusion (data not shown). Levels of expression were highest with purified inducers, i.e., sixfold higher (167.4 $\pm$ 17.9 Miller units for genistein) than those with siratro seed exudate (26.7 $\pm$ 1.2 U). To investigate the role of the three transcriptional activators, NodD1, NodD2, and NolA, in the regulation of *nodA*, the levels of expression of the *nodA-lacZ* fusion in different genetic backgrounds were determined (Table 2). A low level of basal expression was detected in the wild type, and a 20-fold induction was seen in the presence of the inducer genistein. This induction did not require $nodD_1$, $nodD_2$, or *nolA* (BspA3ΔX). The level of basal expression was increased in both $nodD_1$ and $nodD_2$ ($P < 0.001$) backgrounds. This increase in expression was not seen in a $nodD_1$ $nodD_2$ background. Induced levels of expression were increased in BspAD2SK, but the increase was less than twofold. Expression levels in the $nodD_1$ strain (BspAD1N), the *nolA* strain (BspAD15), and the $nodD_1$ $nodD_2$ *nolA* strain (BspA3ΔX) were unchanged compared with wild-type levels. These results suggest that the expression of *nodA* is under both positive and negative regulation and that this reg-

TABLE 2. β-Galactosidase activities of *nod* gene-*lacZ* translational fusions

Strain	Relevant genotype	Reporter plasmid	β-Galactosidase activity (U)[a]	
			− Inducer	+ Inducer
NC92	Wild type	*nodA-lacZ*	4.9 ± 2.4	167.4 ± 17.9
BspAD1N	*nodD$_1$*	*nodA-lacZ*	13.0 ± 0.8[b]	171.4 ± 12.2
BspAD2SK	*nodD$_2$*	*nodA-lacZ*	16.5 ± 2.7[b]	260.9 ± 18.6[b]
BspAD15	*nolA*	*nodA-lacZ*	6.3 ± 2.7	170.4 ± 12.3
BspAD1D2	*nodD$_1$ nodD$_2$*	*nodA-lacZ*	2.1 ± 0.7	142.8 ± 11.8
BspA3ΔX	*nodD$_1$ nodD$_2$ nolA*	*nodA-lacZ*	2.4 ± 1.5	174.8 ± 17.0
NC92	Wild type	*nodD$_1$-lacZ*	0.3 ± 0.1	1.1 ± 0.3
BspAD1N	*nodD$_1$*	*nodD$_1$-lacZ*	0.2 ± 0.1	1.8 ± 0.3
BspAD2SK	*nodD$_2$*	*nodD$_1$-lacZ*	0.3 ± 0.1	0.9 ± 0.8
BspAD15	*nolA*	*nodD$_1$-lacZ*	0.2 ± 0.1	1.4 ± 0.6
BspAD1D2	*nodD$_1$ nodD$_2$*	*nodD$_1$-lacZ*	0.1 ± 0.1	0.6 ± 0.2
BspA3ΔX	*nodD$_1$ nodD$_2$ nolA*	*nodD$_1$-lacZ*	0.2 ± 0.1	0.4 ± 0.4
NC92	Wild type	*nodD$_2$-lacZ*	3.1 ± 0.8	4.4 ± 0.5
BspAD1N	*nodD$_1$*	*nodD$_2$-lacZ*	13.7 ± 2.0[b]	15.6 ± 2.3[b]
BspAD2SK	*nodD$_2$*	*nodD$_2$-lacZ*	50.6 ± 4.6[b]	44.8 ± 4.1[b]
BspAD15	*nolA*	*nodD$_2$-lacZ*	3.1 ± 1.2	2.6 ± 1.2
BspAD1D2	*nodD$_1$ nodD$_2$*	*nodD$_2$-lacZ*	25.1 ± 2.8[b]	26.7 ± 2.3[b]
BspA3ΔX	*nodD$_1$ nodD$_2$ nolA*	*nodD$_2$-lacZ*	2.6 ± 0.4	2.4 ± 0.5
NC92	Wild type	*nolA-lacZ*	1.5 ± 0.1	3.6 ± 0.9
BspAD1N	*nodD$_1$*	*nolA-lacZ*	13.8 ± 1.6[b]	15.6 ± 0.6[b]
BspAD2SK	*nodD$_2$*	*nolA-lacZ*	23.8 ± 7.1[b]	23.1 ± 5.2[b]
BspAD15	*nolA*	*nolA-lacZ*	0.6 ± 0.2[b]	0.7 ± 0.4[b]
BspAD1D2	*nodD$_1$ nodD$_2$*	*nolA-lacZ*	15.2 ± 1.9[b]	16.8 ± 1.4[b]
BspA3ΔX	*nodD$_1$ nodD$_2$ nolA*	*nolA-lacZ*	0.9 ± 1.0[b]	0.8 ± 1.0[b]

[a] Units were calculated as described previously (38). − Inducer, no inducer added; + Inducer, genistein added to a final concentration of 2 μM. Data are means ± standard deviations.

[b] Value is significantly different ($P < 0.001$) from the value of the same reporter in the wild-type background.

ulation is achieved through a complex interaction of factors, which include, but are not limited to, NodD1 and NodD2.

The level of *nodD$_1$* expression is low in the presence and absence of inducers and is not altered in any mutant genetic background tested. Both basal and induced levels of expression from the *nodD$_1$-lacZ* reporter construct approached the level of detectability. These levels were not affected by the genetic background of the host strain (Table 2).

***nodD$_2$* and *nolA* expression is increased in *nodD$_1$*, *nodD$_2$*, and *nodD$_1$ nodD$_2$* strains.** Low levels of *nodD$_2$* and *nolA* basal expression are detectable in the wild-type strain. The presence of seed extracts (data not shown) or purified genistein (Table 2) did not affect these levels. Expression from both the *nodD$_2$-lacZ* and *nolA-lacZ* fusion constructs was increased in *nodD$_1$*, *nodD$_2$*, and *nodD$_1$ nodD$_2$* backgrounds. This expression was independent of the presence of genistein. *nodD$_2$* appears to contribute more to the control of its own promoter than does *nodD$_1$*; *nodD$_2$* expression is increased 4-fold in BspAD1N and 10-fold in BspAD2SK. The finding that the levels of control of either reporter construct are not additive (compare the levels of expression of either *nodD$_2$* or *nolA* in BspAD1N and BspAD2SK with that in BspAD1D2) suggests that other factors are involved in the control of these genes. This idea is supported by the observation that *nolA* is required for the expression of both genes in a *nodD$_1$ nodD$_2$* background (BspAD1D2 versus BspA3ΔX).

DISCUSSION

Studies of the nodulation process as it occurs on the molecular level in the broad-host-range species of the genus *Bradyrhizobium* have revealed similarities to as well as differences from the process as it occurs in *Rhizobium* species (20). In this report we describe the identification, isolation, and characterization of the common *nod* locus from *Bradyrhizobium* (*Arachis*) sp. strain NC92, a member of the cowpea miscellany group of *Bradyrhizobium* (32). This agronomically significant organism (2) shares several hosts, a notable exception being soybean, with the well-characterized *B. japonicum* USDA 110 and nodulates an additional host plant, peanut. We have constructed strains with mutations in the genes present in the common *nod* region and analyzed the effects of these mutations on the bacterium's ability to nodulate known host plants.

nolA is required for the efficient nodulation of host plants by *Bradyrhizobium* (*Arachis*) sp. strain NC92. Strains lacking *nolA* (BspAD15 and BspA3ΔX) are delayed in nodulating cowpea and siratro and induce significantly fewer nodules than does the wild type. Complementation of strain BspAD15 with *nolA* provided in *trans* indicates that the absence of *nolA* is the major defect in this strain. The discovery of *nolA* in *Bradyrhizobium* (*Arachis*) sp. strain NC92 is interesting in that this gene was first identified by its ability to bestow upon *B. japonicum* serogroup 123 the ability to nodulate soybeans with serogroup 123-restricting genotypes. The predicted NolA proteins share a putative helix-turn-helix motif characteristic of many DNA-binding proteins, suggesting a regulatory role for these proteins. A *nolA* mutant has not been reported for *B. japonicum*; however, several lines of evidence indicate that *nolA* also plays a major role in this species. The presence of extra copies of *nolA* represses the expression of a *nodC-lacZ* fusion and improves the nodulation abilities of *B. japonicum* strains with deletions of *nodD$_1$*, *nodD$_2$*, and *nolA* (14).

The mild nodulation defect (1- to 2-day delay) of a *Bradyrhizobium* (*Arachis*) sp. strain NC92 $nodD_1$ mutant indicates that NodD1 is a relatively minor factor in the nodulation of host plants by this organism. This contrasts with the situation in many rhizobia, in which NodD1 is essential (28, 29), has a major role in efficient nodulation (4, 22, 28), or contributes to the host range (22, 23, 28, 65, 68). The lack of a host-specific defect for strain BspAD1N also contrasts with the situation in *B. japonicum*, in which a $nodD_1$ mutation strongly affects nodulation of siratro and mung bean and only slightly delays soybean nodulation (22). A role for other NodD proteins in substituting for the loss of $nodD_1$ in strain BspAD1N is unlikely, since deletion of the only other *nodD* gene detected in *Bradyrhizobium* (*Arachis*) sp. strain NC92 (by Southern analysis) has no effect on the nodulation of the plants used in these experiments. This is shown by the results obtained with BspAD2SK, a $nodD_2$ mutant, and BspAD1D2, a $nodD_1$ $nodD_2$ mutant. These strains do not differ from the wild type and BspAD1N, respectively, in their host range or in their timing and extent of nodulation of siratro. The lack of a phenotype for $nodD_2$ mutants has also been reported for *B. japonicum* (22). The high degree of sequence conservation and the detected expression of $nodD_2$ in *Bradyrhizobium* (*Arachis*) sp. strain NC92 suggest that NodD2 has a function not revealed in the experiments reported here.

Recent reports have indicated that *nodYABCSUIJ* gene expression in *B. japonicum* is under the complex control of at least three gene products (14, 58). In order to understand the regulation of *nod* genes in *Bradyrhizobium* (*Arachis*) sp. strain NC92, we created *lacZ* translational fusions to monitor expression of the genes in this region in various genetic backgrounds.

As expected, on the basis of the nodulation data, expression of the *nodA-lacZ* reporter was inducible in the wild type and in $nodD_1$, $nodD_2$, and $nodD_1$ $nodD_2$ mutants. This induction in a strain devoid of all detectable *nodD* genes (BspAD1D2) indicates that another pathway exists for isoflavone-dependent induction of the *nodA* operon. This also agrees with the results of nodulation experiments using a strain lacking the *nodA nod* box (BspABgl), in which all host plants tested were nodulated. Unexpectedly, the levels of basal expression of the *nodA* reporter were increased in both $nodD_1$ and $nodD_2$ backgrounds. These results suggest that $nodD_1$ and $nodD_2$ have a negative effect on *nodA* gene expression. NodD proteins are members of the LysR family of transcriptional activators; most of the members of this family are activators, but repressor activity has been documented for several (for a review, see reference 59). Although NodD can exhibit negative regulation (autoregulation [51]), it has not been reported as a repressor of the common *nod* genes. Several additional cases of negative regulation of nodulation genes have been reported for *Rhizobium* species (8, 34, 35, 60, 61, 64). Recently, negative regulation has also been reported for a species of *Bradyrhizobium* (14), in which expression of the $nodD_1$ and *nodYABCSUIJ* operons is mediated by NolA.

For *Bradyrhizobium* (*Arachis*) sp. strain NC92, basal *nodA* expression is increased in $nodD_1$ and $nodD_2$ mutants, but no increase was seen in BspAD1D2 or BspA3ΔX. This suggests that one *nodD* gene is necessary for the observed increase in constitutive *nodA* expression. One possibility which is consistent with these data is that increased expression of *nolA* in the $nodD_1$ and $nodD_2$ backgrounds is due to the disruption of a NodD1-NodD2 heterodimer required for repression. Recent evidence suggests that NodD proteins bind the *nod* box as dimers (18); however, there is currently no evidence for or against the existence of heterodimers. The finding that BspA3ΔX and BspAD15 have wild-type levels of *nodA* expression is interesting, considering their severe phenotypes. Disparity between *nod* gene expression and nodulating phenotype was also seen in a *B. japonicum* mutant analogous to BspA3ΔX (14). BspA3ΔX may produce an altered set of nodulation factors, perhaps due to altered expression of genes controlled by NolA. This possibility has been explored for *B. japonicum*, but normal types and amounts of nodulation factors are produced by the strain lacking $nodD_1$, $nodD_2$, and *nolA* (58). Another possibility is that the timing of nodulation factor production in these strains is altered.

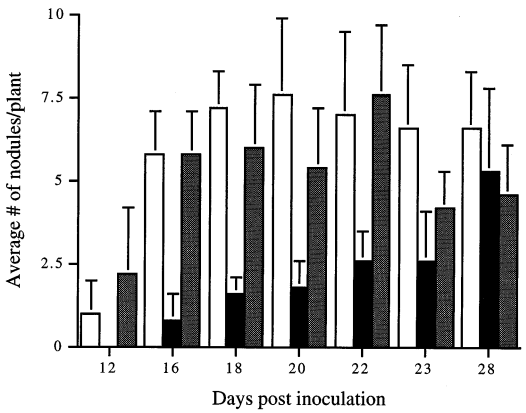

FIG. 4. Complementation of the *nolA* defect in BspAD15. Five plants were harvested at each time point. White bars, NC92 (pVK102); black bars, BspAD15 (pVK102); stippled bars, BspAD15 (pVKNOLA). NC92 (pVK102) and BspAD15 (pVKNOLA) were not statistically different from each other but were both different from BspAD15 (pVK102) at 20 dpi ($P = 0.01$).

In contrast to the case with *B. japonicum*, there is no evidence that *nolA* from *Bradyrhizobium* (*Arachis*) sp. strain NC92 encodes a repressor. Levels of expression of a *nodA-lacZ* fusion are not significantly different between BspA3ΔX and BspAD1D2, nor are levels of expression different between BspAD15 and the wild type. However, our data indicate that *nolA* can affect the expression of $nodD_2$ and *nolA* (Fig. 4). As with the *nodA-lacZ* fusion, expression from the $nodD_2$- and *nolA-lacZ* fusions was increased in $nodD_1$ and $nodD_2$ backgrounds. This expression was also increased in the $nodD_1$ $nodD_2$ strain, but not in strain BspA3ΔX. This result indicates that this increase in expression is dependent upon *nolA* and independent of $nodD_1$ and $nodD_2$. The identical expression patterns observed for the *nolA* and $nodD_2$ fusions in the various genetic backgrounds tested suggest common regulation of these two genes. One common feature between the two promoters is the presence of a conserved 33-bp sequence (85% identical); however, the sequences are located at positions −322 and −16 with respect to the putative start codons of the $nodD_2$ and *nolA* genes, respectively. This sequence is also present in *B. japonicum* and *B. elkanii* in the analogous positions, but there is no evidence of coordinated expression of $nodD_2$ and *nolA* in *B. japonicum*: expression of $nodD_2$ is not detectable. No expression data are available for *B. elkanii.*

The lack of detectable expression from the $nodD_1$-*lacZ* fusion raises the possibility that $nodD_1$ does not function in *Bradyrhizobium* (*Arachis*) sp. strain NC92 and that the $nodD_1$ phenotype is due to disruption of one or both of the ORFs originating within $nodD_1$ and transcribed in the opposite direction. However, it is also possible that the conditions used in these experiments were not optimal for detection of $nodD_1$ expression.

The importance of optimal expression of the *nod* genes has

been shown for *Rhizobium* species. The repressor NolR is present in the majority of *Rhizobium meliloti* strains (35). Strains lacking *nolR* show increased expression of a *nodC-lacZ* fusion and are less efficient in nodulation (34). The results with *B. japonicum* and *Bradyrhizobium* (*Arachis*) sp. strain NC92 indicate that optimal *nod* gene expression is important in *Bradyrhizobium* species as well.

With a few exceptions, the organization of the common *nod* region and the predicted protein products are well conserved with other *Bradyrhizobium* species (13, 22, 42, 54, 62, 70). Although *Bradyrhizobium* (*Arachis*) sp. strain NC92, *B. japonicum* (42, 54), and *B. elkanii* (13) are very similar in the organization of the genes linked to the common *nod* locus, the lack of a clear *nodY* or *nodK* homolog in the *nodA-nodD*$_1$ intergenic region of *Bradyrhizobium* (*Arachis*) sp. strain NC92 is a notable difference. The two ORFs present in this region are not essential for nodulation, as strain BspABgl was able to nodulate all host plants used in this study. This result extends the observation that NodD proteins are not the sole activators of the *nodYABCSUIJ* operon in *B. japonicum* to another *Bradyrhizobium* species; strain BspABgl has no *nodA nod* box and does not contain detectable *nodD* genes, results which are similar to those seen with the analogous mutant of *B. japonicum* (22). For *B. japonicum*, it has been shown that in addition to *nodD*$_1$, *nodW* activates the *nodYABCSUIJ* operon (58). *nodW* belongs to the response regulator class of prokaryotic two-component regulatory proteins, and a *B. japonicum* USDA 110 *nodW* mutant is unable to nodulate mung bean, cowpea, and siratro but retains the ability to nodulate soybean (22). A null phenotype is obtained only in *nodD*$_1$ *nodW* double mutants, since either NodD1 or NodW can activate the common *nod* genes (14). The lack of a phenotype associated with the *nodVW* mutant in this study may be due to the presence of a functional homolog, as several bands of hybridization with a *nodVW* probe were observed upon Southern analysis of *Bradyrhizobium* (*Arachis*) sp. strain NC92.

This work has revealed that despite the high level of conservation between the common *nod* regions of *B. japonicum*, there are distinct differences. *nodD*$_1$ is not critical for the nodulation of any of the legumes tested and has a repressive effect on basal *nodA* expression. *nodD*$_2$, although having no detectable role in the experiments conducted here, is expressed (in contrast to the situation in *B. japonicum*); seems to be under coordinated control with the downstream gene, *nolA*; and negatively affects the expression of the *nodA* promoter. *nolA* is required for the efficient nodulation of host plants by NC92 and does not, in contrast to *nolA* in *B. japonicum*, act to repress *nod* gene expression. *Bradyrhizobium* (*Arachis*) sp. strain NC92 also shares many traits with the two currently described *Bradyrhizobium* species, *B. japonicum* and *B. elkanii*, both symbionts of soybean, which is not nodulated by *Bradyrhizobium* (*Arachis*) sp. strain NC92. The organizations of the ORFs in the common *nod* region are similar in these organisms, as are the sequences of the identified genes. Using mutational analysis and plant testing, we have shown that, as is the case with *B. japonicum*, *Bradyrhizobium* (*Arachis*) sp. strain NC92 does not require a *nod* box and functional *nodD* gene in order to nodulate host legumes.

Bacteria of the genera *Rhizobium* and *Bradyrhizobium* have evolved a complex regulatory pattern through which they interact specifically with compatible legumes in the formation of an effective symbiosis. The work with the broad-host-range *Bradyrhizobium* (*Arachis*) sp. strain NC92 described here has shown that this member of the cowpea miscellany is no exception. As evidenced by the results of the *nod-lacZ* fusion experiments, the control of *nod* gene expression is complex; it involves both positive and negative regulation of complexity similar to that recently described for *B. japonicum*. These findings indicate that *Bradyrhizobium* species will be useful in understanding the complex and varied mechanisms underlying the nodulation process in the *Bradyrhizobium*- and *Rhizobium*-legume symbioses.

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