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nodZ, a Unique Host-Specific Nodulation Gene, Is Involved in the Fucosylation of the Lipooligosaccharide Nodulation Signal of *Bradyrhizobium japonicum*

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The nodulation genes of rhizobia are regulated by the *nodD* gene product in response to host-produced flavonoids and appear to encode enzymes involved in the production of a lipo-chitose signal molecule required for infection and nodule formation. We have identified the *nodZ* gene of *Bradyrhizobium japonicum*, whose product is required for the addition of a 2-*O*-methylfucose residue to the terminal reducing *N*-acetylglucosamine of the nodulation signal. This substitution is essential for the biological activity of this molecule. Mutations in *nodZ* result in defective nodulation of siratro. Surprisingly, although *nodZ* clearly codes for nodulation function, it is not regulated by NodD and, indeed, shows elevated expression in planta. Therefore, *nodZ* represents a unique nodulation gene that is not under the control of NodD and yet is essential for the synthesis of an active nodulation signal.

Leguminous plants are infected (nodulated) by the gram-negative, soil bacteria *Rhizobium*, *Azorhizobium*, and *Bradyrhizobium* species, which establish a nitrogen-fixing symbiosis within the roots. There is now considerable information available concerning the genetic traits of these bacteria that are required for the establishment of nitrogen-fixing symbiosis. A number of nodulation (*nod*, *nol*) genes have been identified (48). These genes fall into two general groups: the common *nod* genes (e.g., *nodABC*) that are essential for nodulation of any host and the host specificity genes that appear to determine specific nodulation of a narrow range of hosts (reviewed in references 13, 14, and 21). Recent evidence indicates that the function of many, if not all, of the *nod* genes is to synthesize or modify lipooligosaccharide signal molecules that initiate many of the initial nodulation responses in the plant (13, 14, 55). For example, *Rhizobium meliloti* has been shown to synthesize a sulfated lipooligosaccharide that induces many of the early events of nodulation when applied to alfalfa roots (13, 14). Recent results indicate that the protein products of the host specificity genes of *R. meliloti*, *nodHPQ*, are involved in the specific sulfation reaction leading to the synthesis of NodRm-1 (e.g., see references 4, 41, and 50). We recently identified the lipooligosaccharide nodulation signals produced by *Bradyrhizobium japonicum* USDA110 and USDA135 and *Bradyrhizobium elkanii* USDA61 (9, 47). *B. elkanii* is the new taxonomic designation for strains previously classified into group II *B. japonicum* (31). The *B. japonicum* and *B. elkanii* nodulation signals identified were found to be tetramers or pentamers of β1-4-linked *N*-acetylglucosamine acylated at the nonreducing end and substituted at the reducing end with a 2-*O*-methylfucose (or fucose) residue (9, 47). The common factor present in all strains (i.e., BjNod-V [$C_{18:1}$ 2-*O*-methylfucose]) was substituted with an 18:1 fatty acid.

Expression of the *nod* genes is controlled by a positive regulatory protein, NodD (reviewed in references 21 and 28). Evidence suggests that the NodD protein recognizes and binds specific flavonoid compounds produced by the host. This is a necessary step for activation of *nod* gene expression. NodD appears to activate transcription by binding to a conserved *nod* box sequence 5′ of each *nod* operon (21, 28). The action of NodD may partially determine host specificity, since the NodD of each *Rhizobium* species specifically recognizes flavonoids produced by the compatible legume host (reviewed in reference 34).

Soybean, an important agricultural plant, is nodulated by *B. japonicum*. *Bradyrhizobium* species are phylogenetically distinct from *Rhizobium* and *Azorhizobium* species (65). In general, *Bradyrhizobium* species exhibit a broader nodulation host range than *Rhizobium* species. One exception to this generality is *Rhizobium* sp. strain NGR234 (also called MPIK3030), which can nodulate a remarkable number of different legume species and the nonlegume *Parasponia andersonii* (59). Bachem et al. (3) and Bassam et al. (7) isolated clones from strain NGR234 that could confer on other *Rhizobium* species the ability to nodulate siratro (*Macroptilium atropurpureum*), one of the many host plants of NGR234 and *B. japonicum*. Subsequently, it was shown that the primary host range determinant encoded by this cloned DNA was NodD (6, 25, 33). This host range locus is one of three isolated by Lewin et al. (33). Apparently, each of these host range loci controls the ability of NGR234 to nodulate a particular subset of hosts. Previously, Nieuwkoop et al. (37) reported the isolation of a siratro-specific nodulation locus from *B. japonicum*. This re-

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gion was first detected and subsequently cloned by hybridization to the NGR234 host specificity region isolated by Bachem et al. (3). Subsequent characterization of this region found no homology to *nodD*, suggesting that an additional siratro-specific genetic trait had been detected in *B. japonicum*.

In this study, we present a detailed characterization of the siratro-specific nodulation locus of *B. japonicum* and the identification of the *nodZ* gene encoded by this locus. This gene is unusual, relative to other *nod* genes, since its expression is not regulated by NodD. Mutations in *nodZ* affect only nodulation; plants that are nodulated by a *nodZ* mutant exhibit wild-type levels of nitrogen fixation. Chemical analysis of the lipooligosaccharide nodulation signals produced by a *nodZ* mutant suggests that NodZ is essential for the fucosylation of the terminal reducing *N*-acetylglucosamine residue.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are summarized in Table 1. Aerobic *B. japonicum* cultures were grown with the appropriate antibiotics (150 μg of kanamycin per ml and 100 μg of spectinomycin, streptomycin, and/or tetracycline per ml) in YS medium or RDY medium, which have been previously described (53). The nitrate medium used in some studies is a modified RDY medium with a reduced amount of yeast extract (0.25 g/liter) and 10 mM KNO_3. *Escherichia coli* cultures were grown in Luria-Bertani medium (10), except for strain JM101, which was grown in TYE medium (46). The antibiotic concentrations (per milliliter) used for *E. coli* were 100 μg of ampicillin, 15 μg of chloramphenicol, 25 μg of kanamycin, 20 μg of nalidixic acid, 100 μg of spectinomycin, 30 μg of streptomycin, and/or 20 μg of tetracycline. *Rhizobium* sp. strain NGR234 was grown in TY medium (8).

DNA isolation. Plasmid DNA was isolated on a large scale as described by Davis et al. (10) or Sambrook et al. (44). Single-stranded DNA from phage was isolated by the method described by Messing et al. (36). *B. japonicum* chromosomal DNA was isolated as previously described (15).

Cloning procedures. Cosmid pRjUT10 carrying the common nodulation genes of *B. japonicum* (42) was digested with *Hin*dIII and electrophoresed in agarose. A fragment of 3.4 kb with homology to the region II *hsn* genes from *Rhizobium* sp. strain NGR234 (Fig. 1) was isolated from the agarose gel by electroelution. The expression vector pKK223-3 was linearized with *Hin*dIII, ligated to the 3.4-kb fragment, and transformed into *E. coli* CRS603, selecting for Amp^r colonies on Luria-Bertani medium containing 50 μg of ampicillin per ml. The resulting plasmids from such transformations were designated pRP2 and pRP14 (fragment in both orientations) and were used as a source of DNA for subsequent subcloning. The plasmids pRP2 and pRP14 were subsequently transformed into *Escherichia coli* DH1 to be used in *E. coli* maxicells. The resulting strains were designated RP36(pRP2) and RP45 (pRP14). Plasmids pRP2 and pRP14 were digested with *Bgl*II, religated, and transformed into *E. coli* DH1. The resulting Amp^r transformants were screened for the loss of a 1.0-kb *Bgl*II fragment. Two isolated colonies carrying such *Bgl*II deletions were designated pAM40 and pAM41, respectively. Likewise, plasmid pRP2 was partially digested with *Xho*II, ligated, and transformed into *E. coli* DH1. A transformant (Amp^r) lacking the 290-bp *Xho*II fragment was isolated and designated pZB51.

Southern blotting and hybridization. Plasmid pRP2 was restricted with several enzymes (i.e., *Eco*RI, *Pst*I, *Xho*I, *Hin*dIII, *Bgl*II) in single and double digests, electrophoresed in 0.7% agarose gels, and transferred to nitrocellulose filters as described previously (54). A 2.7-kb *Eco*RI-*Pst*I fragment of plasmid pCB507 (3) was isolated, labeled by the random primer method of Feinberg and Volgelstein (18), and used as a hybridization probe. This fragment contains the *Hsn*II region of *Rhizobium* sp. strain NGR234 (3). Hybridizations were carried out in 50% formamide buffer at 37°C by the method of Kondorosi et al. (29). Filters were washed at 37°C twice for 1 h each in 2× SSC (1× SSC contains 15 mM sodium citrate and 150 mM NaCl [pH 7.0]) and 0.1% sodium dodecyl sulfate (SDS) and twice for 1 h with 2× SSC. Exposure to X-ray film was done as described by Sambrook et al. (44).

Enzymes and isotopes. DNA restriction endonucleases and modifying enzymes were purchased from Bethesda Research Laboratories (BRL [Gaithersburg, Md.]) and Promega (Madison, Wis.) and were used as specified by the manufacturers. Radioactive nucleotides used for hybridization and sequencing were obtained from New England Nuclear Corporation (Wilmington, Del.).

DNA sequencing. DNA sequences were determined by the dideoxy chain termination method (46). A 2.0-kb *Pst*I-*Hin*dIII fragment was sequenced in both orientations by using the Sequenase 2.0 kit from U.S. Biochemical Corp. according to the manufacturer's instructions and employing either ^{32}P or ^{35}S. The sequencing was repeated in both orientations with the heat-stable *Taq* polymerase from the Taquence kit (U.S. Biochemical Corp.). The M13 phage derivatives used for sequencing were WS18 and WS19 carrying the 2.0-kb *Pst*I-*Hin*dIII fragment in the polylinkers of mp18 and mp19, respectively (Table 1). In addition to the M13 universal primer, 13 synthetic primers were used to sequence the entire fragment.

Site-directed mutagenesis. Tn*5* mutagenesis was carried out in *E. coli* EG47 by the method of Quinto and Bender (40) or by Tn*5-lac* mutagenesis (37). Mutated fragments were conjugated from *E. coli* to *B. japonicum* USDA110 by the triparental mating procedure described by Ditta et al. (16). The Tn*5*-mutated fragments were marker exchanged for the corresponding wild-type DNA by the method of Ruvkun and Ausubel (43). The fidelity of each marker exchange was confirmed by hybridizing *Eco*RI-digested genomic DNA isolated from these mutants with pSUP1011, which harbors Tn*5*. These data confirmed the location of Tn*5* insertions mapped on pRjUT10 (data not shown).

Plant tests. Seeds (*Glycine max* cv. Essex) were surface sterilized and germinated as described previously (42). Nodulation and nitrogen-fixing ability were assayed as described previously (63). For delayed-nodulation assays and comparison of nodulation ability on different soybean cultivars (i.e., *Glycine max* cv. Essex, Harosoy, Williams, Forest, and Peking), seedlings were sprouted as before and then grown three to a pack in clear plastic pouches (Dispo Seed Pack; Northrup King Seed Co.) as described previously (24).

Siratro (*M. atropurpureum*) plant assays were carried out either in test tubes on nitrogen-free medium (29) or in plastic pouches as described above.

Identification of NodZ protein. The presence of NodZ protein was identified by using the *E. coli* maxicell system (45). *B. japonicum* DNA fragments cloned into the expression vector pKK223-3 (see Fig. 3) were analyzed with SDS-polyacrylamide gel electrophoresis (PAGE) gels and compared with protein standards.

RNA isolation and dot blot hybridization. Total RNA from *B. japonicum* USDA110 free-living cells was extracted by the hot phenol method described by Aiba et al. (1). Cultures (500 ml) were grown in YS medium under aerobic conditions to an

TABLE 1. Bacterial strains, plasmids, and bacteriophages

Strain, plasmid, or phage	Relevant characteristic(s)	Source or reference
Bacteria		
Bradyrhizobium sp. strain 32H1 (cowpea)	Wild type	P. van Berkum
B. parasponia ANU289	Wild type	B. Rolfe
B. japonicum		
USDA6	Wild type	P. van Berkum
USDA76	Wild type	
USDA94	Wild type	
USDA122	Wild type	
USDA123	Wild type	
USDA46	Wild type	
USDA135	Wild type	
61A101c	Wild type	LiphaTech, Inc.
USDA110	Wild type	30
BSL104	USDA110, pMGS104	This study
BSL111	USDA110, pMS95::Tn*3-lacZ*, insert in *nodZ* but in wrong orientation	This study
NAD138	USDA110, *nodZ*::Tn*5*	37
ZB939	USDA110, *nodZ*::Tn*5-lacZ*	This study
2101	USDA110, *nifA*::Tn*5*	J. Sommerville, Michigan State University
BSL123	2101, pMGS104	This study
GS300	pRj1025, USDA110	This study
RP36	USDA110, pRP2	This study
RP45	USDA110, pRP14	This study
Rhizobium spp.		
Rhizobium sp. strain NGR234	Wild type	A. Kondorosi
R. meliloti AK631	Wild type	A. Kondorosi
R. leguminosarum bv. *viciae* 897	Wild type	N. Brewin
R. leguminosarum bv. *trifolii* ANU843	Wild type	B. Rolfe
R. fredii USDA194	Wild type	H. Keyser
E. coli		
DH1	F$^-$, *recA1 endA1 gyrA96 thi-1 hsdR17 supE44*	44
JM101	*lac proAB supE thi/f1 traD36 lacI*^{q}, ZM15	36
CRS603	*thr-1 leuB6 proA2 phr-1 recA1 argE3 thi-1 uvrA6 ara-14 lacV1 galK2 xyl-5 mtl-1 gyrA98 rpsL31 tsx-33 supE44* λ^-	58
C2110	*polA*, Nal^r	58
EG47	*hsdR lac gal rpsL594*	23
Plasmids		
pRjUT10	pHC79 clone of *B. japonicum nod* region	42
pRP2, pRP14	pKK233-3 clones with 3.3-kb *Hin*dIII in both orientations	This study
pZB51	*Xho*I deletion of insert DNA of pRP2	This study
pAM40	*Bgl*II deletion of insert DNA of pRP2	This study
pPP375	pRK290 with λVX polylinker in the *Eco*RI site	P. Putnoky, Szeged, Hungary
pMS95	pPP375 containing the 9.5-kb *Eco*RI fragment from pRjUT10	This study
pAM41	*Bgl*II deletion of insert DNA of pRP14	This study
pKK233-3	Expression vector	2
pCB507	pLAFR1 containing the Hsn regions of *Rhizobium* sp. strain NGR234	3
pRK2073	*tra*$^+$ (RK2:ColE1)	32
pMGS104	pMS95::Tn*3*HoHo1, *nodZ-lacZ*	This study
pRj1025	pRK290::*nifD-lacZ*	H. Hennecke
pSup1011	Km^r, Nm^r (by Tn*5*), Cm^r, *oriT* (Rp4)	51
Phages		
λ::Tn*5*	Km^r	D. Berg
P1::Tn*5*	Km^r	40
P1clr100	Cm^r	D. Kaiser
pms201 and 202	mp19 containing the 2.0-kb *Pst*I-*Hin*dIII fragment from pRP2 in both orientations	This study
pms203 and 204	mp19 containing the 0.5-kb *Eco*RI-*Hin*dIII fragment from pRP2 in both orientations	This study
pms208 and 210	mp19 containing the 1.0-kb *Bgl*II fragment from pRP2 in both orientations	This study
WS18 and WS19	2.0-kb *Pst*I-*Hin*dIII fragment in mp18 and mp19, respectively	This study
pms210	mp19 containing the 0.6-kb *Alu*I fragment from pRP2	This study

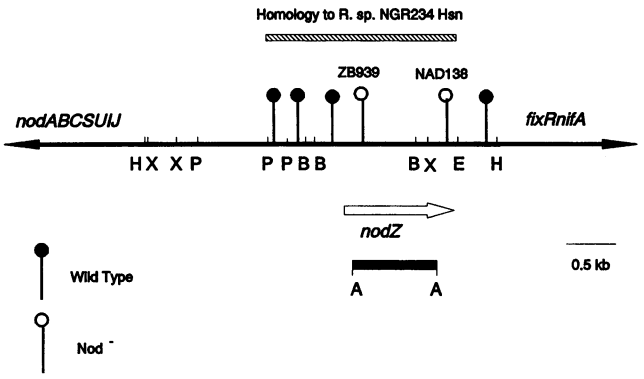

FIG. 1. Physical map of the 3.4-kb *Hin*dIII fragment of *B. japonicum* USDA110 encoding the *nodZ* gene. The approximate region showing hybridization to the *Hsn*II probe of *Rhizobium* (R.) sp. strain NGR234 is shown at the top of the figure. The locations of Tn*5* and Tn*5-lacZ* insertions (i.e., ZB939) are shown by the vertical lines. Open circles represent a defective nodulation phenotype on siratro; closed circles represent a wild-type phenotype. The solid bar shows the location of the 895-bp *Alu*I fragment used as a *nodZ*-specific hybridization probe. Abbreviations: A, *Alu*I; B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; X, *Xho*I. The region sequenced lies between the rightmost *Hin*dIII site and the nearest *Pst*I site.

A_{600} of 0.4. Cells were pelleted and then frozen under liquid nitrogen. Cell pellets were resuspended in diethylpyrocarbonate-treated 0.02 M $NaCH_3COOH$–0.5% SDS–1 mM EDTA–vanadyl ribonucleoside complex added to a 100-μM concentration, and then equal volumes of hot phenol (65°C) were added and the tubes were set at 65°C for 5 min. The phases were separated by centrifugation, and the extraction was repeated once more at 65°C and then one time at room temperature. RNA was precipitated out of the aqueous phase by the addition of 1/10 of a volume of 3 M $NaCH_3COOH$ and 3 volumes of ethanol. The RNA pellet was resuspended in sterile diethylpyrocarbonate-water and stored at −80°C. Bacteroids were isolated from soybean nodules 21 days after plant inoculation. Nodules (approximately 1 g) were macerated with a homogenizer in 0.5 ml of bacteroid extraction buffer (i.e., 0.5 M mannitol, 20 mM sodium succinate, 5 mM sodium dithionite, 25 mM Tris-HCl [pH 7.5]). Plant debris was collected by centrifugation at 1,500 rpm for 5 min (Beckman SS-34 rotor). The supernatant was then transferred to a clean tube and centrifuged at 14,000 rpm for 10 min to collect the bacteroids. The bacteroid cell pellet was then treated as in the free-living procedure outlined above.

Dot blots were prepared by spotting different amounts of RNA (i.e., 0.1, 1, 3, and 6 μg) with a BRL dot blot system. The RNA was bound to a Hybond nylon membrane (Amersham Co.), and Northern (RNA) hybridizations were carried out according to the manufacturer's specifications. An 895-bp *Alu*I fragment internal to *nodZ* was used as a hybridization probe. After hybridization at 42°C for 48 h, blots were washed with 100-ml aliquots of 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]) for two washes and then were washed once with 1× SSPE and twice with 0.1× SSPE–0.01% SDS. After exposure to X-ray film, the spots were cut out of the nylon filter and placed in scintillation vials containing 5 ml of aqueous scintillation cocktail (Scintiverse I; Fisher Scientific), and samples were counted in a Beckman LS3800 scintillation counter.

Determination of the *nodZ* transcriptional start. The transcriptional start site of the *nodZ* gene was determined by primer extension. An oligonucleotide was synthesized complementary to the region adjacent to the promoter (see Fig. 4). Prior to annealing to the RNA, the oligonucleotide was end labeled with 4 U of polynucleotide kinase (BRL) in the presence of [γ-^{32}P]ATP (240 μCi) at 37°C for 1 h in a total volume of 25 μl. The labeled oligonucleotide was precipitated by the addition of an equal volume of 4 M ammonium acetate followed by 5 volumes of ethanol. After centrifugation, the pellet was dried and resuspended in 25 μl of water. The oligonucleotide was then precipitated as described above, and the pellet was dried under a vacuum.

The primer extension method used was performed as described by Kassavetis and Geiduschek (26). Twenty micrograms of *B. japonicum* USDA110 bacteroid RNA was mixed with 0.01 pmol of end-labeled primer (5×10^4 cpm), and these were precipitated together by the addition of 4 M ammonium acetate and ethanol. The pellet was dried, resuspended in 30 μl of hybridization buffer (20 μM Tris [pH 8.0], 200 mM NaCl, 0.1 mM EDTA), and denatured at 100°C for 3 min. The primer was allowed to anneal at 63°C for 1.5 h. The sample was diluted to 100 μl in extension buffer (6 mM $MgCl_2$, 6 mM dithiothreitol, 500 μM deoxynucleoside triphosphates). After addition of 3 μl (15 U) of avian myeloblastosis virus reverse transcriptase (BRL), the extension was carried out at 37°C for 1 h. The reaction was terminated by the addition of 2 μl of 0.25 M EDTA–1 μl of RNAse A (1 mg/ml), and the mixture was incubated at 37°C for 30 min. The sample was extracted with 125 μl of phenol-$CHCl_3$ and then was precipitated by the addition of 100 μl of 2.5 M ammonium acetate and 300 μl of ethanol. The pellet was dried and resuspended in 3 μl of 1× Tris-EDTA buffer and 4 μl of sequencing stop solution (0.03% deionized formamide, 20 mM EDTA). The samples were heated to 95°C for 2 min prior to running in a 6% acrylamide–8 M urea denaturing gel. The results of the primer extension reactions were compared with those of a sequencing ladder generated by using the same primer annealed to the 3.4-kb *Hin*dIII fragment contained in plasmid pRP2.

Construction of *nodZ::lacZ* fusions. Several *lacZ* fusions were generated randomly within the *nodZ* region by Tn*3*-HoHo1 mutagenesis (58). The 9.5-kb *Eco*RI fragment from pRjUT10 was cloned into pPP375 to generate target plasmid pMS95 used for mutagenesis. Plasmid DNA was isolated from each of the presumptive Tn*3*-HoHo1 mutants and digested with *Eco*RI. The position and orientation of the transposon within the target gene (i.e., *nodZ*) were mapped by Southern blot hybridization with a DNA fragment containing the *nodZ* gene (1-kb *Bgl*II [Fig. 1]). Single *nodZ*::Tn*3* insertions were transferred by conjugation into *B. japonicum* USDA110 and tested for activity. A single active *nodZ*::Tn*3-lacZ* insertion (contained on plasmid pMGS104) was chosen for further analysis. Plasmid pMGS104 was also conjugated from *E. coli* into *B. japonicum* mutant 2101 (*nifA*).

β-Galactosidase activity of the *nodZ*::Tn*3-lacZ* fusions. β-Galactosidase activity of *Bradyrhizobium* transconjugants was determined as described by Banfalvi et al. (5). Bacterial cultures were grown in nitrate medium under aerobic and anaerobic conditions with or without other additions as described. Aerobic cultures were grown in 1.5 ml of liquid with antibiotics (50 μg of tetracycline per ml and 50 μg of spectinomycin per ml) at 220 rpm to an A_{600} of 0.4. Anaerobic cultures were grown with gentle shaking for 5 days in 15-ml screw-cap tubes filled to the top. Other than those in the inoculum, no antibiotics were added to the anaerobic cultures since they severely restricted growth.

The β-galactosidase activity of the *nodZ-lacZ* fusion in the symbiotic state was determined after inoculating 3-day-old

soybean seedlings with 1-ml cultures (approximately 10^8/ml) of the fusion strains. Plants were grown in plant nutrient solution (63) in growth pouches for 21 days. Bacteroids were isolated from soybean nodules as previously described for RNA isolation. The bacteroid cell pellet was resuspended in 0.5 ml of RDY medium and sonicated at 4°C for a total of 30 s. The lysate was then assayed for β-galactosidase activity in the same way used for the free-living method. Protein concentrations of the extracts were determined with a Bradford assay kit (Bio-Rad).

Detection of nodulation signals by thin-layer chromatography. Detection of nodulation signals on reverse-phase thin-layer chromatography plates was done as described previously (47, 56).

Purification of the lipooligosaccharide nodulation signals of *B. japonicum*. The lipooligosaccharide nodulation signals were purified from *B. japonicum* USDA110 and mutant strain NAD138 (*nodZ*) as described previously (9, 47). Cultures were induced for *nod* gene expression by the addition of soybean seed extract (5, 52).

Chemical analysis of the Nod factor. Glycosyl composition was determined by gas chromatography-mass spectrometry analysis of alditol acetates prepared as described by York et al. (64). The gas chromatography analysis was performed with a 30-m DB1 fused silica capillary column from J&W Scientific. The alditol acetates were identified by comparison of their retention times with those of authentic standards. Inositol was added as an internal standard. Fast atom bombardment-mass spectrometry analysis was carried out with a VG ZAB-SE instrument at an accelerating voltage of 8 kV in the positive mode. Thioglycerol was used as the matrix. The samples were dissolved in dimethyl sulfoxide, and approximately 2 to 10 μg in 1 μl was applied to the probe. The location of the double bond in the fatty acyl residue of the USDA110 lipooligosaccharide was examined by the procedure of Yruela et al. (66). The lipooligosaccharide was saponified in 4 M KOH for 4 h at 100°C. The solution was acidified, and the fatty acid was extracted into methylene chloride. The double bond of the fatty acid was determined by the preparation and analysis of dimethyl disulfide ethers of the fatty acid methyl esters (66).

Bioassay of the nodulation signals. Seeds of *Glycine soja* PI468397 and siratro were surface sterilized and germinated as previously described (37). Root deformation activity of the purified nodulation signals was tested as described previously (47), with serial dilutions of the different factors in 50% acetonitrile. Assays for cortical cell division utilized the spot inoculation method originally described by Turgeon and Bauer (62). In this case, 2-day-old seedlings (*G. soja*) were placed in plastic growth pouches containing 5 ml of plant nutrient solution and were allowed to grow overnight in the dark. At the time of inoculation, the position of the smallest emergent root hairs visible in a dissecting microscope at ×50 magnification and the root tip were marked on the top face of the plastic pouch. The top face of the pouch was slit with a razor blade and rolled back to expose the roots. Prior to inoculation, a single Amberlite bead (Fluka XAD-4) was transferred with fine forceps to a position approximately 80% between the two marks and proximal to the root tip. Droplets containing different amounts of the purified lipooligosaccharide nodulation factors in a volume of 30 to 50 nl were delivered with a micropipet to the same position as the Amberlite bead. The droplets were allowed to dry on the root surface for approximately 10 to 15 min, and the pouches were taped closed. To avoid undesirable binding of the nodulation signals to the plastic, a sterile straw was placed between the root and pouch for the first 2 h after inoculation. Plants were then transferred to a growth room with a light-dark photoperiod of 16 h of light to 8 h of dark.

Roots were analyzed for cortical cell division and nodule formation by the clearing method described by Truchet et al. (61). Whole roots were immersed in a solution containing 5.25% hypochlorite for 15 min under a vacuum and an additional 15 min at atmospheric pressure. Roots were then rinsed thoroughly in distilled water and stained with methylene blue (0.01%) for 45 s. After several washes in distilled water, roots were observed with a dissecting microscope.

Nucleotide sequence accession number. The DNA sequence of *nodZ* has been submitted to GenBank (accession no. L22756).

RESULTS

Identification of a DNA region homologous to *Hsn*II of *Rhizobium* sp. strain NGR234. Southern hybridization experiments, with the two Hsn regions *Hsn*II and *Hsn*III isolated from *Rhizobium* sp. strain NGR234 as hybridization probes, previously identified a region of apparent similarity (i.e., a 3.4-kb *Hin*dIII fragment) in *B. japonicum* USDA110 (37). This 3.4-kb *Hin*dIII fragment was isolated from pRjUT10 and subcloned into pKK233-3 to yield pRP2. A physical map of the cloned fragment was established by using four restriction enzymes (Fig. 1).

Hybridization of the *Rhizobium* sp. strain NGR234 Hsn probes to Southern blots of single or double digests of pRP2 was used to further define the region of similarity. Hybridization of the 2.7-kb *Eco*RI-*Pst*I fragment of plasmid pCB507 (*Hsn*II) identified a 1.5-kb *Eco*RI-*Pst*I fragment internal to the *B. japonicum* 3.4-kb *Hin*dIII fragment. No hybridization was seen when the *Hsn*III region (i.e., the 3.8-kb *Eco*RI fragment encoding NodD [6, 33]) of pCB507 was used as a probe (data not shown). The approximate extent of the similarity between the *B. japonicum* DNA and the *Hsn*II region is indicated by the bar above the map in Fig. 1.

Identification of the *hsn* locus. Site-directed Tn*5* mutagenesis of the DNA region contained within pRjUT10 previously identified several symbiotic genes (37). One Tn*5* insertion mutant, NAD138, mapped within the 3.4-kb *Hin*dIII fragment (Fig. 1). The phenotype of this mutation was previously described as normal nodulation of soybean but no nodule formation on siratro (37). To further delimit this symbiotic region, additional Tn*5* insertions in the 3.4-kb *Hin*dIII fragment were isolated and confirmed, and their symbiotic phenotypes on soybean and siratro were determined. The positions and phenotypes of these Tn*5* insertions are indicated in Fig. 1. Insertions to the left and right of the ZB939 (a Tn*5-lacZ* insertion) and NAD138 insertions (Fig. 1) exhibited a wild-type nodulation and nitrogen fixation phenotype in both plants, delimiting a region of 1.5 kb between them. Therefore, the siratro-specific nodulation locus is unlikely to be longer than 1.5 kb and is located near the unique *Eco*RI site of the 3.4-kb *Hin*dIII fragment.

Plant test and nodulation phenotype of mutant NAD138. Previously published work indicated that mutant NAD138 exhibited a wild-type symbiotic phenotype on soybean. However, this work involved only one variety of soybean (i.e., cv. Essex). Therefore, we sought to further define the phenotype of NAD138 by testing for nodulation and nitrogen fixation on different soybean cultivars and siratro. This mutant was found to have differing nodulation abilities on several soybean varieties (Table 2). Similar to the wild-type strain, mutant NAD138 produced nitrogen-fixing (i.e., assayed by C_2H_2 reduction) nodules on *G. max* cv. Essex and Harosoy within 11

TABLE 2. Nodulation ability of NAD138 on different soybean cultivars

G. max var.	Day of 100% nodulation with strain[a]:		No. of nodules/plant with strain[b]:	
	USDA110	NAD138	USDA110	NAD138
Essex	12	13	11	6
Harosoy	11	11	10	12
Williams	10	14	16	12
Forest	13	16	13	11
Peking	15	24	3	1

[a] Data indicate the first day postinoculation on which nodules appeared on all plants.

[b] Data represent the average number of nodules calculated from 15 to 20 plants 20 days postinoculation. The plant test was carried out with plastic growth pouches (see Materials and Methods). Sixty to 80% of the nodules formed by NAD138 were on the lateral roots compared with 20 to 30% induced by USDA110.

days after inoculation. However, in the case of Essex, the number of nodules produced by the mutant was reduced compared with that produced by the wild type. A delay in nodule formation by the mutant was observed when *G. max* cv. Williams, Forest, and Peking were tested. In the case of cv. Williams and Forest, the delay was 4 days with respect to the wild-type nodulation pattern, and in the case of cv. Peking, the delay was more pronounced (i.e., 9 days). The number of nodules per plant was about the same, but in the last two varieties (Forest and Peking), 60 to 80% of the nodules were found on the lateral roots, compared with the 20 to 30% induced by the wild-type bacteria (Table 2).

In addition, the nodulation ability of NAD138 depended not only on the host itself but on the growth condition of the plants (Fig. 2). Siratro plants grown on agar slants in tubes cannot be infected by NAD138 until 26 days after inoculation. These results are similar to those reported earlier (37). However, when the siratro plants are grown in pouches, NAD138 induced nodule formation with only a 3-day delay compared with the wild type. However, under these conditions, the number of nodules was reduced (Fig. 2).

Identification of the NodZ protein and sequence analysis of the *nodZ* gene. The 3.4-kb *Hin*dIII fragment of *B. japonicum* USDA110 was subcloned into the expression vector pKK223-3 in two orientations (pRP2 and pRP14) and analyzed in *E. coli* maxicells. A single, novel protein with a molecular weight of approximately 35,800 was expressed in cells containing pRP2 (Fig. 3). The protein band of approximately 25 kDa seen in all the lanes corresponds to β-lactamase encoded by the vector.

In order to establish the approximate location of the translational start site, deletions utilizing the *Xho*I and *Bgl*II sites were constructed (Fig. 3). The *Xho*I deletion had no effect on protein expression; however, the novel 35.8-kDa protein was not expressed in cells containing the *Bgl*II deletion. These results suggested that the translational start of the 35.8-kDa protein lies within the 1.0-kb *Bgl*II fragment.

The complete DNA sequence of the 2.0-kb *Pst*I-*Hin*dIII fragment encompassing this region was determined (Fig. 4 [GenBank accession no. L22756]). Analysis of the DNA sequence identified a 970-bp open reading frame yielding a protein of approximately 36.6 kDa, similar to that detected in *E. coli* maxicells. The direction of translation of this open reading frame is also consistent with the maxicell data. The presumptive translational start was located within the 1.0-kb *Bgl*II fragment at position 505. This putative open reading frame was termed *nodZ*. The NodZ stop codon (TGA), at

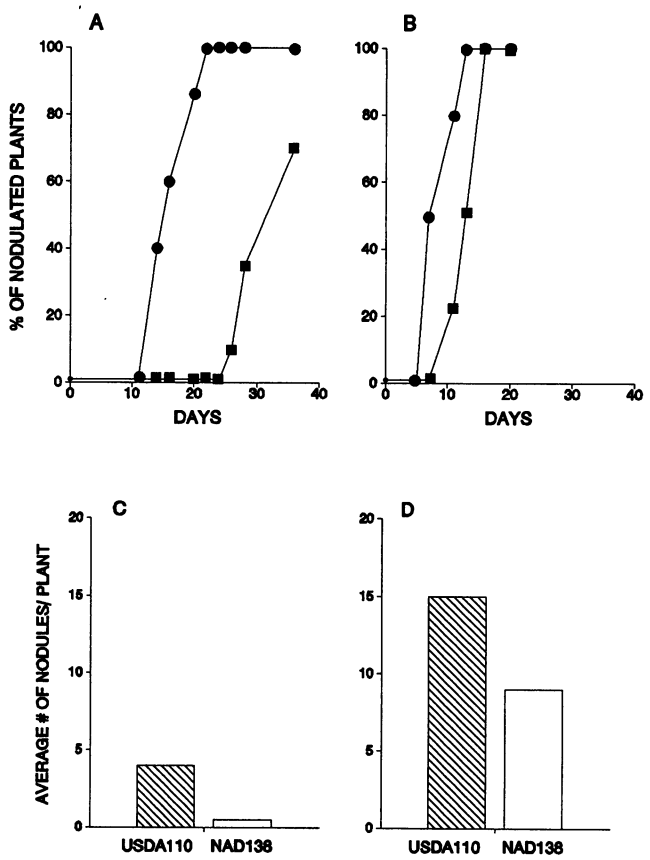

FIG. 2. (A and B) Comparison of the nodulation kinetics of the wild-type USDA110 (●) and the mutant NAD138 (■) on siratro plants grown on agar slants in test tubes (A) or in plastic growth pouches (B). (C and D) Comparison of the average number of nodules per siratro plant formed by the wild-type USDA110 or mutant NAD138 when plants were grown on agar slants in test tubes (C) or in plastic growth pouches (D). The average number of nodules per plant was calculated from 20 plants at 20 days after inoculation. Deviation was ±20%.

position 1477, predicts a protein of 323 amino acids. Comparison of the NodZ coding sequence with those in the GenBank DNA and protein and Swissprot data bases was performed with the TFastA and FastA programs provided in the University of Wisconsin Genetics Computer Group software package (version 7.2; GenBank release 12/92). No striking similarities were detected.

The occurrence of *nodZ* in other *Bradyrhizobium* and *Rhizobium* species was examined by hybridizing the internal 895-bp *Alu*I fragment to *Eco*RI-digested chromosomal DNA from a variety of strains (data not shown). All of the *B. japonicum* strains tested (i.e., USDA110, USDA6, USDA76, USDA94, USDA122, USDA123, USDA46, and 61A101c) showed positive hybridization, although, in some cases, the bands differed in size (data not shown). Other *Bradyrhizobium* species (i.e., strain 32H1 [cowpea] and strain ANU289 [*Parasponia* sp.]) also hybridized. Of the five *Rhizobium* species tested (i.e., *R. meliloti* AK631, *R. leguminosarum* bv. *trifolii* ANU843, *R. leguminosarum* bv. *viciae* 897, *Rhizobium* sp. NGR234, and *R. fredii* USDA194), only two, *Rhizobium* sp. strain NGR234 and *R. fredii*, showed weak hybridization to the *nodZ* probe (data not shown). These results suggest that *nodZ*

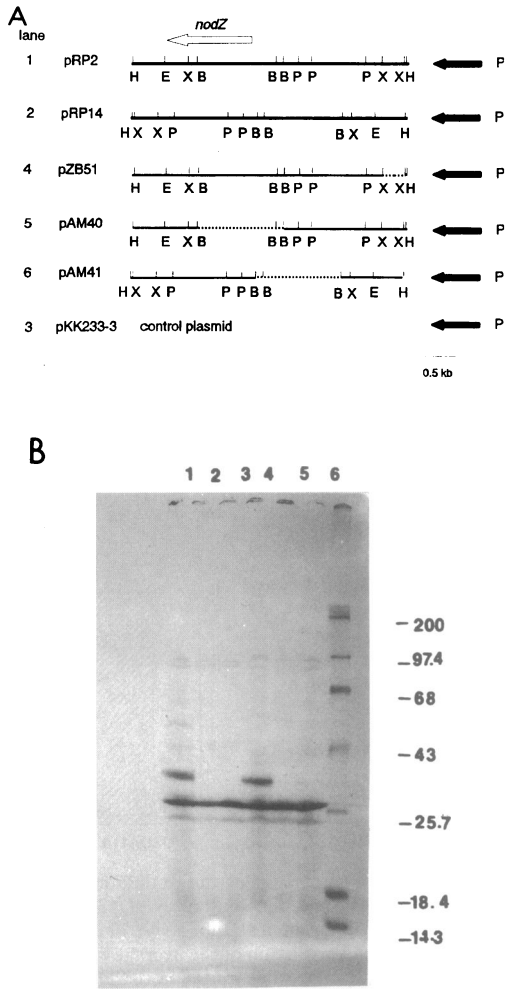

FIG. 3. (A) Physical map of the plasmid constructs used to detect the NodZ protein upon expression in *E. coli* maxicells. Deleted DNA is represented by a broken line. pKK233-3 is the control and represents the expression vector without insert DNA. (B) SDS-PAGE gel showing expression of proteins in *E. coli* maxicells containing the plasmid constructs shown in panel A. Lanes: 1, pRP2; 2, pRP14; 3, pKK233-3; 4, pZB51; 5, pAM40; 6, pAM41. Note the 35.8-kDa protein band found only in lanes 1 and 4.

```
1     CTGCAGGATTGGAGTGCGCTGGCTGCGTCATTGCCGCCGTACGCATCCCTTCATCGTGTT 60
61    CGCTCCCACATAGCGCCAGATCGGCAGGAAACGGTCTGCGAGATCCGCATGGGACATTCC 120
121   GCCCACTCTAGTATGCGTATCTCACCTGAGCTTTTCGAGATCTTGATGCGAATCGAGGGC  180
181   GAGGCGTCGCTCGGGTCGCTTTTCGACACAGCCCTGCTGGACCAGGCCAAGCGTGAAGAT 240
241   CTTGTGAAGGAATTGCGGCTCGTATGGGAGCTTCGGGGCGTTCGCCTGCATCCGCTGCAC 300
301   GCGGCTTGCCGTCACGATAATGTACAAAGCGGAACATAGCCATGACTCATGGCGCATGGC 360
361   CACGCCCATGCGTGCATGTGAGGGGACCAGGCCATCCGATGCAGAGCGGGGCATCGGGCA 420
421   TCGGGCTTCGGGCAGGTCGATGCAACACCGTTGAAGAAAGGACGATCGTATTGAGAGAAC 480
481   GCGGCCCGTCACGGATAGCCCTGCTATGAAGTTCTACCGATGCAGCTCGCCGGCACCGCG 540
                                M  K  F  Y  R  C  S  S  P  A  P  R
541   ATTTCAGGCCGTGACGCCGGGCGAGAAGATACGCGAGACGTCTGTACTGACGTCTCTTGT 600
      F  Q  A  V  T  P  G  E  K  I  R  E  T  S  V  L  T  S  L  V
601   CCAACCAGGAGCGCGCGAAAAGGCAAGGCAGATGGTTAGCGGTTCGAGCAATGATCGGTT 660
      Q  P  G  A  R  E  K  A  R  Q  M  V  S  G  S  S  N  D  R  F
661   CGTAGTGTCCCGGCGACGTACGGGGTTTGGCGACTGTCTGTGGTCACTCGCGGCAGCCTG 720
      V  V  S  R  R  R  T  G  F  G  D  C  L  W  S  L  A  A  A  W
721   GCGATTTGCAAAGCAGACAGGACGGACGCTAGCCATTGATTGGCGGGGATCTTGTTATCT 780
      R  F  A  K  Q  T  G  R  T  L  A  I  D  W  R  G  S  C  Y  L
781   TGATGAACCATTCACCAATGCCTTTCCAGTCTTCTTCGAACCGGTTGAAGACATTGGCGG 840
      D  E  P  F  T  N  A  F  P  V  F  F  E  P  V  E  D  I  G  G
841   AGTGCGAGTAATTTGCGATGACGACATCAATACGCGTTCGTTTCCAGGACCATTCTTTCC 900
      V  R  V  I  C  D  D  D  I  N  T  R  S  F  P  G  P  F  F  P
901   AACATGGTGGAACAAGCCATCTTTCGATTGCATCTATCGACCGGACGAGCAGATTTTCAG 960
      T  W  W  N  K  P  S  F  D  C  I  Y  R  P  D  E  Q  I  F  R
961   GGAACGCGATCAACTCGATCAACTGTTCCAAAGTCAGAGAGATAGTGACGCTAACACGGT 1020
      E  R  D  Q  L  D  Q  L  F  Q  S  Q  R  D  S  D  A  N  T  V
1021  TGTGTGTGATGCCTGCCTGATGTGGCGCTGCGATCAGGAAGCGGAACGCGAAATCTTCCG 1080
      V  C  D  A  C  L  M  W  R  C  D  Q  E  A  E  R  E  I  F  R
1081  GAGTATTAAGCCGCGGCCTGAAATTCAGGCCCGGATTGATGCTATCTACCGCGAGCACTT 1140
      S  I  K  P  R  P  E  I  Q  A  R  I  D  A  I  Y  R  E  H  F
1141  TGAGCCGTACAGCGTAATCGGCATTCATGTCCGGCATGGCAATGGCGAAGATATTATGGC 1200
      E  P  Y  S  V  I  G  I  H  V  R  H  G  N  G  E  D  I  M  A
1201  ATGCCCCTTACTGGGCGGACACGGAGCCGCGTTGCGGCAGATCTACAATGCCATTGATGA 1260
      C  P  L  L  G  G  H  G  A  A  L  R  Q  I  Y  N  A  I  D  E
1261  GGCCAGGAGTTTATCCCACGCAAAACCTGTGAGGGCGTTCCTGTGCACGGACAGTGCGCT 1320
      A  R  S  L  S  H  A  K  P  V  R  A  F  L  C  T  D  S  A  L
1321  CGTCCTCGAGCAGGTTTCGGTTAAATTTCCCGATGTCTTCGCGATTCCAAAGCAGTTCCA 1380
      V  L  E  Q  V  S  V  K  F  P  D  V  F  A  I  P  K  Q  F  Q
1381  GGCGCCTCAAGCGGGCCCATTGCACCATCCGCCCTCGGAGCTGAAGGCGGCTTTTCTGCC 1440
      A  P  Q  A  G  P  L  H  H  P  P  S  E  L  K  A  A  F  L  P
1441  CTCACCGAGATGTATCTCCTTGCCCGCTGCGACACGGTGATCCGTTTTCCGCCAACGAGC 1500
      S  P  R  C  I  S  L  P  A  A  T  R  *
1501  GCTTTTACGCGCTATGCGCGTCTTTTCGCCCCACGCGTTATAGAATTCGATTTGAACGAT 1560
1561  CCGGGCCGGTTGATCCTGATTGAAGACAACTCGCAAGCGCTTATGGCTTCGTGAAAGTCG 1620
1621  CAAACCCGCGTGATCGATCTCACCGCCGCTCTTTACGTCCGATAGCCGTGGAAGTTCTTC 1680
1681  TCCGGGCGACGATTTTCTGGCCGCGGGCTGTCATTGCCGCAACGTTCCAGGGGCGAAGAG 1740
1741  AACGAAGCCGCGGCCGAATGCGTTCGGCTAAGGCGCGGCTTCGACAACAGAGCCCTAAAG 1800
1801  TGCCGCTGCGTTTTACAGAAGGAGCACGCCTTCGTCTTCGCTTCGGGCGGGCCGCAGACG 1860
1861  GAAGCTAAACAACGCTCAATCACGGATGAACATTTCAGCTCGCCAGCTCATGCTTGGAAC 1920
1921  TAGCGCCGCGAAATCCAGTGCCGCTCAGAGCATGGCGCGATAGATCAAAAGTGGCCGCCG 1980
1981  ACGAATCTAACGTGCAAGCTT  2001
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FIG. 4. DNA sequence of the 2.0-kb *Pst*I-*Hin*dIII fragment and deduced amino acid sequence of NodZ. The transcriptional start site is indicated by an arrow. The region complementary to the oligonucleotide used for primer extension is underlined. The asterisk indicates the translational stop codon.

is common to *Bradyrhizobium* species and to *Rhizobium* species with an overlapping host range with *B. japonicum.*

Mapping of the *nodZ* promoter. Since a functional *nodZ* gene is essential for efficient nodulation, one would expect its expression to depend on NodD. However, analysis of the 5′-flanking DNA of *nodZ* reveals the lack of the characteristic *nod* box sequence found in other *nod* genes (Fig. 4). Further sequence analysis of the two *Pst*I fragments located upstream from this region (0.2 and 0.7 kb, respectively) also failed to show *nod* box homology (35).

The transcriptional start site of *nodZ* was determined by primer extension with an oligonucleotide complementary to the mRNA between nucleotide positions 633 and 655 (Fig. 4). Since it was established (see below) that the *nodZ* gene was expressed at the highest levels in the bacteroid state, 21-day-old root-nodule bacteroid RNA was annealed to the oligonucleotide and extended with reverse transcriptase. The primer extension product was resolved on a sequencing gel, and its sequence was compared with the DNA sequence of the region (Fig. 5). The transcriptional start corresponds to the cytosine residue at position 363 (Fig. 4).

Expression of *nodZ*. Northern hybridization to mRNA dot blots was performed as an initial screening method to look for conditions of high *nodZ* expression. Total RNA was isolated from *B. japonicum* USDA110 grown aerobically in RDY medium or from bacteroids. After hybridization with a *nodZ* internal DNA probe, an 895-bp *Alu*I fragment, it was found that the gene was expressed at high levels only in the symbiotic state (Fig. 6). These results were confirmed by measuring β-galactosidase expression with a *nodZ-lacZ* fusion. Plasmid pMGS104, containing a *nodZ*::Tn*3-lacZ* fusion, was conju-

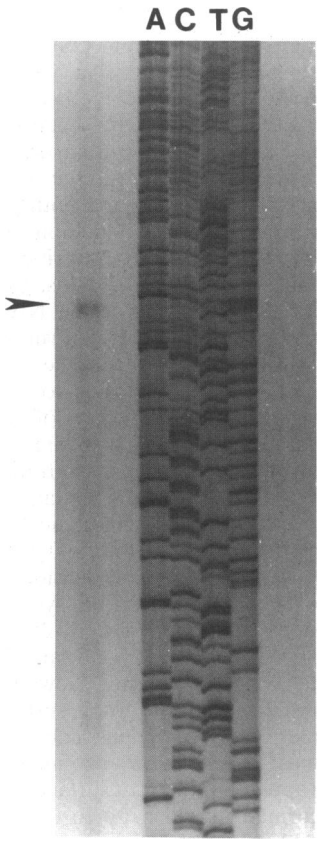

FIG. 5. Determination of the transcriptional start of *nodZ* by primer extension. The sequence derived by primer extension of *nodZ* mRNA is compared with that of a DNA sequence ladder. The transcriptional start site is indicated by the arrowhead.

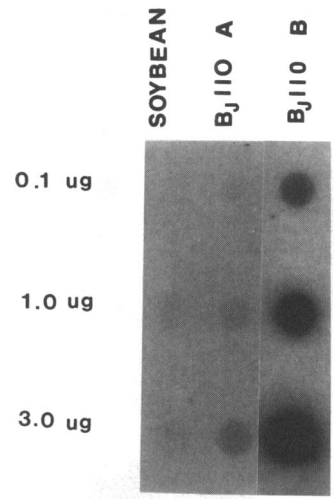

FIG. 6. RNA dot blot hybridization of a *nodZ* internal DNA probe (i.e., an 895-bp *AluI* fragment) to different RNA species. As indicated, 0.1, 1, or 3 μg of RNA was blotted to the filter. Lanes: soybean, soybean leaf total RNA (control); Bj110 A, total RNA isolated from aerobically grown *B. japonicum* cultures (RDY medium); Bj110 B, total RNA isolated from *B. japonicum* bacteroids. Approximately equal amounts of RNA were loaded in each lane as judged by hybridization to a 23S rRNA from *Pseudomonas aeruginosa* (19 [data not shown]).

gated to *B. japonicum* USDA110 and 2101 (USDA110, *nifA*). As a control, a *nifD-lacZ* fusion (encoded on pRj1025) was also mated into these strains. The level of β-galactosidase expression in these strains was subsequently measured (Table 3). The fusion was expressed in the wild type at a relatively high level in the symbiotic state. A significant level of constitutive expression was found in cells grown aerobically in nitrate medium. The *nodZ-lacZ* fusion was not expressed at a significantly higher level under anaerobic conditions. However, under similar conditions, the *nifD-lacZ* fusion was expressed, as expected, at a very high level. Consistent with the lack of increased expression under anaerobiosis, the *nifA* mutation had no effect on *nodZ* expression (Table 3). As expected, the lack of a functional NifA significantly reduced *nifD-lacZ* expression. Since a *nifA* mutation affects bacteroid development (20), it was not possible to test the role of NifA in symbiotic expression of *nodZ*. Consistent with the lack of a *nod* box, no differences in β-galactosidase expression were found when pMGS104 was conjugated into a NodD$^-$ strain (i.e., AN314 [data not shown]).

Structure of the lipooligosaccharide nodulation factors produced by *B. japonicum* NAD138 (*nodZ*). Given the phenotype of a *nodZ* mutant strain and the postulated role of nodulation genes in the synthesis of the nodulation factors, we sought to examine the profile of nodulation factors produced by strain NAD138. As shown in Fig. 7, induction of the *nodZ* mutant strain with 2 μM genistein or soybean seed extract resulted in the production of a nodulation metabolite that migrates slightly more slowly on a reverse-phase thin-layer chromatography plate than the wild-type factor. These data suggested that the NodZ mutant produced a metabolite that differed in structure from that of the wild type.

To explore this possible difference, we purified the nodulation metabolite from strain NAD138 after induction of cells with soybean seed extract. Figure 8A shows a fast atom bombardment-mass spectrum of the wild-type compound (i.e., BjNod-V [$C_{18:1}$; 2-*O*-methylfucose]) (47) with an inset showing a similar spectrum of the purified compound from strain NAD138. As can be seen, the molecular ion of the factor from NAD138 differs from that of the wild type by 160 mass units (i.e., 1416 versus 1256 mass units). This difference in mass would correspond to a nodulation factor identical to that of the wild type but lacking the 2-*O*-methylfucose residue (i.e., BjNod-V [$C_{18:1}$]) (Fig. 8A). In order to confirm this prediction, alditol acetate derivatives of the wild-type and NAD138 factors were prepared and analyzed by gas chromatography. As can be

TABLE 3. β-Galactosidase activity of a *Bradyrhizobium japonicum* *nodZ-lacZ* fusion under different growth conditions

Strain	β-Galactosidase activity (Miller units/OD_{600}) in[a]:		β-Galactosidase activity (Miller units/mg of protein) in bacteroids
	Aerobic conditions	Anaerobic conditions	
USDA110	10	6	232
USDA110			
nodZ-lacZ	52	64	1,330
nifD-lacZ	336	10,947	NT[b]
USDA110 NifA$^-$			
nodZ-lacZ	35	68	NA[c]
nifD-lacZ	337	490	NA

[a] Cells were grown in nitrate medium.
[b] NT, not tested.
[c] NA, not applicable. NifA$^-$ mutants are defective in nodulation (see text).

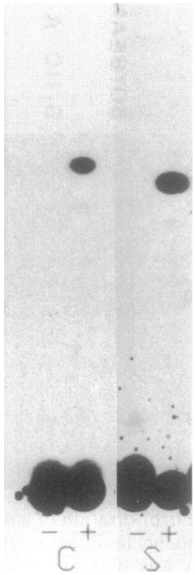

FIG. 7. Separation and detection of radiolabeled nodulation factors produced by induced cultures of strain USDA110 and the *nodZ* mutant NAD138. The factors were separated by reverse-phase, thin-layer chromatography as previously described (47, 56). C, *B. japonicum* wild-type USDA110; S, *B. japonicum* mutant NAD138; −, no inducer added; +, cultures induced with 2 μM genistein. Note that the major spot produced by strain NAD138 is slightly more hydrophobic than the corresponding spot in the USDA110 lane.

seen in Fig. 8B, 2-*O*-methylfucose and *N*-acetylglucosamine in the predicted 5:1 ratio can be seen in the wild-type preparations. However, as predicted, no 2-*O*-methylfucose could be detected in the NAD138 preparations (Fig. 8B). These results were confirmed with another mutant strain, WS1 (53a), with a deletion lacking the *nodZ* gene (i.e., deletion of the two *Bgl*II fragments within the 3.4-kb *Hin*dIII fragment [data not shown]).

A previous paper of ours (47) reported that the lipooligosaccharide from strain USDA110 (the parent of strain NAD138) had a $C_{18:1}$ fatty acyl residue in which the double bond was located between carbons 9 and 10, i.e., oleic acid. In contrast to that result, the $C_{18:1}$ fatty acyl residue in other reported lipooligosaccharides was found to be $C_{18:1\Delta11}$, i.e., vaccenic acid, including the lipooligosaccharides from other *B. japonicum* strains (9). Since that earlier report, a procedure was developed to ensure maximum release of the fatty acyl residue from the lipooligosaccharide. Thus, the location of the double bond in the $C_{18:1}$ fatty acyl residue of the USDA110 lipooligosaccharides was reexamined with the procedure of Yruela et al. (66). Following this procedure, analysis by gas chromatography-mass spectrometry gave one major fatty acid derivative, a dimethyl disulfide methyl ester of $C_{18:1}$ in which the major fragment ions were of *m/z* 145 and 245 (data not shown). These ions result from cleavage between the carbons that carry the methyl sulfide groups and show that the double bond is between carbons 11 and 12. Thus, the $C_{18:1}$ fatty acyl residue from the USDA110 lipooligosaccharides is vaccenic acid. A small amount of the dimethyl disulfide derivative of oleic acid methyl ester was also present—probably because of slight contamination of the lipooligosaccharide preparation or because of the presence of a contaminant in the solvents used in the derivatization process. This contaminant probably accounts for the presence of oleic acid previously reported in the lipooligosaccharide preparation from USDA110.

Plant response to the nodulation factor from strain NAD138. The NodBj-V ($C_{18:1}$; 2-*O*-methylfucose) factor produced by *B. japonicum* USDA110 has been shown previously to induce root hair deformation and cortical cell division in soybean (9, 47). Since both *B. japonicum* and *Rhizobium* sp. strain NGR234 nodulate soybean and both possess the 2-*O*-methylfucose derivative on their nodulation signals, it seemed likely that this substituent would be critical for biological activity on soybean. In order to test this, we examined the ability of the purified factor from strain NAD138 ($NodZ^-$; NodBj-V [$C_{18:1}$]) to induce root hair deformation and cortical cell division on soybean and siratro. The nodulation factor purified from strain NAD138 was able to induce significant root hair deformation at concentrations of 10^{-9} M on both siratro and *G. soja*. This compares with similar activity by the USDA110 wild-type compound at concentrations of 10^{-11} M on *G. soja* and 10^{-12} M on siratro (47 [data not shown]).

In bioassays using *G. soja* to detect cortical cell division, the wild-type nodulation signal (i.e., NodBj-V [$C_{18:1}$; 2-*O*-methylfucose]) induced foci of cortical cell division and nodule-like structures. With the spot inoculation system described in Materials and Methods, approximately 25% of the plants treated with 1.5 ng of the wild-type factor and 70 to 75% of the plants treated with 15 ng of the factor showed the appearance of nodule-like swellings on the root surface (Fig. 9). In addition, clear meristematic regions could be detected in cleared roots of these plants. In order to increase the levels of nodulation factor applied to the root surface, additional experiments were performed in which up to 0.5 μl of a 1-mg/ml solution of the wild-type nodulation factor in 25% dimethyl sulfoxide was applied to the root surface. The amount of nodulation signal in this volume would be equivalent to 500 ng. However, this volume is in excess of what can be retained on the root surface, and, therefore, significant amounts of the factor were lost because of overflow onto the plastic growth pouch. We estimate that no more than one-fifth of the volume was retained on the root surface (approximately 100 ng of factor). In this assay, 18 days postinoculation, approximately 75% of the plants showed up to four or five nodule-like swellings per root. However, additional early meristematic regions could also be detected in cleared roots. Interestingly, these early meristems appeared to develop in the ground meristem region of the root immediately above the vascular tissue. This is the position of the first divisions in indeterminate nodules (27). However, soybean forms determinate nodules in which the first cell divisions occur in the subepidermal cortical cells. The meristematic regions formed seemed to cease division at some point and appeared disorganized. The inside of these structures was devoid of apparent vascular tissue, with abnormally large cells and large intercellular spaces (Fig. 9). Therefore, these structures are quite different from nodules induced by bacterial infection.

None of the plant roots inoculated with a similar amount of the nodulation signal purified from NAD138 exhibited any observable swellings, bumps, or nodule-like structures similar to those seen with the wild-type factor (Fig. 9). Cleared roots of these plants failed to show any clear meristematic regions, although a few (one per root) blue-staining regions were seen in approximately 20% of the plants inoculated. It is unclear whether these regions reflect early meristems induced by the mutant nodulation factor. We conclude from these results that the unfucosylated nodulation factor is unable to induce cortical cell division on soybean at concentrations estimated to be 65 times higher than are required for the wild-type compound.

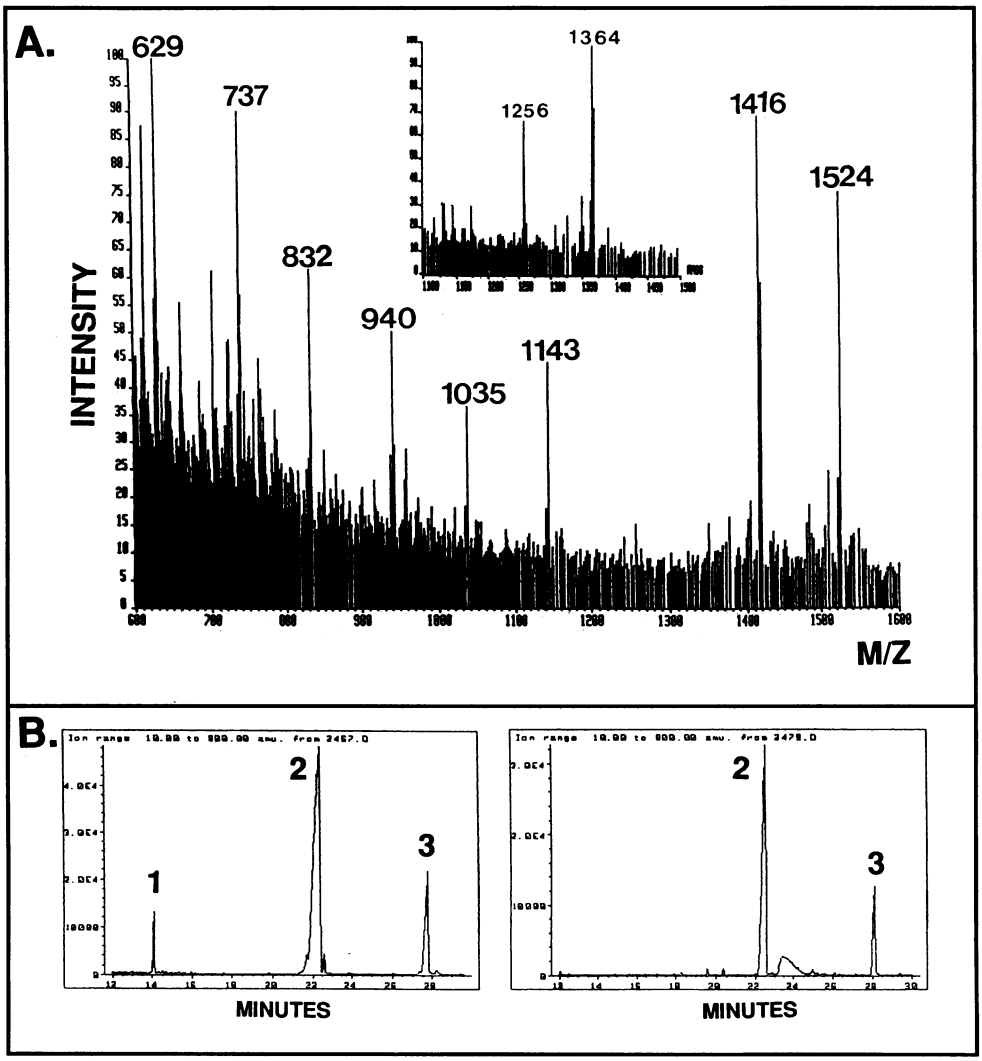

FIG. 8. (A) Fast atom bombardment-mass spectrum of the Nod factor from strain USDA110 and strain NAD138 (inset). Thioglycerol adducts (+108 a.m.u.) were observed for all $(M + H)^+$ ions and for all fragment ions. Thioglycerol adducts are observed whenever the Nod factor contains an unsaturated fatty acyl substituent. Thus, the molecular ion results for the USDA110 Nod factor are $(M+H)^+$ m/z = 1,416, and those for the NAD138 factor are m/z = 1,256. The respective thioglycerol adducts are m/z = 1,524 and m/z = 1,364. (B) Gas chromatography profiles of the alditol acetates of the USDA110 Nod factor (left) and the NAD138 Nod factor (right). The peaks are the alditol acetate derivatives of the following sugar residues: 1, 2-*O*-methylfucose; 2, inositol (the internal standard); and 3, *N*-acetylglucosamine.

DISCUSSION

The *nodZ* gene of *B. japonicum* was identified by hybridization to the *Hsn*II region isolated from *Rhizobium* sp. strain NGR234 that is required for nodulation of siratro (3). Indeed, *nodZ* mutants of *B. japonicum* are defective in siratro nodulation (37). Such mutants also show altered nodulation ability on a few varieties of soybean, but not with others (e.g., cv. Essex). Therefore, NodZ function exhibits host-specific character at both the species and cultivar level. In addition to this species specificity, the nodulation phenotype of a *nodZ* mutant is also dependent on the plant growth conditions used, being more severe when siratro plants are grown on agar slants. A similar situation has been reported for *nodH* mutants of *R. meliloti*, in which the nodulation phenotype is less severe when plants are grown in plastic pouches than when they are grown on agar slants (38). NodH is known to be essential for sulfation of the terminal reducing residue of the *R. meliloti* nodulation factors and would appear to be needed for nodulation. Apparently, in the cases of both NodZ and NodH, specific substitutions of the lipooligosaccharide signal are not absolutely required for nodulation under some plant growth conditions.

The sequence of *nodZ* suggests a translation product of approximately 36.6 kDa. Indeed, a protein product of approximately this size was detected in an *E. coli* maxicell expression system. However, although mutations in *nodZ* clearly have a nodulation-defective phenotype and synthesis of the nodulation factors is affected, *nodZ* expression is not dependent on NodD, unlike other characterized *nod* genes of *B. japonicum*. The *nodZ* gene is expressed constitutively under both aerobic and anaerobic growth conditions as measured both by mRNA hybridization and assay of a *nodZ-lacZ* fusion. Although we failed to identify conditions in which *nodZ* was induced during free-living growth, at this point, we cannot rule out that certain, at present unknown, conditions, perhaps characteristic of the

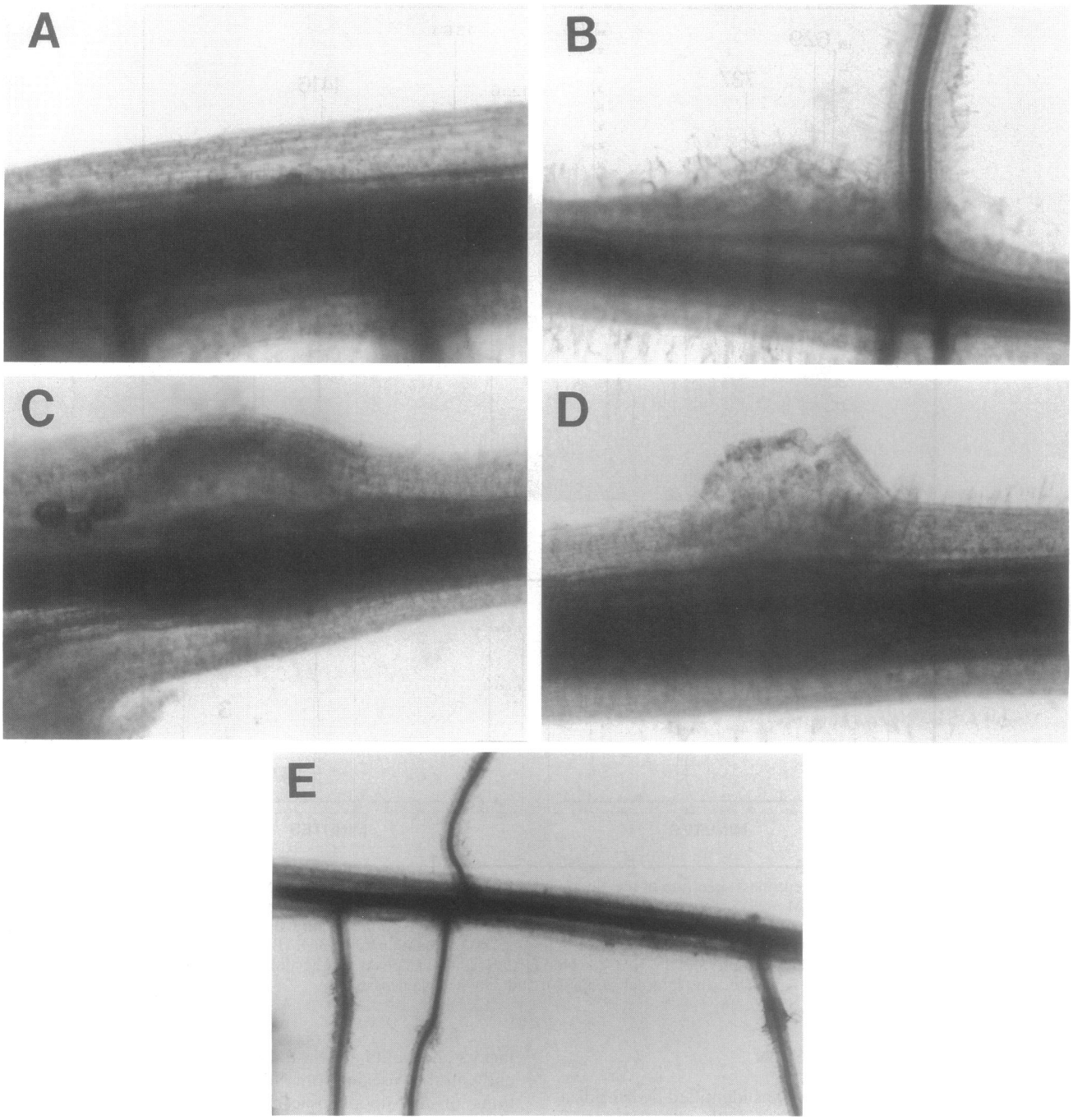

FIG. 9. Meristem formation and development induced by NodBj-V ($C_{18:1}$ [2-*O*-methylfucose]) on roots of *G. soja*. Roots were spot inoculated with purified compound as described in Materials and Methods and were observed with a dissecting microscope after clearing and staining. (A) Young meristems start to develop at different locations on the root. (B and C) The meristems develop, giving rise to bumps and swellings resembling nodules. (D) These nodule-like structures show anatomical disorganization and apparent cell death. (E) Representative root spot inoculated with the purified nodulation factor from NAD138 (*nodZ*). In each case, 0.5 μl of a 1-mg/ml solution was applied to the root as described in Materials and Methods. Magnifications: A, ×43; B, ×26; C, ×34; D, ×34; E, ×10.

rhizosphere environment, could be found to activate higher levels of *nodZ* expression. A puzzling characteristic of *nodZ* regulation is the existence of higher levels of expression in isolated bacteroids. However, NifA does not appear to be required for *nodZ* expression, nor does *nodZ* expression appear to be influenced by oxygen concentration. Therefore, one can postulate that a unique symbiotic regulator may be involved in regulating *nodZ* expression in planta.

Our initial analysis of the ^{14}C-acetate-labeled *nod* factors produced by the NodZ$^-$ mutant suggested that NodZ could be involved in the synthesis of such factors. These results were confirmed by the purification and structural elucidation of

these factors. The results suggest that NodZ is involved in fucosylation of the core lipooligosaccharide nodulation structure. All of the wild-type *B. japonicum* nodulation factors that we have isolated (9, 47), as well as the *nod* factors isolated from *Rhizobium* sp. strain NGR234 (39) (also capable of soybean nodulation), have a 2-*O*-methylfucosyl (or fucose) residue linked to C-6 of the reducing *N*-acetylglucosamine residue. This suggests that this residue is critical for soybean nodulation. Indeed, unlike the wild-type situation, bioassay of the nodulation signal from NAD138 showed that this factor could not induce root cortical cell division. However, our data clearly indicate that *nodZ* mutants are fully competent for nodulation of most soybean genotypes and can also nodulate siratro under the appropriate growth conditions. Therefore, we are left with an apparent paradox in that the 2-*O*-methylfucose substituent is essential for the biological activity of the nodulation signal but not for nodulation. A possible solution of this paradox could be that the 2-*O*-methylfucose substituent is vital to soybean nodulation, but other functions in *B. japonicum* are able to compensate for the loss of this substituent. Precedent for such a situation is provided by the *nodO* gene of *R. leguminosarum* bv. *viciae* (11, 17, 49). This gene, under the appropriate conditions, can phenotypically complement mutations in the *nodFE* genes. NodFE proteins are critical for pea nodulation under most conditions and are thought to be involved in the synthesis of the polyunsaturated fatty acyl substituent on the terminal nonreducing *N*-acylglucosamine residue of the *R. leguminosarum* bv. *viciae* nodulation factor (12, 22, 55, 57). The presence of the polyunsaturated fatty acyl substitution is critical to the ability of this signal to induce cortical cell division on the pea (12, 55). In contrast, NodO does not appear to be involved in the synthesis of the nodulation signals and is a calcium binding protein that has been proposed to form a membrane pore (17, 60). It is clearly a puzzle as to how such a protein can phenotypically compensate for a change in the fatty acyl composition of the *R. leguminosarum* bv. *viciae* nodulation signal. By analogy, a presently unidentified gene in *B. japonicum* may be able to compensate for the structural changes in the nodulation factors resulting from a NodZ mutation. The fact that both *nodH* mutants of *R. meliloti* and *nodZ* mutants of *B. japonicum* exhibit a conditional nodulation phenotype on their respective hosts even though they appear to be critical for active nodulation factor synthesis suggests that additional unknown factors are involved in nodulation specificity.

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REFERENCES

1. **Aiba, H., S. Adhya, and B. de Crombrugghe.** 1981. Evidence for two functional gal promoters in intact *Escherichia coli* cells. J. Biol. Chem. **256:**11905–11910.
2. **Amann, E., and J. Brosius.** 1985. ATG vectors for regulated high-level expression of cloned genes in *Escherichia coli*. Gene **40:**183–190.
3. **Bachem, C. W. B., Z. Banfalvi, E. Kondorosi, J. Schell, and A. Kondorosi.** 1986. Identification of host range determinants in the *Rhizobium* species MPIK3030. Mol. Gen. Genet. **203:**42–48.
4. **Banfalvi, Z., and A. Kondorosi.** 1989. Production of root hair deformation factors by *Rhizobium meliloti* nodulation genes in *Escherichia coli*: HsnD (NodH) is involved in the plant host-specific modification of the NodABC factor. Plant Mol. Biol. **13:**1–12.
5. **Banfalvi, Z., A. J. Nieuwkoop, M. G. Schell, L. Besl, and G. Stacey.** 1988. Regulation of nod gene expression in *Bradyrhizobium japonicum*. Mol. Gen. Genet. **214:**420–424.
6. **Bassam, B. J., M. A. Djordjevic, J. W. Redmond, M. Batley, and B. G. Rolfe.** 1988. Identification of a *nodD*-dependent locus in the *Rhizobium* strain NGR234 activated by phenolic factors secreted by soybeans and other legumes. Mol. Plant-Microbe Interact. **1:**161–168.
7. **Bassam, B. J., B. G. Rolfe, and M. A. Djordjevic.** 1986. *Macroptilium atropurpureum* (siratro) host specificity genes are linked to a *nodD*-like gene in the broad host range *Rhizobium* strain NGR234. Mol. Gen. Genet. **203:**49–57.
8. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84:**188–198.
9. **Carlson, R. W., J. Sanjuan, U. R. Bhat, J. Glushka, H. P. Spaink, A. H. M. Wijfjes, A. A. N. van Brussel, T. J. W. Stokkermans, N. K. Peters, and G. Stacey.** 1993. The structures and biological activities of the lipo-oligosaccharide nodulation signals produced by type I and type II strains of *Bradyrhizobium japonicum*. J. Biol. Chem. **268:**18372–18381.
10. **Davis, L. C., M. D. Dibner, and J. F. Battey.** 1986. Methods in molecular biology. Elsevier/North Holland Publishing Co., New York.
11. **de Maagd, R. A., A. H. M. Wijfjes, H. P. Spaink, J. E. Ruiz-Sainz, C. A. Wijffelman, R. J. H. Okker, and B. J. J. Lugtenberg.** 1989. *nodO*, a new *nod* gene of the *Rhizobium leguminosarum* biovar *viciae* Sym plasmid pRL1JI, encodes a secreted protein. J. Bacteriol. **171:**6764–6770.
12. **Demont, N., F. Debelle, H. Aurelle, J. Denarie, and J. C. Prome.** 1993. Role of the *Rhizobium meliloti nodF* and *nodE* genes in the biosynthesis of lipo-oligosaccharide nodulation factors. J. Biol. Chem. **268:**20134–20142.
13. **Denarie, J., and J. Cullimore.** 1993. Lipo-oligosaccharide nodulation factors—a minireview: new class of signaling molecules mediating recognition and morphogenesis. Cell **74:**951–954.
14. **Denarie, J., F. Debelle, and C. Rosenberg.** 1992. Signaling and host range variation in nodulation. Annu. Rev. Microbiol. **46:**497–531.
15. **Deshmane, N., and G. Stacey.** 1989. Identification of *Bradyrhizobium nod* genes involved in host-specific nodulation. J. Bacteriol. **171:**3324–3330.
16. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria; construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
17. **Economou, A., W. D. O. Hamilton, A. W. B. Johnston, and J. A. Downie.** 1990. The *Rhizobium* nodulation gene *nodO* encodes a Ca-2+-binding protein that is exported without N-terminal cleavage and is homologous to haemolysin and related proteins. EMBO J. **9:**349–354.
18. **Feinberg, A., and B. Vogelstein.** 1982. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
19. **Festl, H., W. Ludwig, and K. H. Schleifer.** 1986. DNA hybridization probe for the *Pseudomonas fluorescens* group. Appl. Environ. Microbiol. **52:**1190–1194.
20. **Fischer, H.-M., A. Alverez-Morales, and H. Hennecke.** 1986. The pleiotropic natures of symbiotic regulatory mutants: *Bradyrhizobium japonicum nifA* gene is involved in control of *nif* gene expression and formation of determinant symbiosis. EMBO J. **5:**1165–1173.
21. **Fisher, R. F., and S. R. Long.** 1992. Rhizobium-plant signal exchange. Nature (London) **357:**655–660.
22. **Geiger, O., H. P. Spaink, and E. P. Kennedy.** 1991. Isolation of the *Rhizobium leguminosarum* NodF nodulation protein: NodF carries a 4′-phosphopantetheine prosthetic group. J. Bacteriol. **173:**2872–2878.

23. **Goldberg, R. B., R. A. Bender, and S. L. Streicher.** 1974. Direct selection for P1-sensitive mutants of enteric bacteria. J. Bacteriol. **118:**810–814.
24. **Halverson, L. J., and G. Stacey.** 1984. Host recognition in the *Rhizobium*-soybean symbiosis. Detection of a protein factor present in soybean root exudate which is involved in the nodulation process. Plant Physiol. **74:**84–89.
25. **Horvath, B., C. W. B. Bachem, J. Schell, and A. Kondorosi.** 1987. Host-specific regulation of nodulation genes in *Rhizobium* is mediated by plant-signal, interacting with the *nodD* gene product. EMBO J. **6:**841–848.
26. **Kassavetis, G. A., and E. P. Geiduschek.** 1982. Bacteriophage T4 late promoters: mapping 5′ ends of T4 gene 23 mRNAs. EMBO J. **1:**107–114.
27. **Kijne, J.** 1991. The *Rhizobium* infection process, p. 349–398. *In* G. Stacey, R. H. Burris, and H. J. Evans (ed.), Biological nitrogen fixation. Chapman and Hall, New York.
28. **Kondorosi, A.** 1992. Regulation of nodulation genes in rhizobia, p. 325–340. *In* D. P. S. Verma (ed.), Molecular signals in plant-microbe communications. CRC Press, Boca Raton, Fla.
29. **Kondorosi, A., E. Kondorosi, E. C. Pankhurst, W. J. Broughton, and Z. Banfalvi.** 1982. Mobilization of a *Rhizobium meliloti* megaplasmid carrying the nodulation and nitrogen fixation genes into other rhizobia and *Agrobacterium*. Mol. Gen. Genet. **188:**433–439.
30. **Kuykendall, L. D., and G. H. Elkan.** 1976. *Rhizobium japonicum* derivatives differing in nitrogen-fixing efficiency and carbohydrate utilization. Appl. Environ. Microbiol. **32:**511–519.
31. **Kuykendall, L. D., B. Saxena, T. E. Devine, and S. E. Udell.** 1992. Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. Can. J. Microbiol. **38:**501–505.
32. **Leong, S. A., G. S. Ditta, and D. R. Helinski.** 1982. Heme biosynthesis in *Rhizobium*. Identification of a cloned gene coding for delta-aminolevulinic acid synthetase from *Rhizobium meliloti*. J. Biol. Chem. **257:**8724–8730.
33. **Lewin, A., C. Rosenberg, H. Meyer, C. H. Wong, L. Nelson, J.-F. Manen, J. Stanley, D. N. Dowling, J. Denarie, and W. J. Broughton.** 1987. Multiple host-specificity loci of the broad host-range *Rhizobium* sp. NGR234 selected using the widely compatible legume *Vigna unguiculata*. Plant Mol. Biol. **8:**447–459.
34. **Long, S. R.** 1989. *Rhizobium*-legume nodulation: life together in the underground. Cell **56:**203–214.
35. **Luka, S., J. Sanjuan, R. W. Carlson, and G. Stacey.** 1993. *nolMNO* genes of *Bradyrhizobium japonicum* are co-transcribed with *nodY-ABCSUIJ*, and *nolO* is involved in the synthesis of the lipooligosaccharide nodulation signals. J. Biol. Chem., in press.
36. **Messing, J., R. Crea, and R. H. Seeburg.** 1981. A system for shotgun DNA sequencing. Nucleic Acids Res. **9:**309–321.
37. **Nieuwkoop, A. J., Z. Banfalvi, N. Deshmane, D. Gerhold, M. G. Schell, K. M. Sirotkin, and G. Stacey.** 1987. A locus encoding host range is linked to the common nodulation genes of *Bradyrhizobium japonicum*. J. Bacteriol. **169:**2631–2638.
38. **Ogawa, J., H. L. Brierley, and S. R. Long.** 1991. Analysis of *Rhizobium meliloti* nodulation mutant WL131: novel insertion sequence IS*Rm3* in *nodG* and altered *nodH* protein product. J. Bacteriol. **173:**3060–3065.
39. **Price, N. P. J., B. Relic, F. Talmont, A. Lewin, D. Prome, S. G. Pueppke, F. Maillet, J. Denarie, J.-C. Prome, and W. J. Broughton.** 1992. Broad-host-range *Rhizobium* species strain NGR234 secretes a family of carbamoylated and fucosylated nodulation signals that are O-acetylated or sulphated. Mol. Microbiol. **6:**3575–3584.
40. **Quinto, M., and R. A. Bender.** 1984. Use of bacteriophage P1 as a vector for Tn*5* insertion mutagenesis. Appl. Env. Microbiol. **47:**436–438.
41. **Roche, P., F. Debelle, F. Maillet, P. Lerouge, C. Faucher, G. Truchet, J. Denarie, and J.-C. Prome.** 1991. Molecular basis of symbiotic host specificity in Rhizobium meliloti: *nodH* and *nodPQ* genes encode the sulfation of lipo-oligosaccharide signals. Cell **67:**1131–1143.
42. **Russell, P., M. G. Schell, K. K. Nelson, L. J. Halverson, K. M. Sirotkin, and G. Stacey.** 1985. Isolation and characterization of the DNA region encoding nodulation functions in *Bradyrhizobium japonicum*. J. Bacteriol. **164:**1301–1308.
43. **Ruvkin, G. B., and F. M. Ausubel.** 1981. A general method for site-directed mutagenesis in procaryotes. Nature (London) **289:**85–88.
44. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
45. **Sancar, A., A. M. Hack, and W. D. Rupp.** 1979. Simple method for identification of plasmid-coded proteins. J. Bacteriol. **137:**692–693.
46. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
47. **Sanjuan, J., R. W. Carlson, H. P. Spaink, U. R. Bhat, W. M. Barbour, J. Glushka, and G. Stacey.** 1992. A 2-O-methylfucose moiety is present in the lipo-oligosaccharide nodulation signal of *Bradyrhizobium japonicum*. Proc. Natl. Acad. Sci. USA **89:**8789–8793.
48. **Sanjuan, J., S. Luka, and G. Stacey.** 1993. Genetic maps of *Rhizobium* and *Bradyrhizobium* species, p. 2.136–2.146. *In* S. O'Brien (ed.), Genetic maps. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
49. **Scheu, A. K., A. Economou, G. F. Hong, S. Ghelani, A. W. B. Johnston, and J. A. Downie.** 1992. Secretion of the *Rhizobium leguminosarum* nodulation protein NodO by haemolysin-type systems. Mol. Microbiol. **6:**231–238.
50. **Schwedock, J., and S. R. Long.** 1990. ATP sulphurylase activity of the *nodP* and *nodQ* gene products of *Rhizobium meliloti*. Nature (London) **348:**644–646.
51. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vitro genetic engineering: transposon mutagenesis in Gram negative bacteria. Biotechnology **1:**784–791.
52. **Smit, G., V. Puvanesarajah, R. W. Carlson, W. M. Barbour, and G. Stacey.** 1992. *Bradyrhizobium japonicum nodD1* can be specifically induced by soybean flavonoids that do not induce the *nodYABC-SUIJ* operon. J. Biol. Chem. **267:**310–318.
53. **So, J.-S., A. L. M. Hodgson, R. Haugland, M. Leavitt, Z. Banfalvi, A. J. Nieuwkoop, and G. Stacey.** 1987. Transposon-induced symbiotic mutants of *Bradyrhizobium japonicum*: isolation of two gene regions essential for nodulation. Mol. Gen. Genet. **207:**15–23.

53a. **Song, W., and G. Stacey.** Unpublished observations.

54. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
55. **Spaink, H. P.** 1992. Rhizobial lipo-oligosaccharides: answers and questions. Plant Mol. Biol. **20:**977–986.
56. **Spaink, H. P., A. Aarts, G. Stacey, G. V. Bloemberg, B. J. J. Lugtenberg, and E. P. Kennedy.** 1992. Detection and separation of *Rhizobium* and *Bradyrhizobium* Nod metabolites using thin layer chromatography. Mol. Plant-Microbe Interact. **5:**72–80.
57. **Spaink, H. P., O. Geiger, D. M. Sheeley, A. A. N. van Brussel, W. S. York, V. N. Reinhold, B. J. J. Lugtenberg, and E. P. Kennedy.** 1991. The biochemical function of the *Rhizobium leguminosarum* proteins involved in the production of host specific signal molecules, p. 142–149. *In* H. Hennecke and D. P. S. Verma (ed.), Advances in molecular genetics of plant-microbe interactions, vol. 1. Kluwer Academic Publishers, Dordrecht, The Netherlands.
58. **Stachel, S. E., G. An, C. Flores, and E. W. Nester.** 1985. A Tn3 *lacZ* transposon for the random generation of β-galactosidase gene fusions: application to the analysis of gene expression in *Agrobacterium*. EMBO J. **4:**891–898.
59. **Stanley, J., and E. Cervantes.** 1991. Biology and genetics of the broad host range *Rhizobium* sp. NGR234. J. Appl. Bacteriol. **70:**9–19.
60. **Sutton, M. J., E. J. A. Lea, S. Crank, R. Rivilla, A. Economou, S. Ghelani, A. W. B. Johnston, and J. A. Downie.** 1993. NodO: a nodulation protein that forms pores in membranes, p. 163–167. *In* E. W. Nester and D. P. S. Verma (ed.), Advances in molecular genetics of plant-microbe interactions. Kluwer Academic Publishers, Dordrecht, The Netherlands.
61. **Truchet, G., D. G. Barker, S. Camut, F. Debilly, J. Vasse, and T. Huguet.** 1989. Alfalfa nodulation in the absence of *Rhizobium*. Mol. Gen. Genet. **219:**65–68.

62. **Turgeon, B. G., and W. D. Bauer.** 1982. Early events in the infection of soybean by *Rhizobium japonicum*. Time course and cytology of the initial infection process. Can. J. Bot. **60:**152–161.
63. **Wacek, T. J., and W. J. Brill.** 1976. Simple, rapid assay for screening nitrogen fixing ability of soybean. Crop Sci. **15:**519–523.
64. **York, W. S., A. G. Darvill, M. McNeil, T. T. Stevenson, and P. Albersheim.** 1985. Isolation and characterization of plant cell walls and cell wall components. Methods Enzymol. **118:**3–40.
65. **Young, J. P. W.** 1991. Phylogenetic classification of nitrogen-fixing organisms, p. 43–86. *In* G. Stacey, R. H. Burris, and H. J. Evans (ed.), Biological nitrogen fixation. Chapman and Hall, New York.
66. **Yruela, I., A. Barbe, and J. O. Grimalt.** 1990. Determination of double bond position and geometry in linear and highly branched hydrocarbons and fatty acids from gas chromatography-mass spectrometry of epoxides and diols generated by stereospecific resin hydration. J. Chromatogr. Sci. **28:**421–427.