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The NodD Protein Does Not Bind to the Promoters of Inducible Nodulation Genes in Extracts of Bacteroids of *Rhizobium leguminosarum* Biovar viciae

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In a previous study, we showed that in bacteroids, transcription of the inducible *nod* genes does not occur and expression of *nodD* is decreased by 65% (H. R. M. Schlaman, B. Horvath, E. Vijgenboom, R. J. H. Okker, and B. J. J. Lugtenberg, J. Bacteriol. 173:4277–4287, 1991). In the present study, we show, using gel retardation, that in crude extracts of bacteroids of *Rhizobium leguminosarum* biovar (bv.) viciae, NodD protein does not bind to the *nodF*, *nodM*, and *nodO* box and that it binds only weakly to the *nodA* box. Binding of NodD from bacteroids to *nod* box DNA could be restored by mild proteinase K treatment, indicating that NodD is present in bacteroids in an altered form or complex which prevents its binding to *nod* box DNA. In addition, a novel *nodA* box DNA-protein complex was found which is specific for the *nodA* promoter region. This novel complex was formed neither with material from cultured bacterial cells nor with an extract from uninfected roots, and it did not contain NodD but another protein. These results are consistent with the hypothesis that the protein present in the novel retardation complex acts as a transcriptional repressor causing the decreased *nodD* expression in bacteroids. Such a repressor also explains the lack of *nodABCIJ* transcription despite the weak NodD binding to the *nodA* box.

Rhizobia form nitrogen-fixing nodules on leguminous plants in a host-specific way, i.e., effective nodules are formed on pea (*Pisum sativum*) and *Vicia* spp. by *Rhizobium leguminosarum* bv. viciae and on alfalfa and *Melilotus* spp. by *Rhizobium meliloti*. The nodulation process consists of many steps, starting with bacterial attachment to root hairs, followed by root hair curling and the formation and growth of infection threads, in which the bacteria multiply and concomitantly induce meristematic activity in the root cortex. Finally, the bacteria are released from the infection thread into the newly formed nodule cells and differentiate into bacteroids, which fix atmospheric nitrogen.

Bacterial *nod* (for nodulation) genes, localized on the Sym (for symbiosis) plasmid, play a crucial role in nodulation. The *nod* genes of *R. leguminosarum* bv. viciae consist of the positive regulatory gene *nodD*, which is constitutively transcribed in free-living cells, and of the inducible *nod* operons *nodABCIJ*, *nodFEL*, *nodMNT*, and *nodO*. The promoters of *nodD* and *nodA* are read divergently and overlap (28, 33). The inducible *nod* operons are transcriptionally activated in the rhizosphere of the host plant, a process which requires both the bacterial NodD protein and flavonoids released by the host plant (6, 21, 24, 40, 42). The promoter regions of the inducible *nod* operons each contain a highly conserved DNA sequence, designated the *nod* box (25, 31, 33), which is essential for promoter function. It has been demonstrated that NodD binds to *nod* box DNA in the presence as well as in the absence of flavonoids (7, 10, 15), but the molecular mechanism of transcription activation by the NodD-*nod* box complex is still unknown.

In bacteroids of *R. leguminosarum* bv. viciae and *R. meliloti*, the inducible *nod* genes are no longer expressed (29, 32), and it has been shown that this expression stops before the bacteria are released from the infection thread (29). Although the level of *nodD* expression is reduced in bacteroids, to approximately 35% in *R. leguminosarum* bv. viciae (29) and to practically zero for *nodD1* and *nodD3* in *R. meliloti* (32), lack of NodD protein or depletion of flavonoids is not the cause of this expression stop (29).

In this paper, it is shown that the NodD protein of bacteroid extracts of *R. leguminosarum* bv. viciae does not bind to the *nodF*, *nodM*, and *nodO* promoters and binds only very weakly to the *nodA* promoter region. This might be because NodD is present in bacteroids in an altered form or complex. Furthermore, we describe a novel bacteroid-specific protein-DNA complex which specifically contains DNA sequences of the *nodABCIJ-nodD* intergenic region. We suggest that the protein involved is responsible for the reduced *nodD* transcription in bacteroids as well as for inhibition of *nodABCIJ* transcription.

MATERIALS AND METHODS

Bacterial strains and crosses. The bacterial strains and plasmids used are listed in Table 1. During cloning procedures, plasmids were propagated in *Escherichia coli* JM101. Plasmids were transferred from *E. coli* to *Rhizobium* strains by tripartite mating with pRK2013 as the helper plasmid (5).

Isolation of bacteroids and preparation of bacteroid lysates. To obtain nodules, seeds of *P. sativum* were sterilized, inoculated with cells of *Rhizobium* strain RBL1402 (pMP280), a *nodD* mutant strain with a plasmid containing cloned *nodD* from pRL1JI (34), or wild-type *R. leguminosarum* bv. viciae strain 248, and cultured as described previously (22). For reference, use was made of uninfected pea plants, which were grown on gravel with Raggio medium (23) supplemented with 10 mM NH_4NO_3.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
R. leguminosarum		
248	Wild-type bv. viciae, containing Sym plasmid pRL1JI	11
RBL1391	248 Rif^r, cured of the Sym plasmid	This study
RBL1402	248(pRL1JI), *nodD2*::Tn*5*	39
E. coli JM101	Δ(*lac-pro*) *supE thi* (F′ *traD36 proAB lacI*^{q}ZΔM15)	41
Plasmids		
pMP230	IncP, 333-bp *Sma*I-*Bcl*I fragment of pRL1JI containing pr. *nodF-nodF′*	H. P. Spaink
pMP233	IncP, 876-bp *Sal*I-*Eco*RV fragment of pRL1JI containing pr. *nodM-nodM′*	H. P. Spaink
pMP238	IncP, containing entire *nodD* without its own pr., under control of pr. *nodA*	30
pMP280	IncP, containing pr. *nodD-nodD*	36
pMP300	IncP, 110-bp *Bgl*II-*Bcl*I fragment of pRL1JI containing *nodA* promoter region and part of the overlapping *nodD* promoter	33
pMP448	IncP, 291-bp *Pst*I-*Bam*HI fragment of pRL1JI containing pr. *nodO-nodO′*	A. H. M. Wijfjes
pMP1070	IncP, containing pr. *nodA*	H. P. Spaink
pMP2010	IncColE1, containing pr. *lac-nodD*	29
pMP2066	IncColE1, containing 110-bp PCR product with *nodF* box	This study
pMP2068	IncColE1, containing 110-bp PCR product with *nodM* box	This study
pMP2069	IncColE1, containing 110-bp PCR product with *nodO* box	This study

[a] All *nod* sequences are from pRL1JI. pr., promoter; Rif, rifampin.

Bacteroids were isolated from pea nodules 22 days postinoculation, essentially by the method of Katinakis et al. (12). The entire procedure was performed in the cold with ice-cold buffers and precooled materials. The nodules from 35 plants (approximately 2.7 g [fresh weight]) were ground in a mortar in 2.5 ml of isolation buffer (0.4 M sucrose, 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5], 2.5 mM $MgCl_2$, 10 mM KCl, 4% [wt/vol] polyvinylpyrrolidone) supplemented with 1.0 mM dithiothreitol (DTT) and 1.4 mM phenylmethylsulfonyl fluoride (PMSF). The procedure was terminated at the step at which bacteroids, still containing the peribacteroid membrane, were obtained. Such a bacteroid preparation contains approximately 5% bacteria (29). These bacteroids were suspended in 500 μl of isolation buffer supplemented with 0.1 mM DTT, 800 μM PMSF, 40 μg of leupeptin per ml, and 200 μg of soybean trypsin inhibitor per ml. Subsequently, 1.2 ml of 50 mM Tris-HCl (pH 7.5) was added, and the bacteroids were lysed by sonication (four times for 15 s each and subsequently twice for 5 s each). Small aliquots were stored at −20°C until use.

Preparation of lysates from cultured cells. *Rhizobium* cells were grown in TY medium (2) supplemented with 20% B^- medium (38) and, when *nod* genes were to be induced, also with 1.0 μM naringenin, until an A_{620} of 0.6 was reached. The cells were collected by centrifugation, and the bacterial pellet was resuspended in ice-cold 20% (wt/vol) sucrose in lysis buffer (50 mM Tris-HCl [pH 8.5], 5 mM EDTA, 0.1 mM DTT, 500 μM PMSF, 10 μg of leupeptin per ml, 50 μg of soybean trypsin inhibitor per ml). Cells were lysed by three passages through a French press at 1,550 lb/in^2; 200 μg of lysozyme per ml was added, and after incubation on ice for 40 min, the sucrose was diluted to 7% by adding lysis buffer. Unbroken cells were removed by low-speed centrifugation, and the supernatant fluid (cleared lysate) was stored at −20°C and used for binding studies.

Preparation of root extract. Roots (16 g), obtained from uninfected 22-day-old pea plants, were frozen in liquid nitrogen and blended twice for 5 s each at low speed and twice for 15 s each at high speed in a Waring blender. Subsequently, 20 ml of ice-cold isolation buffer containing 1.0 mM DTT and 800 μM PMSF was added. The roots were blended again for a few seconds and filtered over two layers of Miracloth. Debris was removed by low-speed centrifugation, and leupeptin (40 μg/ml) and soybean trypsin inhibitor (200 μg/ml) were added to the supernatant fluid. The crude root extract was stored in small aliquots at −20°C.

DNA fragments for binding studies. A 110-bp DNA fragment spanning the *Bgl*II-*Bcl*I region of pRL1JI and containing the *nodA* promoter region (Fig. 1) was isolated by digestion of pMP300 (33). To obtain DNA fragments of identical sizes and with the same intramolecular locations of the *nod* box of either *nodF*, *nodM*, or *nodO*, the polymerase chain reaction (PCR) method was used. Oligonucleotides that were homologous over at least 16 bases with sequences upstream and downstream of the conserved *nod* box sequences were designed (Fig. 1). Terminal *Sal*I sites and extra nucleotides were added for cloning, and PCR was performed with pMP230, pMP233, or pMP448 as the template to obtain *nodF*, *nodM*, and *nodO* promoter regions, respectively. PCR-generated fragments were digested with *Sal*I and cloned into pIC20H (20), yielding pMP2066 (with *nodF* box), pMP2068 (with *nodM* box), and pMP2069 (with *nodO* box) (Fig. 1). The nucleotide sequences were verified by the dideoxy chain termination method with double-stranded DNA (26).

Binding assay. Digested plasmids were labeled by filling in 3′ recessive ends with Klenow DNA polymerase and [α-^{32}P]dCTP (10 mCi/ml, 3,000 Ci/mmol) by standard methods (26). The labeled DNA fragments containing the *nod* box sequences were isolated from polyacrylamide gels in Tris-borate-EDTA buffer (26), and the amount which was recovered was estimated in a scintillation counter.

The binding assay was a DNA retardation assay and was performed in a final volume of 15 μl of binding buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 50 mM KCl, 0.1 mM DTT, 50 mg of bovine serum albumin per ml, 5% [vol/vol] glycerol) containing 60 μg of bacteroid protein or 15 μg of protein from cultured cells, 0.6 to 1.0 nM labeled DNA, and 500 μg of herring sperm DNA per ml. When lysates from bacteroids or from uninfected roots were used, 2.5 mM EDTA was added as well. In experiments in which antibodies were included, the protein extract was incubated with 3 μl of antiserum and herring sperm DNA in binding buffer for 25 min on ice before the labeled DNA was added.

In the tests for the involvement of RNA or protein, the

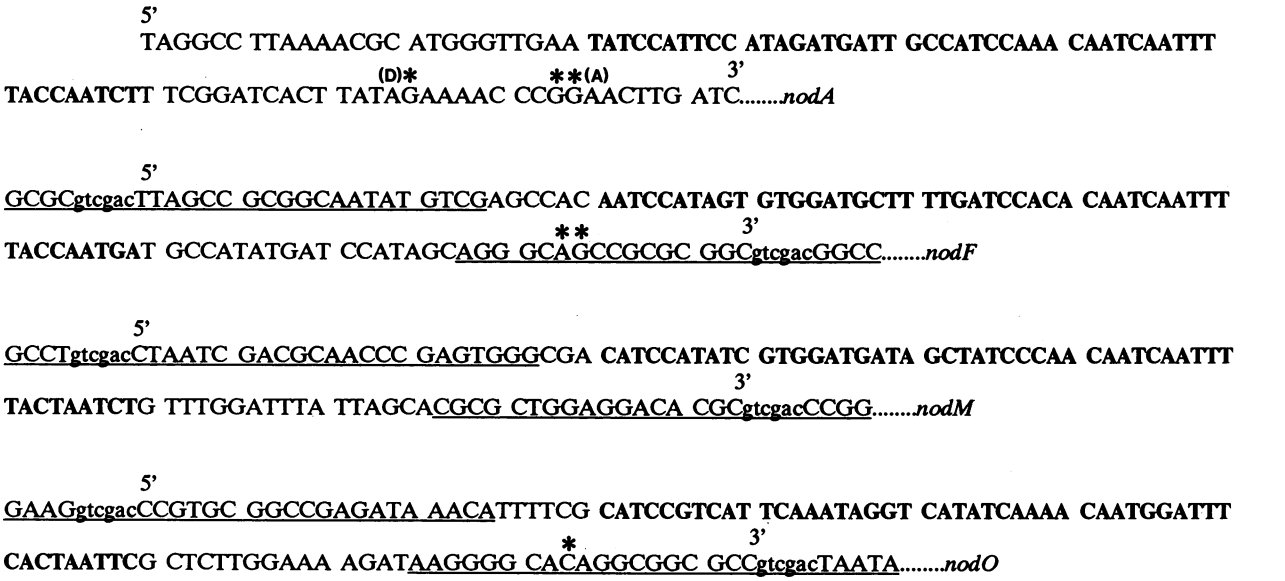

FIG. 1. DNA sequences of fragments containing a *nod* box used in binding studies. From top to bottom, the sequences containing the *nodA*, *nodF*, *nodM*, and *nodO* boxes of pRL1JI are shown. Relevant sequences are present between the indications 5′ and 3′. Only the coding strand of the respective genes is shown. The region overlapping the consensus *nod* box sequence (33) is shown in boldface, and flanking sequences are shown in normal type. Primers used in PCR consist of the underlined nucleotides, which were homologous to pRL1JI sequences (capital letters) or not homologous (*Sal*I site, lowercase letters). The known transcription initiation sites of *nodA* (35), *nodF* (35), *nodO* (4), and *nodD* (28) are indicated by an asterisk above the nucleotide(s).

lysates were treated in one of the following six ways: (i) incubation with a mixture of RNase A (1.0 mg/ml) and RNase T_1 (1,000 U/ml) for 20 min at 37°C; (ii) boiling for 10 min; (iii) incubation with 0.5% sodium dodecyl sulfate (SDS) for 20 min at 37°C; (iv) incubation with trypsin (12.5 mg/ml) in 6.0 mM Tris-HCl (pH 7.9)–2.5 mM $CaCl_2$ for 60 min at 37°C; (v) incubation with proteinase K (50 μg/ml) in 10 mM Tris-HCl (pH 8.4) for 30 min at 37°C; or (vi) incubation with proteinase K (50 μg/ml) in the control buffer 10 mM Tris-HCl [pH 7.8]–5 mM EDTA–0.5% SDS. Each binding reaction was performed at 21.5°C for 40 min, after which the tubes were immediately placed on ice and 4.5 μl of binding buffer containing 25% (vol/vol) glycerol and xylene cyanol FF and bromophenol blue dyes was added. Subsequently, the samples were loaded on a prerun 5% polyacrylamide gel (acrylamide–*N*,*N*′-methylene bisacrylamide, 30:0.8) in Tris-borate-EDTA buffer (26), and electrophoresis was performed at 4°C at 10 V/cm. Finally, the gels were dried and exposed to Fuji X-ray films, using intensifying screens.

Preparation of antibodies against NodD protein. To obtain antibodies against the entire NodD protein of *R. leguminosarum* bv. viciae, pMP2010 (29) was constructed. This plasmid contains the complete *nodD* sequence under control of the *lac* promoter in pIC19H (20). *E. coli* cells containing pMP2010 were grown in LB medium (26) containing 40 μg of isopropyl-β-D-galactopyranoside (IPTG) per ml for 16 h at 37°C and lysed as described above for cultured cells of *Rhizobium*. The low-speed centrifugation step delivered almost exclusively the NodD protein in the pellet, from which it was extracted by preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (18). Rabbits were immunized and antibodies were obtained as described previously (30).

Protein analysis. Protein analysis by SDS-PAGE (18) and Western immunoblotting were performed as described previously (30).

Protein concentrations were estimated by the procedure of Markwell et al. (19), with bovine serum albumin as the standard.

Miscellaneous. Super *Taq* DNA polymerase was obtained from Sphaero Q (Leiden, The Netherlands) and used according to the instructions of the manufacturer. All other enzymes for DNA manipulations were from LKB-Pharmacia (Woerden, The Netherlands). RNase A and RNase T_1 were obtained from Boehringer (Mannheim, Germany), and radioactive nucleotides were from Amersham International plc (Amersham, United Kingdom). Cloning and nucleotide sequencing were performed by standard methods (26).

Protease inhibitors, proteases, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

NodD protein from bacteroids does not bind to any of the inducible *nod* promoters except weakly to the *nodA* promoter. Since *nod* gene expression in bacteroids is severely reduced compared with that in free-living cells, we investigated whether this could be explained either by an altered NodD binding or by a new *trans*-acting factor acting on *nod* promoters in bacteroids. Therefore, DNA retardation studies were performed with lysates from bacteroids of *R. leguminosarum* RBL1402(pMP280), which contains the *nodD* gene of pRL1JI on a multicopy plasmid. Since the amount of NodD in bacteroids is only 35% of that in free-living cells (29), fourfold more bacteroid protein than protein from cultured cells was used in order to obtain at least the same signal for NodD complexes in case such complexes were formed normally. As a positive control for the formation of the NodD-containing complex, lysates were used from cultured cells of *R. leguminosarum* strains carrying a plasmid-borne copy of *nodD*, namely RBL1402 (pMP280), RBL1391(pMP280), and RBL1391(pMP238); the

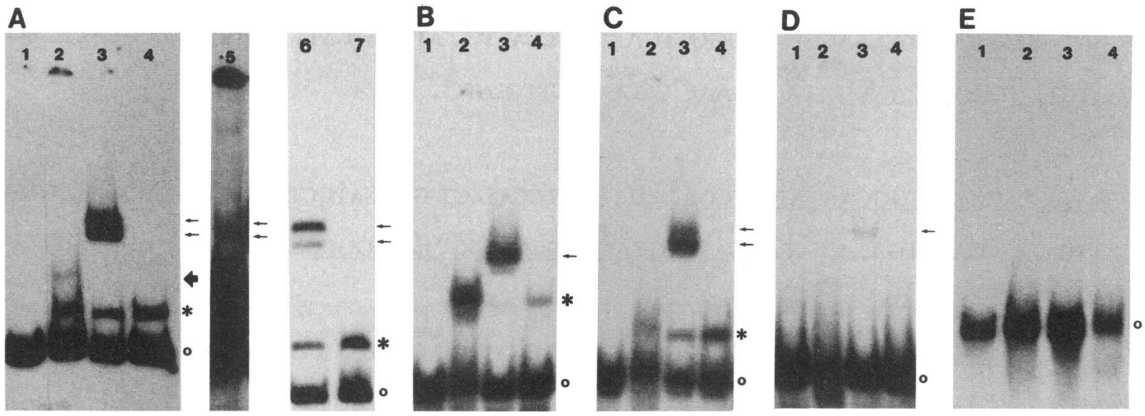

FIG. 2. Binding characteristics to the *nodA* (A), *nodF* (B), *nodM* (C), and *nodO* (D) promoter regions and to a 150-bp *Hin*dIII-*Nhe*I fragment internal to *nodA* (E). The following additions were made in the indicated lanes: 1, no bacterial material; 2, bacteroid lysate of *nodD*::Tn*5* strain RBL1402 harboring *nodD* sequences on plasmid pMP280; 3, lysate from cultured cells of the Sym plasmid-cured strain RBL1391 overproducing NodD from pMP238; 4, lysate from cultured cells of RBL1391 with the control plasmid pMP1070, lacking *nodD* sequences. Lane 5 of panel A represents the same lane as lane 2 but after a much longer exposure to visualize the two NodD-containing complexes. For the other retardation complexes and the unbound fragment, this film is overexposed. Lysates from cultured cells of strains RBL1391 and RBL1402, each producing NodD from pMP280, gave the same pattern of retardation complexes as shown in lanes 3, and that from strain RBL1402 with the control plasmid pMP1070 was identical to that in lanes 4. This is shown for the *nodA* promoter region with lysates from strain RBL1402(pMP280) (lane 6) and from strain RBL1402(pMP1070) (lane 7). The bacteroid-specific complex is indicated by a large arrow, the NodD-containing complexes are indicated with thin arrows, the Sym-independent complex is marked with an asterisk, and the unbound DNA is marked with an open circle.

last strain overproduces the NodD protein (30). As negative controls, *R. leguminosarum* RBL1402(pMP1070) and RBL1391(pMP1070) were used. They both contain a plasmid homologous to pMP238 which lacks the *nodD* open reading frame.

Since the size of the DNA fragment used in DNA retardation assays severely influences the rate of migration and therefore also the detection of the complexes formed, all the *nod* promoter fragments were made to be identical in size. The *Bgl*II-*Bcl*I restriction fragment containing the *nodA* promoter region had the small size of 110 bp, and this was used without alteration. The *nodF*, *nodM*, and *nodO* promoter regions lack appropriate restriction sites, and therefore *Sal*I restriction sites were introduced by PCR, resulting in DNA fragments of 110 bp each. The design of the introduced *Sal*I sites was such that each *nod* promoter DNA probe had the same orientation of the *nod* box within the fragment and contained the same length of flanking sequences (Fig. 1).

As shown in Fig. 2, different retardation complexes were formed by lysates from bacteroids and from cultured cells. With the promoter regions of *nodA*, *nodF*, and *nodM* but not with that of *nodO*, a fast-migrating complex (indicated with an asterisk in Fig. 2) is found. In the case of the *nodA* and *nodM* boxes, this complex migrates even faster than that with the *nodF* box, to a position very close to unbound DNA. These retardation complexes are formed with lysates from free-living *Rhizobium* cells regardless of whether *nodD* is present as well as with bacteroid lysates, and they are therefore unrelated to NodD. They represent complexes with a protein which is not encoded by the Sym plasmid, and they are therefore designated Sym-independent complexes. It is not known, however, whether the Sym-independent complex with the *nodF* promoter region contains a protein identical to that which forms the Sym-independent complex with the *nodA* and *nodM* promoters. These complexes were also formed by a bacteroid lysate (Fig. 2, lanes 2), indicating that DNA-binding proteins in the bacteroid lysate are not inactivated by the isolation procedure.

The retardation complexes with low mobilities in the gel (indicated with thin arrows in Fig. 2) consist of two complexes migrating very closely to each other in the case of the *nodA* and *nodM* promoter regions (Fig. 2A and C); it seems to be a single complex in the case of the *nodF* and *nodO* promoters (Fig. 2B and D). These complexes are likely to contain NodD protein, since they are formed with lysates from cultured cells of RBL1391(pMP238), RBL1402 (pMP280), and RBL1391(pMP280) (Fig. 2, lanes 3 and lane 6, and legend to Fig. 2) but not with lysates from RBL1391 (pMP1070) or RBL1402(pMP1070), which lack *nodD* (Fig. 2, lanes 4 and 7) (see also below). The fact that NodD forms two complexes with both the *nodA* and *nodM* promoter regions indicates that NodD can bind with these sequences in at least two different ways, resulting in two complexes with a different surface charge and/or size; this might be due to an interaction of NodD with other proteins in the extract.

The two NodD-containing complexes with the *nodA* promoter region were also formed by bacteroid lysates, although they were only visible after prolonged exposure (Fig. 2A, lanes 2 and 5). Unexpectedly, such a NodD-containing complex formed by bacteroids was never found with any of the other *nod* promoters (Fig. 2B, C, and D, lanes 2), not even after prolonged exposure or when twice the normal amount of bacteroid material was used. These results show that NodD in bacteroids binds only weakly (in the case of the *nodA* box) or not at all (in the case of the other inducible *nod* promoters) to *nod* boxes.

A novel retardation complex with the *nodA* promoter region is formed with lysates of bacteroids. In addition to the two very weak NodD-containing complexes found with the *nodA* promoter region and the Sym-independent complex, a fourth retardation complex is formed by bacteroid lysates. It migrates between the NodD-containing and the Sym-independent complexes. The novel complex, indicated in Fig. 2 with

a large arrow, is only formed with material from bacteroids and not with lysates from cultured cells, not even when eightfold-more protein than usual was used (Fig. 2A, lane 2). The formation of this complex is not restricted to strain RBL1402(pMP280), since a complex with the same mobility was also found with lysates from bacteroids of *R. leguminosarum* bv. viciae wild-type strain 248 (data not shown).

To test whether this complex is specific for bacteroids and not due to a factor of plant origin, lysates from noninfected pea roots were used in a retardation assay with DNA fragments containing either *nodA* box sequences or sequences from the coding region of the *nodA* gene. No retardation complexes were found at all (data not shown), indicating that a root lysate does not contain a factor which binds to the DNA used under these circumstances. Therefore, the fourth retardation complex found with the *nodA* promoter region is bacteroid specific.

Since the *nodA* promoter region contains not only the *nodA* box but also the overlapping *nodD* promoter (33), it was of interest to test whether the formation of the bacteroid-specific retardation complex is a general phenomenon for all known *nod* boxes. Incubation of bacteroid lysates with either the *nodF* or the *nodM* promoter region resulted in only the Sym-independent complexes (Fig. 2B and C, lanes 2; indicated with an asterisk), whereas no retardation complexes at all were formed with the *nodO* promoter. Since prolonged exposure times did not change these results, it is concluded that a bacteroid-specific retardation complex is not formed with any of the other *nod* box-containing DNA fragments and that this complex is therefore specific for the *nodA-nodD* promoter region.

To test the specificity of the bacteroid lysate for the *nodA* promoter region further, several DNA fragments from the coding regions of *nod* genes were used in a binding assay with bacteroid lysate. Complexes were not formed with any of these fragments (Fig. 2E). Furthermore, the addition of at least a 50-fold molar excess of unlabeled DNA was able to prevent complex formation with the labeled *nodA* box only when the unlabeled DNA contained *nodA* promoter sequences (data not shown), again showing specificity for the *nodA* promoter region.

NodD is not involved in the bacteroid-specific complex with the *nodA* promoter region. The relative amount of NodD protein is reduced in bacteroids of *R. leguminosarum* RBL1402(pMP280) (29), and it appeared that this NodD from bacteroids is still able to bind to *nodA* box DNA (Fig. 2A, lane 2), although to a very much lower extent. To confirm the presence of NodD in the two retardation complexes with the lowest mobility in the gel and to investigate whether NodD protein is involved in the bacteroid-specific retardation complex with the *nodA* promoter region, antibodies against NodD were used.

To obtain antibodies against the entire NodD protein, the *nodD* gene was cloned under the control of the *lac* promoter in pMP2010. Isolation of large quantities of the antigen was relatively simple because the protein was deposited in protein bodies in induced cells of *E. coli* JM101(pMP2010) and these could be solubilized with 0.1% SDS. The specificity of the antibodies against NodD in *Rhizobium* strains was shown by Western blotting (Fig. 3A). Despite cross-reactivity with a few other proteins which were also detected with the preimmune serum, the NodD-specific reaction is clear, since no protein of a similar size reacts with the antiserum in a strain containing a *nodD*::Tn*5* mutation (Fig. 3A, compare lanes 1 and 2). The high sensitivity and specificity of the antiserum against NodD also made it possible to detect

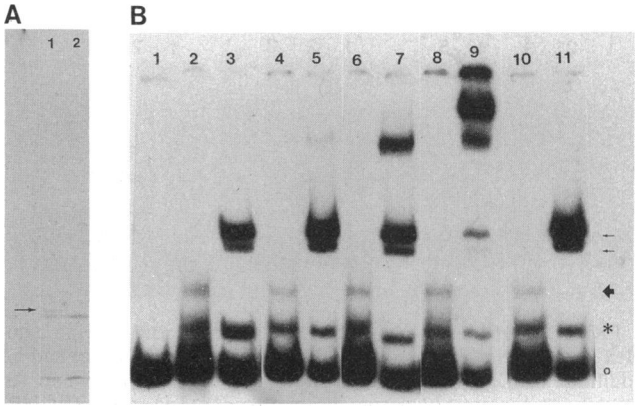

FIG. 3. (A) Western blot showing the specificity of the antibodies against NodD in *Rhizobium* strains. Lanes were loaded with material from *R. leguminosarum* bv. viciae *nodD*::Tn*5* strain RBL1402 either harboring the *nodD*-containing plasmid pMP280 (lane 1) or without any extra plasmid (lane 2). Immunoreactions visible with preimmune serum are the same as in lane 2. The NodD protein is indicated by an arrow. (B) Effect of antibodies against NodD on complex formation with the *nodA* promoter region. Additions to the DNA: none (lane 1), lysate from bacteroids of strain RBL1402(pMP280) (even-numbered lanes) or lysate from cultured cells of strain RBL1391(pMP280) (odd-numbered lanes other than 1). Antiserum against NodD was added 100-fold diluted to the samples in lanes 4 and 5, 10-fold diluted for lanes 6 and 7, and undiluted for lanes 8 and 9. The samples in lanes 10 and 11 are supplemented with undiluted preimmune serum, showing a pattern undistinguishable from those with dilutions of the preimmune serum. Symbols in the right margin are the same as in Fig. 2.

NodD protein in wild-type *Rhizobium* cells (data not shown), in which only very small amounts are present (30).

To test whether NodD occurs in retardation complexes, the polyclonal antiserum against NodD was included in the binding mixture. Preimmune serum was used as a negative control. The result clearly shows that the two slowest-migrating complexes indeed contain NodD, since complexes migrating at higher positions in the gel were observed in the presence of antiserum against NodD. Moreover, these complexes become more pronounced with increasing antiserum concentration, while the amounts of the putative NodD-containing complexes decreased concomitantly (Fig. 3B, lanes 5, 7, and 9). This upward shift of the complexes was not seen when preimmune serum was added in the assay (Fig. 3B, lanes 10 and 11), and it is therefore a NodD-specific effect. Furthermore, since the antiserum against NodD did not have any influence on the bacteroid-specific complex (Fig. 3B, lanes 4, 6, and 8), this complex probably does not contain NodD.

The bacteroid-specific complex contains a novel protein. Only stable complexes can be detected with a gel retardation assay. These complexes can contain protein, RNA, or both. For instance, the bacteroid-specific retardation complex with the *nodA* promoter region may represent a transcription initiation complex of the Sym-independent complex with these DNA sequences. The bacteroid-specific retardation complex with the *nodA* promoter region was therefore investigated for the presence of RNA or protein.

Treatment of the bacteroid lysate with RNase before addition of the DNA probe did not prevent the formation of the bacteroid-specific complex (Fig. 4A, lane 3). In contrast, treatment of the bacteroid lysate with trypsin before the addition of *nodA* box DNA prevented formation of the

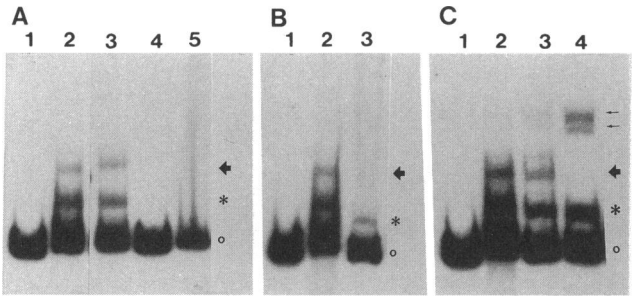

FIG. 4. Test for protein or RNA in the bacteroid-specific complex with the *nodA* promoter region. Lane 1 in each panel had no additions to the DNA and thus shows the unbound fragment. All other lanes contain a bacteroid lysate of *nodD*::Tn*5* strain RBL1402 containing plasmid-borne *nodD* on pMP280, which was treated as follows. (A) Lanes: 2, no further treatments; 3, RNase digestion; 4, 10 min at 100°C; 5, 0.5% SDS added. (B) Lanes: 2, only buffer for trypsin digestion added; 3, trypsin digestion. (C) Lanes: 2, no further treatment; 3, only buffer (without SDS) added; 4, incomplete proteinase K digestion. Symbols are the same as in Fig. 2.

bacteroid-specific complex (Fig. 4B), indicating the involvement of a protein. This assumption was supported by the finding that boiling the bacteroid lysate before the binding assay completely prevented the formation of any retardation complex (Fig. 4A, lane 4) and by the sensitivity of binding to the detergent SDS (Fig. 4A, lane 5). Thus, the bacteroid-specific retardation complex contains at least a bacteroid protein and the *nodA* promoter region.

NodD is present in an altered form or complex in bacteroids. An incomplete degradation of bacteroid proteins by proteinase K was observed when this treatment was performed in a buffer lacking SDS (data not shown). However, it appeared that the bacteroid-specific complex is more sensitive to proteinase K treatment than is NodD. As shown in Fig. 4C, lane 4, the NodD-containing complexes were much more pronounced when the bacteroid-specific complex was hardly present. Also, with the *nodM* promoter region, weak NodD binding was visible after incomplete degradation of bacteroid lysates by proteinase K (data not shown). These results indicate that the NodD protein in bacteroids is present in another form or complex than in bacteria, since the DNA-binding properties which NodD has in free-living cells partly return after mild proteolytic treatment.

To test the relative amounts of compounds which inactivate NodD in bacteroids, a mixture of extracts of NodD-producing free-living cells of strain RBL1391(pMP238) and of bacteroids was used in a binding assay with *nodA* promoter sequences. Lesser amounts of NodD-containing complexes were found in this case than when only the extract of free-living cells was used (data not shown), indicating that the NodD-inactivating compounds may be present in excess.

DISCUSSION

The work described in this article shows that in bacteroid preparations of *R. leguminosarum* bv. viciae, NodD protein does not bind to *nod* box-containing DNA except for the *nodA* promoter region, where very weak binding is observed (Fig. 2, lanes 2). This unexpected result was also obtained even when an excess of bacteroid material was used, indicating that the effect cannot be caused by limiting amounts of NodD protein. Although no NodD binding could be shown, the bacteroid lysates did contain active DNA-binding proteins, as was demonstrated by the formation of Sym-independent retardation complexes with the *nodA*, *nodF*, and *nodM* promoter regions. These results also indicate that the bacteroid lysate does not prevent the probe DNA from binding to protein. Moreover, the binding activity of NodD could be (partially) restored by mild proteolytic digestion of the bacteroid lysate, indicating that the absence of NodD-containing complexes is unlikely to be due to the bacteroid isolation procedure. The different methods used to prepare extracts of bacteroids (sonication) and of free-living cells (French press) do not cause the differences in DNA binding, since extracts of sonicated free-living cells gave results which were indistinguishable from those presented in Fig. 2, lanes 3 and 4 (data not shown).

In the present study, lysates from cultured cells of *R. leguminosarum* RBL1391(pMP280), RBL1402(pMP280), and RBL1391(pMP238) were used as positive controls for the NodD-containing complex with *nod* promoter regions. All these strains formed the same retardation complexes with *nod* boxes. Two NodD-containing complexes were found with the *nodA* and *nodM* promoter regions, but not with those sequences of *nodF* and *nodO* (Fig. 2, lanes 3). The difference between the two NodD-containing complexes is unclear. Since they migrate very close to each other, differences in surface charge and/or size can only be slight. They may represent slightly different binding sites on the DNA, different associations with small components, differences in involved protein(s), or different conformations of the DNA.

Incomplete degradation of bacteroid lysates by proteinase K (partially) restores NodD binding with the *nodA* (Fig. 4C) and *nodM* promoter regions, suggesting that the NodD protein is present in another form or in another complex in bacteroids than in free-living bacteria. This may be the explanation for the findings that NodD in bacteroids does not bind to *nod* boxes and that inducible *nod* gene transcription is absent in bacteroids. The small amount of NodD-containing complexes with the *nodA* promoter region found in bacteroid lysates can be explained by the fact that these sequences have the highest affinity for NodD of all *nod* boxes (27). It is feasible that the compounds which inactivate NodD in bacteroids are present in excess, since a mixture of extracts of bacteroids and of NodD-producing free-living cells resulted in lesser amounts of NodD-containing complexes with the *nodA* promoter region than when only the extract of free-living cells was used. However, this result can also be explained if one assumes that NodD competes with another protein for the same or overlapping DNA-binding sites.

A novel, bacteroid-specific protein was found to bind exclusively to the *nodA* promoter region (Fig. 2A). Since binding of this protein did not occur with any other DNA, it is unlikely that it represents a general DNA-modifying enzyme. Relatively large amounts of bacteroid lysate were necessary to visualize the new complex, which migrated in the gel relatively close to the unbound DNA. Such a migration rate suggests that the protein which forms the complex is a small protein. Alternatively, the binding site within the *nodA* promoter region might be different from that from NodD. This has not been determined yet.

The DNA fragment used as probe for the *nodA* promoter region also contains the overlapping *nodD* promoter (28, 33). Since the novel bacteroid-specific protein forms complexes only with this *nod* sequence and since in bacteroids *nodD* transcription is also reduced (29, 32), the protein is a candidate for a repressor of *nodD* transcription in bac-

teroids. An additional role for the bacteroid protein might be in the inhibition of transcription from the *nodA* promoter. Such transcription might still be possible since (i) some binding of NodD to the *nodA* promoter region still occurs in bacteroids (Fig. 2A) and (ii) inducing flavonoids are present in nodules (29). Apparently, the expression of *nodABCIJ* is unnecessary or undesirable in bacteroids. This is supported by the observations that high constitutive expression of inducible *nod* genes results in impaired nodulation and Fix$^-$ nodules (3, 13). Because the products of the *nodABC* operon are involved in the synthesis of bacterial signal molecules with essential roles in the early stages of symbiosis (17, 35, 37), these compounds are likely not required or desired in bacteroids.

The first report of a repressor of *nod* gene expression in rhizobia was of NolR in *R. meliloti* AK41 (15). The NolR protein influences the transcription of *nodD1*, *nodD2*, and *nodD3* negatively, and it binds to the respective promoters (16). Southern blot analysis showed no DNA homologous to *nolR* in *R. leguminosarum* bv. viciae (16) unless such an analysis was performed under low-stringency conditions (14). Whether the protein in the bacteroid-specific complex which we found in this study is related to the NolR protein is unknown, however.

In summary, the inability of NodD to bind to *nod* boxes in bacteroids seems to be the mechanism by which expression of the inducible *nod* genes is switched off in bacteroids. The novel *nodA* box-binding protein found may be responsible for the decreased *nodD* transcription observed in bacteroids. The same protein may also prevent transcription from the *nodA* promoter, indicating that Nod metabolites are undesirable or unnecessary in bacteroids.

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