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Subcellular Localization of the *nodD* Gene Product in *Rhizobium leguminosarum*

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In *Rhizobium* strains the transcription of symbiosis plasmid-localized *nod* genes, except *nodD*, is induced by plant flavonoids and requires the *nodD* gene product. In order to localize NodD protein in *R. leguminosarum*, a NodD protein-specific antiserum was raised against a *lacZ'-'nodD* gene fusion product. Using these antibodies, we determined that the NodD protein is located exclusively in the cytoplasmic membrane of wild-type *R. leguminosarum* biovar *viciae* cells. This localization is independent of the presence of inducers. In a *Rhizobium* strain that overproduced the NodD protein, the protein was present both in the cytoplasmic membrane and the cytosol, indicating an influence of the protein abundance on its ultimate subcellular localization. It was estimated that 20 to 80 molecules of NodD protein were present per wild-type *Rhizobium* cell. A model which combines the localization and the DNA-binding properties of the NodD protein as well as the observed association of flavonoids with the cytoplasmic membrane is discussed.

Soil bacteria of the genus *Rhizobium* are able to establish a symbiosis with leguminous plants by forming root nodules in which, after differentiation of the bacteria to bacteroids, atmospheric nitrogen is fixed. Differentiation of *Rhizobium* species and biovars is based on their ability to successfully nodulate a particular group of host plants. It has been known for some years that certain bacterial genes which are located on a large Sym (symbiosis) plasmid are involved in important stages of nodule formation. Some of these *nod* genes are functionally interchangeable between different *Rhizobium* species, and they have therefore been designated as common *nod* genes, while other genes determine the host specificity of nodulation (*hsn* genes). The transcription of these Sym plasmid-localized *nod* genes, except *nodD*, is induced by plant flavonoids and requires the presence of the NodD protein. The *nodD* gene, one copy of which is present in *Rhizobium leguminosarum* biovar *viciae* and *R. leguminosarum* biovar *trifolii* and three copies of which are found in *Rhizobium meliloti*, is transcribed constitutively (5).

Although the *nodD* gene has been designated a common *nod* gene, it has recently been established that the response of the *nodD* gene product toward various inducers depends upon its bacterial origin (38). The importance of each of the different *nodD* genes present in *R. meliloti* is reflected by the fact that nodulation of different host plants is impaired by mutations in different *nodD* genes (10, 14, 15). Therefore, a direct interaction between the NodD protein and inducing flavonoids is likely. Studies with homologous recombinants of the *nodD* genes of *R. meliloti* and *R. leguminosarum* biovar *trifolii* (37) support this hypothesis, since several of these *nodD* hybrid genes show novel types of responses toward flavonoids compared with the responses of the parental *nodD* genes.

Conserved DNA sequences, so-called *nod* boxes (29), have been identified upstream of the inducible *nod* genes. These may play a role in transcription activation, as it has been shown by using deletion mutants in this region that the

promoter overlaps the *nod* box (35). Transcriptional start sites of *nodA*, *nodF*, and *nodH* are only 24 to 28 base pairs (bp) downstream of the consensus *nod* box sequence (9, 36). Studies with DNA fragments containing *nod* box sequences and either cell extracts of NodD protein-overproducing strains or partially purified NodD protein have shown that NodD protein binds to *nod* boxes (8, 13). This binding is specific for DNA containing *nod* box sequences and is independent of the presence of an inducer. Another property of NodD protein is autoregulation, which has been found in *R. leguminosarum* biovar *viciae* and *R. leguminosarum* biovar *trifolii* but not, or to a lesser extent, in *R. meliloti* (24, 28, 37). This property is probably caused by binding of the NodD protein to DNA as well.

The nucleotide sequences of the *nodD* genes of *R. leguminosarum* biovar *viciae*, *R. leguminosarum* biovar *trifolii*, *R. meliloti*, *Rhizobium japonicum*, and *Bradyrhizobium* species are highly conserved (1, 6, 32–34) and share homology with several DNA-binding transcriptional activator proteins which constitute the LysR family (12). Homology of the NodD protein with the transcriptional activator AraC protein of *Escherichia coli* has been proposed as well (34), and there exists a great resemblance of the NodD protein with the recently published sequence of the NahR protein, a regulator of the genes involved in naphthalene degradation in *Pseudomonas putida* (31, 44).

The binding of the NodD protein to *nod* boxes and its homology with other transcriptional activator proteins suggest a cytoplasmic localization. We investigated the localization of the NodD protein and found that it is localized exclusively in the cytoplasmic membrane of wild-type *R. leguminosarum* biovar *viciae* cells. This localization is even more interesting since other recent work from our laboratory (27) shows that naringenin, a NodD protein activator, has a very high affinity for the cytoplasmic membrane. In view of these data, we present a model for the interaction of NodD protein, inducing compounds, and regulated *nod* gene promoters.

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TABLE 1. Plasmids used in this study[a]

Plasmid	Relevant characteristics	Reference
pMP97	IncColE1 carrying *Kpn*I, *Cla*I fragment of pRL1JI, which contains the entire *nodD*, 5' parts of *nodA* and *nodF*, and intergenic regions	This study
pMP98	IncP carrying pr. *nodD-nodD'*, which was used as a negative control of pMP238	This study
pMP154	IncQ carrying pr. *nodA-lacZ*	35
pMP235	IncP carrying a deletion in *nodA-nodD* intergenic region	This study
pMP237	IncColE1, IncP carrying pr. *lac-nodD*; pr. *nodA* and pr. *nodD* were both deleted	This study
pMP238	IncP carrying pr. *nodA-nodD*	This study
pMP280	IncP carrying pr. *nodD-nodD*	38
pMP300	IncP carrying a deletion in *nodA-nodD* intergenic region; pr. *nodA* present	35
pMP2001	IncColE1 carrying pr. *lac-lacZ'-'nodD*	This study
pMP2002	Like pMP2001, but the *lacZ* sequences were reversed; used as a negative control of pMP2001	This study
pMP2003	Like pMP237, but pr. *lac* was reversed; used as a negative control of pMP237	This study

[a] All *nod* sequences were from pRL1JI. Abbreviation: pr., Promoter.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* JM101 [*supE thi* Δ(*lac-proAB*) (F' *traD36 proAB lacI*^{q}ZΔM15)] (43) was used for propagation of plasmids and for production of the *lacZ'-'nodD* gene fusion product. *R. leguminosarum* biovar *viciae* wild-type strain 248 (16) and its Sym plasmid pRL1JI-cured derivative RBL1387 (26) were used for protein localization studies. Strain RBL1387 was used as a host for recombinant plasmids. Nodulation assays were performed on *Vicia sativa* var. *nigra* with *R. leguminosarum* RBL5560 (wild-type *nod* genes) and RBL5561 (*nodD*::Tn5) (45), with the latter one carrying a recombinant plasmid containing pRL1JI *nodD*. All plasmids used in this study are listed in Table 1.

DNA manipulation and bacterial crosses. Restriction endonucleases, T4 DNA ligase, nuclease *Bal* 31, DNA primer, and unlabeled nucleotides were purchased from Boehringer GmbH (Mannheim, Federal Republic of Germany). Freeze-dried large fragment (Klenow) of DNA polymerase I was obtained from Bethesda Research Laboratories (Gaithersburg, Md.), and [α-^{35}S]dATP was purchased from Amersham International plc (Amersham, United Kingdom). Nucleotide sequencing was performed as described previously (30). All DNA manipulations were performed essentially as described by Maniatis et al. (19). Transfer of IncP plasmids from *E. coli* JM101 to *R. leguminosarum* RBL1387 was performed by using a triparental mating as described previously (4). Strains carrying plasmids were selected on solid medium supplemented with 100 μg of ampicillin ml^{-1} or 20 and 2 μg of tetracycline ml^{-1} for *E. coli* and *R. leguminosarum*, respectively. Rifampin (20 μg · ml^{-1}) was used for selection against *E. coli* in bacterial crosses.

Construction of *lacZ'-'nodD* gene fusion. Plasmid pMP97, which was derived from pIC20H (21), contained a 2.4-kilobase *Kpn*I-*Cla*I fragment of pRL1JI coding for the entire *nodD* gene and the 5'-terminal parts of *nodA* and *nodF*. The 5'-terminal 192 bp of *lacZ* were cloned as a *Sau*3A fragment of pIC20H in *Bam*HI-digested pMP97, resulting in pMP2001. This vector contained the *lacZ'-'nodD* translational gene fusion downstream of the *lac* promoter (Fig. 1A). Plasmid pMP2002, which was constructed in the same way, contained an inverted *lacZ Sau*3A fragment.

Construction of NodD protein-(over)producing plasmids. A 114-bp *Bcl*I-*Bgl*II fragment containing the *nodA* promoter and part of the *nodD* promoter of pRL1JI was treated with *Bal* 31 starting from the *Bcl*I site (35). After ligation with a *Kpn*I linker at the 3' end and nucleotide sequencing, the IncP plasmid pMP235, which contained a 36-bp fragment with 18

bp in front of the *nodD*-coding region, was isolated. The *Bgl*II fragment of pMP97, which contained *nodD* sequences, was cloned into *Bgl*II-digested pMP235, resulting in pMP236. For overproduction of NodD protein in *E. coli*, pMP237 was constructed by cloning *Kpn*I-linearized pMP236 downstream of the *lac* promoter in pIC20H. Plasmid pMP2003, which was used as a negative control for pMP237, contained the *lac* promoter in the opposite direction (Fig. 1A). For overproduction of NodD protein in *R. leguminosarum*, a *Pst*I-*Kpn*I fragment of pMP300 (35) containing the *nodA* promoter with only 33 adjacent nucleotides 3' of the *nod* box consensus sequence was inserted upstream of the *nodD* gene and its preceding 18 bp in pMP236, resulting in pMP238 (Fig. 1A). Plasmid pMP98, which was used as a negative control, and plasmid pMP280 (38) are both broad-host-range IncP plasmids containing the pRL1JI *nodD* promoter. In addition, pMP98 contained *nodD* sequences upstream of the *Bam*HI site and pMP280 contained the complete *nodD* gene.

Production of antibodies against NodD protein. *E. coli* JM101(pMP2001) was grown for 16 h in LC medium (19) supplemented with ampicillin and 20 μg of isopropyl-β-D-thiogalactopyranoside (IPTG) ml^{-1}. Bacteria were lysed after twofold dilution in sample buffer by boiling them for 10 min. Total cell proteins were separated on sodium dodecyl sulfate (SDS)–11% polyacrylamide gels (18). Gels were stained for 30 min in 0.2% (wt/vol) Coomassie brilliant blue in 10% acetic acid–50% methanol, and after the gels were destained in the same solvent for 30 min, the protein band of 31 kilodaltons (kDa) (Fig. 2A, lane 4, indicated by a solid arrow) was isolated by electroelution by the method of Hager and Burgess (11). An amount of 100 μg of this material, which was suspended in Freund complete adjuvant (1:1), was injected subcutaneously into a New Zealand White rabbit, and a booster injection without adjuvant was given after 1 month. Antiserum was collected 10 days after the booster injection.

Cell fractionation. Wild-type *R. leguminosarum* or strains carrying a recombinant plasmid were grown for 16 h in 400 ml of TYB medium, consisting of TY medium (2) to which 20% (vol/vol) of B$^-$ medium (40) was added, in the presence or absence of 1.0 μM naringenin. After harvesting, the cell pellet was suspended in 10 ml of ice-cold 50 mM Tris hydrochloride (pH 8.5)–20% (wt/vol) sucrose–0.2 mM dithiothreitol supplemented with 200 μg of each of DNaseI and RNaseA (Sigma Chemical Co., St. Louis, Mo.) ml^{-1}. The following protease inhibitors, all of which were purchased

from Sigma, were added to the suspended cells unless indicated otherwise: phenylmethylsulfonyl fluoride (200 µM), soybean trypsin inhibitor (50 µg · ml^{-1}), and leupeptin (20 µM). The bacteria were broken by three passages through a French pressure cell at 15,500 lb/in^2, and cell fractions were isolated as described previously (3). Lysozyme and KCl were added to the cell lysate to final concentrations of 200 µg · ml^{-1} and 0.2 M, respectively, and after incubation on ice for 40 min, membranes were collected by centrifugation for 2 h at 120,000 × g at 4°C. The membrane fraction was suspended in 600 to 800 µl of 15% (wt/wt) sucrose–5 mM EDTA (pH 7.5)–0.2 mM dithiothreitol; and the inner and outer membranes of 500 µl of total membranes were separated in a discontinuous sucrose gradient consisting of 1.5 ml of 60%, 4.0 ml of 40%, 4.5 ml of 25%, and 0.5 ml of 15% (wt/wt) sucrose in 5 mM EDTA (pH 7.5)–0.2 mM dithiothreitol. Fractions of 0.5 ml were collected from the top of the gradient, and their protein contents were determined by measuring the A_{280}. The purity of the membrane fractions was established by measuring NADH oxidase activity (25), a cytoplasmic membrane marker, and by protein pattern analysis in SDS-polyacrylamide gels. These procedures can be used to prove the purity of membrane fractions (3). To concentrate membrane material, sucrose gradient fractions containing either cytoplasmic or outer membranes were pooled and centrifuged for 2 h at 120,000 × g at 4°C. The pellets were suspended in 100 µl of 15% (wt/vol) sucrose–5 mM EDTA (pH 7.5)–0.2 mM dithiothreitol.

Periplasmic and cytoplasmic fractions were isolated as described previously (3).

The proteins that were present in the culture supernatant and in the soluble fraction of broken cells were precipitated by the addition of trichloroacetic acid to a final concentration of 5% (wt/vol) and incubation at 0°C for 60 min. Precipitates were collected by centrifugation for 10 min at 3,000 × g at 4°C and solubilized in 4 and 1.2 ml of 10.0 mM Tris hydrochloride (pH 7.5)–0.2 mM dithiothreitol, respectively. All samples were stored frozen at −20°C until use.

Protein analysis and immunoblotting. Proteins of whole cells or cell fractions were separated on SDS–11% polyacrylamide gels (18) and either stained with fast green (18) or transferred to nitrocellulose (39). The nitrocellulose was blocked with blocking buffer (1% [wt/vol] bovine serum albumin in 10 mM sodium phosphate [pH 7.0]–0.9% [wt/vol] sodium chloride) for 1 h at room temperature. Subsequently, the nitrocellulose sheets were incubated for 2 h with antiserum against the NodD protein or E. coli β-galactosidase (a kind gift from J. van Duyn, Department of Biochemistry, Leiden University, Leiden, The Netherlands) diluted 2,000- and 100-fold, respectively, in blocking buffer. After washing for 1 h with Tween-buffer (0.1% Tween 20 in 10 mM sodium phosphate [pH 7.0]–0.9% [wt/vol] sodium chloride), the blots were incubated for 1 h with 2,000-fold-diluted alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins (Sigma) in blocking buffer. After subsequent washing for 1 h in Tween-buffer, the reaction was visualized by using naphthol AS-MX phosphate and fast red TR salt (both from Sigma) as substrates (41).

Calculation of the number of molecules of NodD protein. The protein p31 (Fig. 2A, lane 4, indicated by a solid arrow) was isolated as described above. To remove the Coomassie brilliant blue stain from the protein, lyophilized powder was solubilized in 100 µl of water and 900 µl of cold acetone (P. A.; Merck AG, Darmstadt, Federal Republic of Germany) was added. After incubation for 30 min at 4°C, the

sample was centrifuged for 15 min at 10,000 × g at 4°C. The pellet was suspended in 100 µl of water, and the acetone precipitation was repeated twice. The resulting white protein pellet was lyophilized and solubilized in water, and its protein content was determined by the method described by Markwell et al. (20) by using bovine serum albumin as a standard. Various known amounts of p31 were electrophoresed on SDS-polyacrylamide gels, and immunological detection was performed as described above. The same gels were also loaded with various dilutions of total membranes of R. leguminosarum biovar viciae wild-type strain 248. The amount of cells, from which this membrane material was derived, was estimated by counting the number of viable cells of the original culture. The number of NodD protein molecules present per wild-type Rhizobium cell was estimated by assuming an equal immunoreactivity of both antigens, p31 and the NodD protein.

RESULTS

Expression of nodD gene in E. coli and production of antibodies against NodD protein. In order to isolate NodD protein for the production of antiserum, pMP237 was constructed (Fig. 1). This plasmid contained the entire nodD gene of pRL1JI under control of the inducible lac promoter. E. coli JM101(pMP237) produced detectable amounts of a 34-kDa protein in the presence, but not in the absence, of IPTG (Fig. 2A, lanes 3 and 8, respectively). This apparent molecular mass is in very good agreement with the predicted size of the NodD protein (34.5 kDa [34]). The 34-kDa protein was not detected in E. coli JM101 carrying the control plasmid pMP2003 (Fig. 2A, lanes 5 and 6). The amount of nodD gene product produced by E. coli JM101(pMP237) was rather low, presumably because of weak translation initiation. In an attempt to enhance the expression level, we constructed pMP2001 (Fig. 1), which contained a lacZ'-'nodD chimeric gene downstream of the lac promoter. Only upon induction of E. coli JM101(pMP2001) with IPTG were six dominant protein bands detected in profiles of whole-cell proteins (Fig. 2A; compare lanes 3 and 4). The apparent molecular size of the slowest-moving protein, which was part of a doublet, corresponded with the predicted size of the fusion protein (33 kDa) and showed the strongest immunoreaction with antibodies against β-galactosidase (data not shown). The control strain E. coli JM101(pMP2002) did not produce any of these six proteins (Fig. 2A, lanes 1 and 2).

The protein with an apparent molecular mass of 31 kDa (designated p31 and indicated by a solid arrow in Fig. 2A) was subsequently used to raise antibodies because it could be excised from the gel with minimal contamination of the other visible proteins.

The smaller proteins produced by E. coli JM101(pMP2001) upon induction were probably degradation products of the entire LacZ'-'NodD fusion protein that arose from specific cleavage by host proteases in vivo, since (i) all these proteins showed an immunoreaction with antibodies against p31 (Fig. 2B, lane 4) and (ii) they did not disappear when bacteria were lysed in the presence of protease inhibitors (data not shown).

Overproduction of NodD protein in R. leguminosarum. Initially, we were not able to detect NodD protein in total cell proteins of wild-type R. legumonisarum biovar viciae 248 using the antiserum raised against p31. Therefore, NodD protein-overproducing Rhizobium strains were constructed. When the copy number of nodD was increased approximately fivefold by introduction of the IncP plasmid pMP280 in R. leguminosarum RBL1387, NodD protein still could not

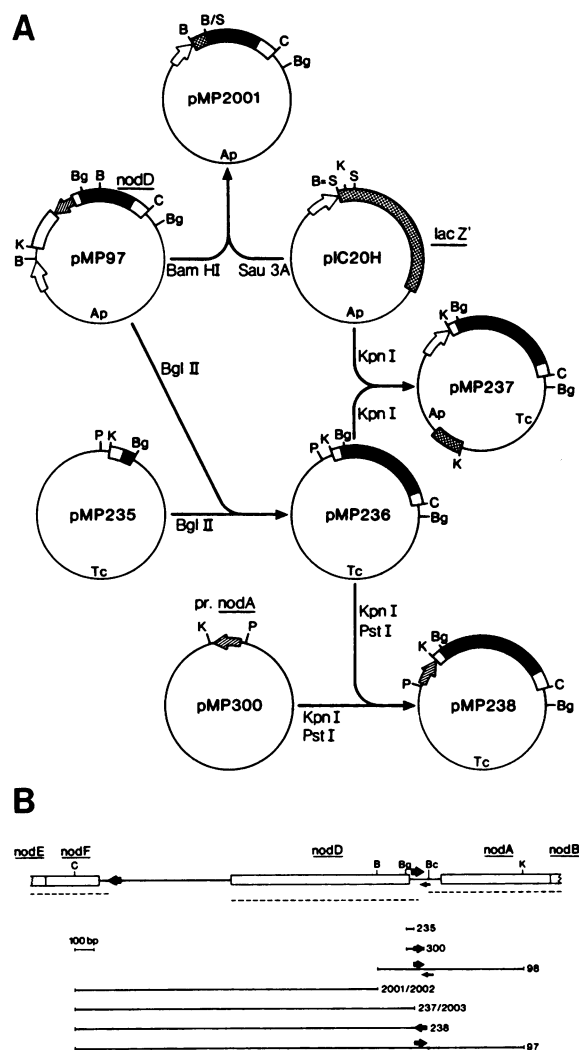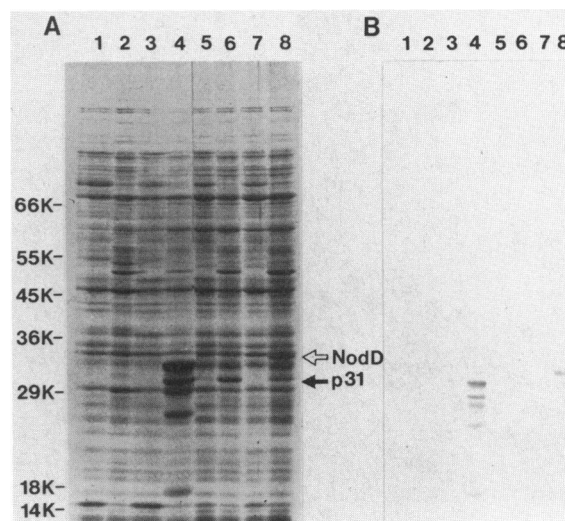FIG. 2. Western blot (immunoblot) analysis of proteins expressed in *E. coli* by using antibodies raised against p31. (A) Profiles of total cell proteins obtained on a SDS-polyacrylamide gel after fast green staining. The positions of p31 and NodD are indicated by solid and open arrows, respectively. (B) Immunological identification of fusion protein bands and the NodD protein directed by pMP2001 and pMP237, respectively, in whole-cell proteins of *E. coli* JM101. The samples represent *E. coli* JM101 containing pMP2002 (lanes 1 and 2), pMP2001 (lanes 3 and 4), pMP2003 (lanes 5 and 6), or pMP237 (lanes 7 and 8) grown in the absence (odd lanes) or presence (even lanes) of IPTG. The positions of molecular weight markers (K indicates 10^3) are given in the left margin.

FIG. 1. (A) Construction of plasmids used in this study. pMP2001 contained the *lacZ'-'nodD* chimeric gene downstream of the *lac* promoter. pMP237 and pMP238 were both expression plasmids for the NodD protein; they both contained the entire *nodD* gene of pRL1JI downstream of the *lac* and *nodA* promoters, respectively. The construction of negative control plasmids was performed in an analogous way (data not shown). In pIC20H, only *Sau*3A sites of interest are indicated. Black boxes indicate *nodD* sequences; dotted boxes indicate *lacZ* sequences. Adjacent *nod* DNA is represented by open boxes. Shaded and open large arrows represent *nodA* and *lac* promoters, respectively. (B) *nod* sequences present in different plasmids. Part of the *nod* region present in pRL1JI is given, with open reading frames indicated by boxes. Dotted lines and shaded and black arrows represent transcripts and inducible and constitutive promoters, respectively. pMP235 and pMP300 both contained deletions of the *nodD-nodA* intergenic region. pMP235 contained the first 37 bp of *nodD* (until the *Bgl*II site) and 18 bp upstream of the *nodD* open reading frame. pMP300 has been described previously (35). Plasmids given in panel A are not drawn to scale. Abbreviations: Ap and Tc, ampicillin and tetracycline resistance regions, respectively. Restriction sites: B, *Bam*HI; B/S, *Bam*HI-*Sau*3A junction; Bc, *Bcl*I; Bg, *Bgl*II; C, *Cla*I; K, *Kpn*I; P, *Pst*I; S, *Sau*3A.

be detected in the protein profiles of total cells. Because inducible *nod* promoters showed a high level of expression upon induction, we cloned the *nodD* gene of pRL1JI downstream of the *nodA* promoter (pMP238 in Fig. 1). *R. legu-*

minosarum RBL1387(pMP238) produced a 34-kDa protein in visible amounts on stained gels, provided that the cells were grown in the presence of one of the inducers naringenin or luteolin (data not shown). This result, in combination with the predicted molecular mass, strongly suggests that the 34-kDa protein is identical to the NodD protein. This notion was further supported by the following experiments, which showed that upon induction a strain carrying pMP238 produces a functional NodD protein with respect to *nod* gene activation and nodulation. (i) *R. leguminosarum* RBL5561, harboring both pMP238 and the *nodA* promoter-*lacZ* transcriptional fusion pMP154, produced 250 U of β-galactosidase (22) in the absence of inducer and 18,000 U of β-galactosidase upon induction with naringenin. (ii) *R. leguminosarum* RBL5561, with a Tn5 insertion in *nodD*, was not able to nodulate *V. sativa*. However, strain RBL5561(pMP238) showed the same nodulation phenotype on *V. sativa* plants as that of wild-type strain RBL5560.

Specificity of antibodies against p31. To test the specificity of the antiserum against p31 in *R. leguminosarum*, total cell proteins of RBL1387(pMP238) were analyzed by using Western blots (immunoblots). Only the putative NodD protein band reacted with the antiserum (Fig. 3, lane 2). No reaction was observed with material derived from *R. leguminosarum* RBL1387(pMP98) (Fig. 3, lane 1) or with total cell proteins of *R. leguminosarum* biovar *viciae* wild-type strain 248 (data not shown). As a control, total cell proteins of *E. coli* JM101(pMP237) were analyzed as well. Several faint bands and one very pronounced band were observed (Fig. 3, lane 4), the last of which corresponded with a protein of 34 kDa, which was absent in crude lysates of *E. coli* JM101(pMP2003) (Fig. 3, lane 3). Thus, the antiserum was specific toward the NodD protein in both *R. leguminosarum* and *E. coli*.
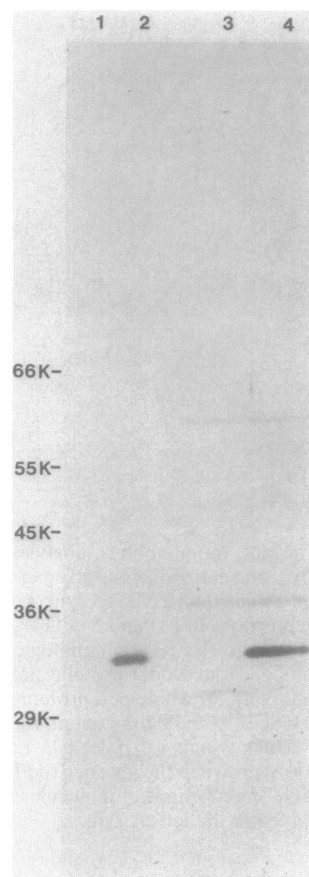
FIG. 3. Specificity of antibodies raised against p31. Proteins of whole cells of *R. leguminosarum* RBL1387(pMP98) (lane 1) and RBL1387(pMP238) (lane 2) and *E. coli* JM101(pMP2003) (lane 3) and JM101(pMP237) (lane 4) were separated on SDS-polyacrylamide gels. Western blots were incubated with antiserum against p31. *Rhizobium* and *E. coli* cells were induced with naringenin and IPTG, respectively. The positions of molecular weight markers (K indicates 10³) are given in the left margin.
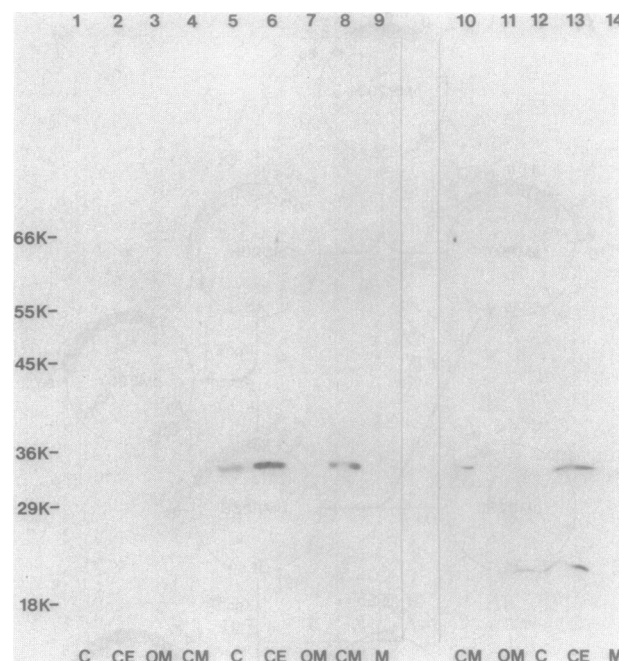

FIG. 4. Western blot (immunoblot) analysis of proteins expressed in *R. leguminosarum* by using antibodies raised against p31. Cell fractions of *R. leguminosarum* RBL1387(pMP238) were prepared as described in the text, and those of wild-type strain 248 were prepared in the presence of leupeptin only. NodD protein was detected in cell fractions of RBL1387(pMP238) after induction with naringenin (lanes 5 to 9) and in wild-type *R. leguminosarum* 248 (lanes 10 to 14). A positive reaction could not be detected in noninduced cell fractions of RBL1387(pMP238) (lanes 1 to 4) or in cell fractions of induced or uninduced RBL1387(pMP98) (data not shown). Abbreviations: C, Soluble cell proteins (lanes 1, 5, and 12); CE, total membranes (lanes 2, 6, and 13); OM, outer membrane (lanes 3, 7, and 11); CM, cytoplasmic membrane (lanes 4, 8, and 10); M, medium (lanes 9 and 14). Lanes containing material belonging to the same strain were each loaded with material which was derived from the same number of cells and therefore represented the total amount of NodD protein present in a given bacterial culture. The positions of molecular weight markers (K indicates 10³) are given in the left margin.

Subcellular localization of NodD protein in an overproducing *Rhizobium* strain. To investigate the localization of the NodD protein in NodD protein-overproducing strains, cultures of RBL1387(pMP238) were initially fractionated into medium components, soluble cell proteins, and total membranes. By using electrophoresis and fast green staining, the NodD protein could only be detected in the total membrane fraction of induced cells (data not shown). Using Western blotting, however, we detected NodD protein both in the membrane fraction and, although to a slightly lesser extent, in the soluble cell-protein fraction as well (Fig. 4, lanes 5 and 6). After separation of the two membranes, the NodD protein was found in the cytoplasmic membrane fraction but not in the outer membrane fraction (Fig. 4, lanes 7 and 8). Subsequent isolation of periplasm and cytoplasm indicated that the NodD protein was present in the cytoplasm but not in the periplasm (data not shown). A positive reaction was not detected on Western blots of cell fractions of noninduced *R. leguminosarum* RBL1387(pMP238) (Fig. 4, lanes 1 to 4), unless the lanes were at least eightfold overloaded, or with the control strain *R. leguminosarum* RBL1387(pMP98) grown either in the presence or in the absence of flavonoid inducers (data not shown). In conclusion, the *nodD* gene product is localized in the cytoplasmic membrane as well as

in the cytoplasm of a NodD protein-overproducing *Rhizobium* strain.

Subcellular localization of NodD protein in wild-type *Rhizobium* cells. After we improved the methods used for the detection of NodD protein in overproducing *Rhizobium* cells, we were able to detect NodD protein in a concentrated cell fraction of wild-type *R. leguminosarum* biovar *viciae* 248. Protein fractions from the culture medium, the combined cytoplasmic and periplasmic fractions, the total membrane fraction, as well as separated inner and outer membrane fractions were electrophoresed on SDS-polyacrylamide gel. A protein with a molecular mass of 34 kDa could only be detected on Western blots in unseparated membranes and in the cytoplasmic membrane fraction (Fig. 4, lanes 10 and 13). No signal was detected in concentrated cell fractions derived from *R. leguminosarum* RBL1387 or RBL5561 (*nodD*::Tn5) (data not shown). Therefore, the 34-kDa protein of *R. leguminosarum* biovar *viciae* observed in Fig. 4 was indeed the NodD protein, which appeared to be localized exclusively in the cytoplasmic membrane of wild-type cells.

In Fig. 4, lanes 12 and 13, which contained soluble cell proteins and unseparated membranes, respectively, a faint
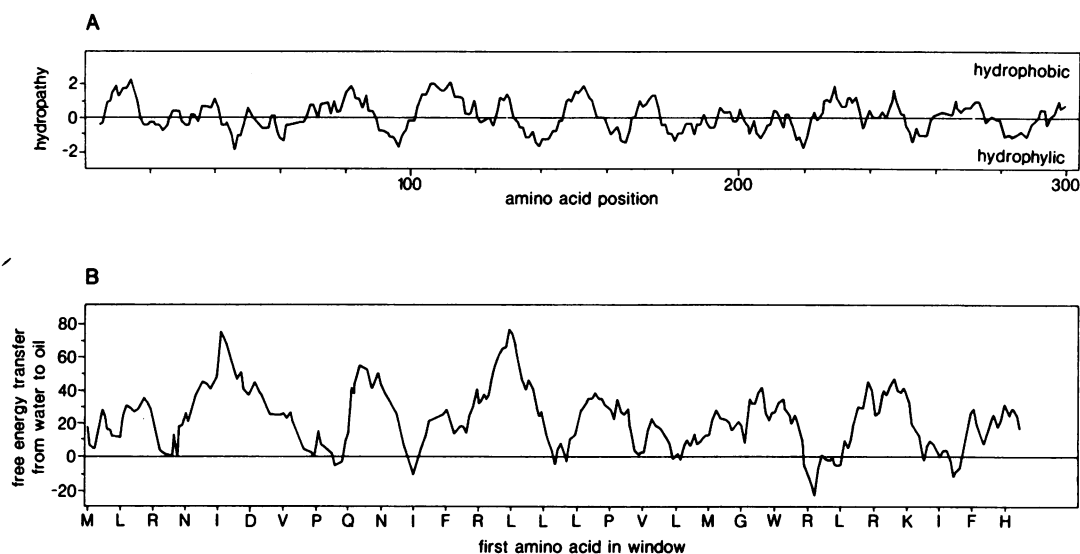
FIG. 5. Analysis of the NodD protein predicted from the nucleotide sequence of *nodD* of pRL1JI (34) with different computer predictions. (A) Hydrophobicity plot determined by the method of Kyte and Doolittle (17). (B) Profile giving the free energy transfer of water to oil with the algorithm of Engelman et al. (7) performed with a 16-amino-acid window width. A value of free energy equal to or less than −20 kcal/mol means a potential membrane-spanning region. The horizontal scales of both drawings are identical.

band corresponding to an apparent molecular mass of approximately 23 kDa could be seen as well. This phenomenon was also observed on Western blots of *R. leguminosarum* RBL1387(pMP238) when cells were fractionated in the absence of all protease inhibitors mentioned in the Materials and Methods. These results indicate that this polypeptide is a degradation product of the NodD protein. The presence of this polypeptide in the soluble cell protein fraction of *R. leguminosarum* biovar *viciae* 248 is probably an experimental artifact since no reaction was detected in this cell fraction after membranes were quantitatively removed by centrifugation for 16 h at 120,000 × *g* in the absence of sucrose, indicating that the 23-kDa protein is membrane bound.

The localization of the NodD protein in the cytoplasmic membrane was independent of the presence of naringenin during growth of the bacteria. We could not detect any 34-kDa protein on Western blots of the soluble cell protein fraction when this fraction was concentrated 15-fold relative to the concentration used for the experiments for which the results are given in Fig. 4, which indicates that at least 94% of the NodD protein present in a wild-type cell is membrane associated. The presence of 1 M NaCl during the collection of membranes did not affect the localization of the NodD protein either, indicating that the membrane localization is not an artifact of electrostatic interactions.

The number of NodD protein molecules present in a wild-type *Rhizobium* cell was estimated to be between 20 and 80, as described in the Materials and Methods.

Prediction of NodD protein localization with computer programs. The amino acid sequence of the *nodD* gene of *R. leguminosarum* biovar *viciae* (34) was analyzed with computer programs for the prediction of several properties of the NodD protein relevant to protein localization. Several hydrophobic regions could be distinguished with the algorithm developed by Kyte and Doolittle (17), whereas the *nodD* gene product as a whole was not extremely hydrophobic (Fig. 5A). Using the prediction of transbilayer helices in membrane proteins (7), we found one dip of free energy transfer with a value of less than −20 kcal/mol (Fig. 5B). This indicates a potential transmembrane position in the

region flanked by Leu at position 224 and Asn at position 240 of the *nodD* sequence. However, this result was only obtained with a window of 16 amino acids instead of the usual window of 20 amino acids, a result which makes a membrane-spanning α-helix at this position unlikely.

DISCUSSION

In fast-growing rhizobia, the *nodD* gene product positively regulates inducible Sym plasmid-localized *nod* genes in the presence of flavonoids. We investigated the localization of the NodD protein as a contribution to the understanding of this process.

Membrane association of NodD protein. In this report we have shown that the NodD protein is localized exclusively in the cytoplasmic membrane of wild-type cells of *R. leguminosarum* biovar *viciae* 248 (Fig. 4, lane 10), and only 20 to 80 molecules of the protein are estimated to be present per cell. A hydrophobicity plot (Fig. 5A) supported a membrane association of the NodD protein. It is unlikely that the NodD protein is a peripheral membrane protein because it could not be solubilized from the membrane fraction with 1 M sodium chloride. In view of the data obtained by using the algorithm of Engelman et al. (7) (Fig. 5B), one or more transmembrane α-helices seems unlikely as well. Analysis of the predicted *nodD1* gene product of *R. meliloti* with this algorithm, which was performed with a window of 20 amino acids, yielded one dip in free energy transfer with a value of less than −20 kcal/mol (data not shown), suggesting that in this case a membrane-spanning α-helix is possible. However, in the region involved (amino acids 259 to 279), four Pro residues were found, indicating several interruptions of the α-helix. A membrane association of the NodD protein caused by acylation is not very likely either, since the consensus sequence Leu-Ala-Gly-Cys in the N termini of lipoproteins (for a review, see reference 42) is not present in the *nodD* gene product. This possibility requires further study, however. Based upon our current knowledge, we postulate that the NodD protein is an amphipathic protein that is inserted in the inner monolayer of the cytoplasmic membrane.
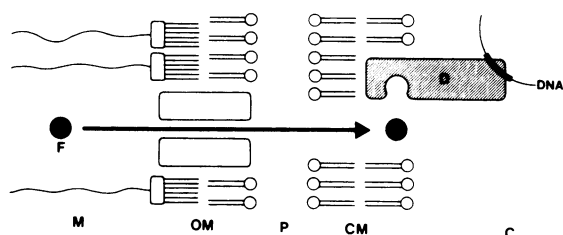
FIG. 6. Model presenting an amphipathic NodD protein localized in the cytoplasmic membrane and its interaction with *nod* box DNA (indicated by a black box) and flavonoids. For further explanation, see text. The presumed flavonoid binding site is not in the membrane part of NodD protein per se. Abbreviations: C, Cytoplasm; CM, cytoplasmic membrane; D, NodD protein; F, flavonoid; M, medium; OM, outer membrane; P, periplasmic space.

Membrane localization and DNA-binding properties of NodD protein. Our finding that the NodD protein is localized in the cytoplasmic membrane was unexpected because the following data suggested that the NodD protein is localized in the cytoplasm. (i) It has been shown that the *nodD* gene product binds specifically at the *nod* box sequences present in the promoter region of inducible *nod* genes of *R. meliloti* (8) and *R. leguminosarum* biovar *viciae* (13). (ii) The NodD protein was isolated from the soluble cell protein fraction, and binding studies between the NodD protein of *R. meliloti* and *nod* box-containing DNA fragments have been performed with presumably soluble cell proteins derived from overproducing strains (8). This is in agreement with our observation of a relatively large amount of NodD protein in the cytosol of our overproducing strain *R. leguminosarum* RBL1387(pMP238) (Fig. 4, lane 5). (iii) The predicted NodD proteins share homology with cytoplasm-localized transcriptional regulator proteins constituting the LysR family (12) and with the *E. coli* AraC protein (34). The localization of the NodD protein is not unique for a regulatory protein, since another membrane-localized regulatory DNA-binding protein, ToxR protein, has been described (23).

NodD protein and flavonoids. It has recently become evident that *nod* gene expression is mediated specifically by the host (10, 15, 38) because of the characteristic responses of *nodD* toward sets of flavonoids. These data strongly suggest a direct interaction between inducing compounds and the *nodD* gene product. Recent results from our laboratory indicate that flavonoids accumulate in the cytoplasmic membrane (27). Because the *nodD* gene product is the only soluble cell protein which binds specifically to *nod* boxes (8), it is unlikely that a second protein is involved in the information transfer between a membrane-localized NodD protein and a DNA-localized NodD protein.

Hence, we propose a model (Fig. 6) in which the NodD protein is an amphipathic protein localized in the cytoplasmic membrane with a substantial domain extending into the cytosol. It is presumed that the predicted direct interaction between the NodD protein and flavonoid inducers takes place in or close to the cytoplasmic membrane. A cytoplasmic domain of the NodD protein is supposed to be constitutively bound to *nod* box DNA. Activation of the NodD protein by inducers presumably causes a conformational change which initiates transcription of genes downstream of *nod* box-containing promoters. Our observation that the NodD protein is localized in the cytoplasmic membrane of *R. leguminosarum* biovar *viciae* 248 in the presence as well as in the absence of inducers supports the notion that there exists a class of DNA-binding proteins which regulates transcription in a protein-membrane-DNA complex.

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