Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 9581–9585, December 1986
Genetics

Expression of the nodulation gene *nodA* in *Rhizobium meliloti* and localization of the gene product in the cytosol

(fusion protein/antibodies/nodules)

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Contributed by Jeff Schell, August 22, 1986

ABSTRACT **The *nodA* gene of *Rhizobium meliloti* encodes a 21.8-kDa protein, which is conserved in several *Rhizobium* species. We overproduced the nodA protein as a fusion product with a portion of the λ *c*I repressor in *Escherichia coli*. This fusion protein was purified from inclusion bodies by gel and hydroxyapatite chromatography in the presence of $NaDodSO_4$. Monospecific polyclonal antibodies against the hybrid protein were used to detect the nodA protein in the cytosol of *E. coli* and *R. meliloti* by immunoblotting. In contrast to experiments with antibodies against the *R. meliloti* nodC membrane protein, the alfalfa–*R. meliloti* nodulation was not affected by the addition of anti-nodA antibodies to medium and inoculum. This suggests that the nodA protein is located within the cell and is therefore not accessible to antibodies. The expression of the *nodA* gene is induced in *R. meliloti* by various compounds present in the exudate of leguminous plants, particularly by the flavone luteolin. We show that the plant hormone trigonelline also has some inducing activity. The nodC protein was further localized in the membrane fraction of *R. meliloti*. Our experiments demonstrate that the nodC transmembrane protein is not necessary for the uptake of the compounds inducing the synthesis of the nodA protein. The nodA and the nodC proteins were also detected in mature nodules. During nodule development, the nodC protein may be processed to a 34-kDa protein.**

In *Rhizobium meliloti* a number of genes involved in nodulation (*nod*) are located on a very large symbiotic plasmid (megaplasmid; refs. 1 and 2). Four *nod* genes (*nodA*, *-B*, *-C*, and *-D*), which are involved in the induction of root hair curling and nodule formation, are clustered in a 4-kilobase region of this megaplasmid (3–5). These genes are highly conserved between different *Rhizobium* species (3, 6–8).

The proteins, which are essential for the induction of nodule formation, are encoded by the *nodABC* operon. Little is known about the biochemical roles of these *nod* gene products. The expression of the *nodABC* operon is positively controlled by the product of the *nodD* gene, which is located adjacent to this operon (9, 10). The *nodD* gene is transcribed divergently from the *nodA*, *-B*, and *-C* genes (5), and its product activates the expression of the *nod* operon only in the presence of plant exudate (9, 10). A complex promoter region including a conserved *nod* box has been found in front of the *nodA* and *nodD* genes (11).

Previously, we provided evidence indicating that the nodC protein is associated with the outer membrane of *R. meliloti* and that this protein may play a role in transmembrane signaling (12). In this paper, we show that the nodC transmembrane protein is not required for the induction of nodA by plant exudate. We found the nodA protein to be localized in the cytosol of *R. meliloti* and its synthesis to be induced by trigonelline, which is a component of root exudates of leguminous plants.

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MATERIALS AND METHODS

Bacterial Strains and Plasmids. *Escherichia coli* DS410 (13) was used as minicell-producing strain. The *lac* repressor-overproducing strain *E. coli* W3110*lacI^q*L8 (14) was used as a host for *lac* and *tac* promoter-containing plasmids. HB101 (15) was used for cell fractionation experiments. *R. meliloti* AK631 (Nod^+, Fix^+) is a compact colony variant of the wild-type *R. meliloti* 41 (1). *R. meliloti* Nod^- mutants AK1655, AK1679, and AK1657 carry Tn*5* insertions in *nodA*, *-B*, and *-C* genes, respectively (16). AK1680 and AK1672 are Nod^- mutants with Tn*5* in the *nodC* gene (17). *R. meliloti* MG107 (Nod^+-delayed; ref. 18) carries a Tn*5* insertion in the *nodD* gene of the megaplasmid pRme41*b*. Unless otherwise stated, the *E. coli* and *Rhizobium* strains were grown in M9 salts (19) supplemented with 0.2% Casamino acids/0.4% glycerol. Plasmid pEA305 carries the *tac* promoter and the *c*I gene of phage λ (20). In plasmids pJS120 and pJS201 (4), the *nodA*, *-B*, and *-C* genes were placed under the control of *E. coli* promoters in pACYC184 (21) and pIN-II-A2 (22). Plasmid pJS123 is a deletion derivative of pJS120 lacking the *nodA* gene (4).

Plant Exudate. Seeds of *Medicago sativa* cv. cardinal were surface-sterilized and germinated for 5 days at 22°C in the dark on nitrogen-free medium (23). The seedlings were washed briefly with sterile water and exudates were prepared with two seedlings per ml of water by gentle agitation for 4 hr in the dark. Exudates were filter-sterilized (Millex-GV) to eliminate plant fragments and tested for contamination by plating on TY agar (24). Uncontaminated batches were freeze-dried, dissolved in water (≈15% of the original volume), filter-sterilized, and stored at −20°C.

Protein Purification. *E. coli* W3110*lacI^q*L8 carrying pJS2023 was induced at OD_{600} = 0.8 by 1 mM isopropyl β-D-thiogalactopyranoside, and 4 hr after induction cells were harvested by centrifugation, washed with 50 mM Tris·HCl (pH 8.0) and resuspended in 50 mM Tris·HCl (pH 8.0) containing 20% sucrose. After treatment of the cells with lysozyme, spheroplasts were collected by centrifugation and resuspended in 20% sucrose containing 3 mM EDTA (pH 7.3). The spheroplasts were disrupted on ice by sonication and inclusion bodies were collected by centrifugation at 8000 rpm (SS-34 rotor) for 30 min. The granules were washed in 20% sucrose/3 mM EDTA, pH 7.3, and centrifuged again. The pellet was dissolved in 0.1 M sodium phosphate buffer (pH 6.8) containing 4% $NaDodSO_4$ and 0.1 M dithiothreitol. The sample was boiled for 5 min. The *c*I–nodA fusion protein was purified by a procedure of gel and hydroxyapatite in the presence of $NaDodSO_4$ as described for another *nod* gene product (12).

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Antibodies and Affinity Purification. Antiserum was raised in rabbits against purified *c*I–nodA fusion protein using the same immunization protocol as described (12). IgG was purified from the antiserum by chromatography on protein A-Sepharose (25) and dialyzed against phosphate-buffered saline (PBS).

For the isolation of monospecific antibodies, an affinity matrix was prepared by coupling the *c*I–nodA hybrid protein to CNBr-activated Sepharose 4B (Pharmacia) according to the instructions of the manufacturer. Antigen affinity chromatography was carried out as described (26). Antibodies were dialyzed against PBS, concentrated (≈1 mg/ml), and assayed for immunoreactivity.

Electrophoresis and Immunoblotting. $NaDodSO_4$/PAGE was performed in 12% polyacrylamide gels (27). Proteins were electrophoretically transferred to nitrocellulose (28). The immobilized proteins were incubated for 1 hr at room temperature in 20 mM Tris·HCl (pH 7.5) containing 500 mM NaCl (TBS) and 3% gelatin. The membrane was washed twice with TBS containing 0.05% Tween-20 (TTBS). The blotted proteins were incubated for 4 hr with a 1:1000 dilution of the monospecific antibodies in TTBS containing 1% gelatin. After three washes with TTBS (10 min each), the blot was incubated for 3 hr with ^{125}I-labeled anti-rabbit antibodies (5 μCi; 1 Ci = 37 GBq; Amersham). The immunoblot was washed three times in TTBS (10 min each), followed by single washes in TTBS containing 2 M NaCl, TBS containing 2 M NaCl, and TBS. The membrane was rinsed briefly with water, air-dried, and exposed to Kodak X-Omat S film at −70°C.

Induction Experiments. M9 medium supplemented with 0.2% Casamino acids/0.4% glycerol was inoculated with 8×10^6 bacteria per ml and cultured for 16 hr at 28°C in the presence of 0.1 vol of concentrated exudate of alfalfa seedlings. Compounds isolated from exudates of peas and sweet peas (29, 30), luteolin, naringenin (Roth, Karlsruhe, F.R.G.; refs. 31 and 32), and the inducer of the *vir* region of *Agrobacterium tumefaciens*, acetosyringone (33), were tested for their ability to induce the nodA protein at concentrations of 10–500 μM. Cells were harvested by centrifugation and 1.5 OD_{600} pellets were analyzed by $NaDodSO_4$/PAGE, immunoblotting, and autoradiography. The autoradiograms were scanned with a laser densitometer (LKB) and peak areas were determined with an integrator (HP 3390A).

Other Procedures. The recombinant DNA techniques were carried out essentially as described by Maniatis *et al.* (34). *E. coli* minicells were isolated and labeled with [^{35}S]methionine (35). Minicells were lysed with $NaDodSO_4$ and proteins were immunoprecipitated as described (12). For the detection of the nodA and nodC proteins in mature nodules, 94 mg of alfalfa nodules were lysed in 500 μl of electrophoresis buffer (27) containing 4% $NaDodSO_4$. The sample was boiled for 15 min prior to loading, and 80 μl per well was analyzed by $NaDodSO_4$/PAGE and immunoblotting.

Cytosol inner and outer membrane fractions of *E. coli* were prepared as described (36), using the spheroplasting procedure, lysis by sonication, and sucrose-gradient centrifugation. *R. meliloti* cells were fractionated into a cytosol and a total membrane fraction using the same procedure except that the duration of lysozyme and ultrasonic treatment was doubled.

Protein concentrations were determined by the method of Bradford (37) using bovine immunoglobulin as the standard. The amount of hybrid protein was determined by laser densitometer scanning of a polyacrylamide slab gel stained with Coomassie blue. Plant nodulation experiments were carried out with alfalfa (*Medicago sativa*) seedlings, which were grown on nitrogen-free medium as described (23).

RESULTS

Construction of Expression Plasmid pJS2023. To obtain sufficient quantities of the nodA protein we constructed a plasmid that expresses a fusion protein that has a portion of the λ *c*I repressor at the amino terminus and ≈92% of the 21.8-kDa nodA protein at the carboxyl end. For this purpose, we used the *tac* promoter vector pEA305, which directs the synthesis of high levels of the λ *c*I repressor (26% of total cellular protein) upon induction with isopropyl β-D-thiogalactopyranoside (20). This plasmid carries two copies of the transcription terminators of the *rrnB* operon (38). The strategy was similar to that reported for the construction of the *c*I–*nodC* gene fusion (12), except that a 12-mer *Hin*dIII linker was attached to the filled-in *Hin*dIII sites of pEA305 leading to the plasmid intermediate pEA305 Δ*Hin*dIII-2. The 12-mer linker was inserted to ligate the *nodA* coding sequence in-frame with the λ *c*I initiation codon on the expression vector.

The *nodA* gene is contained within a 1.2-kilobase *Bgl* II/*Sst* II fragment (4). Digestion with *Pvu* II and *Mbo* II and insertion of the *nodA*-containing fragment into the filled-in *Hin*dIII site of pEA305 Δ*Hin*dIII-2 yielded plasmid pJS2023. Plasmid pJS2023 was stably maintained in the *lac* repressor overproducing strain *E. coli* W3110*lacI*^{q}L8.

Overproduction and Purification of the Fusion Protein. Isopropyl β-D-thiogalactopyranoside-induced *E. coli* cells containing pJS2023 accumulated the *c*I-nodA fusion protein (37.5 kDa) after a 4-hr induction period up to ≈32% of total cellular protein. In contrast to the overproduction of the nodC membrane protein, which drastically reduced the growth rate of the bacteria (12), the synthesis of the *c*I–nodA fusion protein had no significant effect on bacterial growth.

Induced *E. coli* cells harboring pJS2023 were grown to stationary phase and examined by phase-contrast microscopy. The bacteria showed bulging cell walls caused by the presence of inclusion bodies in the cytoplasm. These cells were treated with lysozyme and the spheroplasts were disrupted by sonication. Inclusion bodies were collected from the crude lysate by low-speed centrifugation. The granules were dissolved in $NaDodSO_4$-containing buffer, and the fusion protein was purified by a procedure involving gel and hydroxyapatite chromatography in the presence of $NaDodSO_4$ (12). Gel chromatography separated the fusion protein from considerable amounts of DNA and RNA that were present in the inclusion bodies. After hydroxyapatite chromatography, the fusion protein was >95% pure as judged by $NaDodSO_4$/PAGE. Using antibodies raised against λ *c*I repressor, we could confirm the immunodiffusion tests (39) that this antiserum recognized the *c*I-encoded portion of the purified 37.5-kDa fusion protein.

Immunoprecipitation. Monospecific polyclonal rabbit antibodies against the fusion protein were prepared as described in the text. The specificity was tested by immunoprecipitation of the nodA protein expressed and labeled in *E. coli* minicells.

The nucleotide sequence of the *R. meliloti nod* genes revealed that in the open reading frame of *nodA* two potential initiation codons are found (40). This explains why two proteins of 21.8 and 32.3 kDa could sometimes be detected only in *E. coli* minicells harboring plasmids with strong promoters (e.g., pJS201; ref. 4). As shown in Fig. 1, antibodies against the fusion protein precipitated two proteins with the expected molecular masses (lanes 1 and 2). In minicells containing pJS120, only the 21.8-kDa protein was synthesized from the *nodA* coding region (lane 3), and the antibodies specifically precipitated this protein (lane 4). As a negative control, we used plasmid pJS123, which carries a deletion eliminating the *nodA* coding sequence (lane 5). The

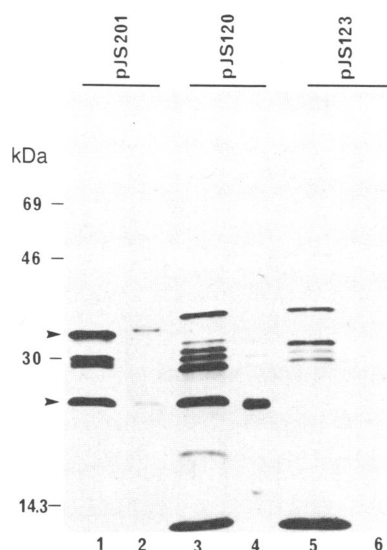

FIG. 1. Immunoprecipitation of nodA protein expressed in *E. coli* minicells containing *nod* genes from *R. meliloti*. Minicells containing the plasmids indicated were isolated and labeled with [^{35}S]methionine as described (35). Cell extracts reacted with affinity-purified antibodies against the *c*I–nodA fusion protein (lanes 2, 4, and 6). Lanes 1, 3, and 5 are cell extracts not precipitated. Cell extracts and immunoprecipitates were analyzed by $NaDodSO_4$/PAGE, fluorography, and autoradiography. The two forms of the nodA protein are indicated by arrowheads.

antibodies did not react with the proteins synthesized from this plasmid (lane 6).

Cellular Localization of the nodA and nodC Proteins. To localize the nodA protein in *E. coli* and *R. meliloti*, we fractionated cells into cytosol and membrane fractions by sucrose gradient centrifugation. $NaDodSO_4$/PAGE of sucrose gradient fractions of *E. coli* HB101 cells harboring pJS120 and subsequent immunolabeling of the immunoblot with antibodies against the nodA protein revealed that the nodA protein is present in the cytosol (Fig. 2*A*, lane 3).

In *R. meliloti* the gene products of *nodA* and *nodC* were localized by using appropriate antibodies and the mutant MG107, which carries the transposon Tn*5* in the *nodD* gene (18). In MG107, a promoter within the transposon causes constitutive expression of the *nodA*, *-B*, and *-C* genes so that induction of the *nod* operon with plant exudate was not essential. Tn*5*-associated promoter activity was recently reported by Berg *et al.* (41). Probably due to this promoter activity in MG107, we found higher expression of *nodABC* than with the wild-type strain AK631, which was induced with plant exudate. Using the cell-fractionation procedure described in the text, we obtained a cytosol and a total membrane fraction from cells of *R. meliloti* MG107. Labeling of the blotted proteins with monospecific antibodies against the nodA and nodC proteins showed that the 21.8-kDa nodA protein is present in the cytosol of *R. meliloti* (Fig. 2*B*, lane 3) and that the 46.8-kDa nodC protein is associated with the membranes (lane 4).

In a further experiment, *R. meliloti* 41 was inoculated onto the host plant *M. sativa* together with antibodies against the nodA protein. In these plant tests, normal nodulation occurred and there was no difference in the number of nodules formed in comparison with the control experiments. Similar plant tests with anti-nodC antibodies resulted in ≈50% inhibition of nodule formation, suggesting that the nodC transmembrane protein was accessible and inactivated by the antibodies (12). Since the bacterial membrane is impermeable to large molecules, we imagine that the nodA protein in the cytosol could not be reached by the antibodies, which thus explains why nodulation was not affected.

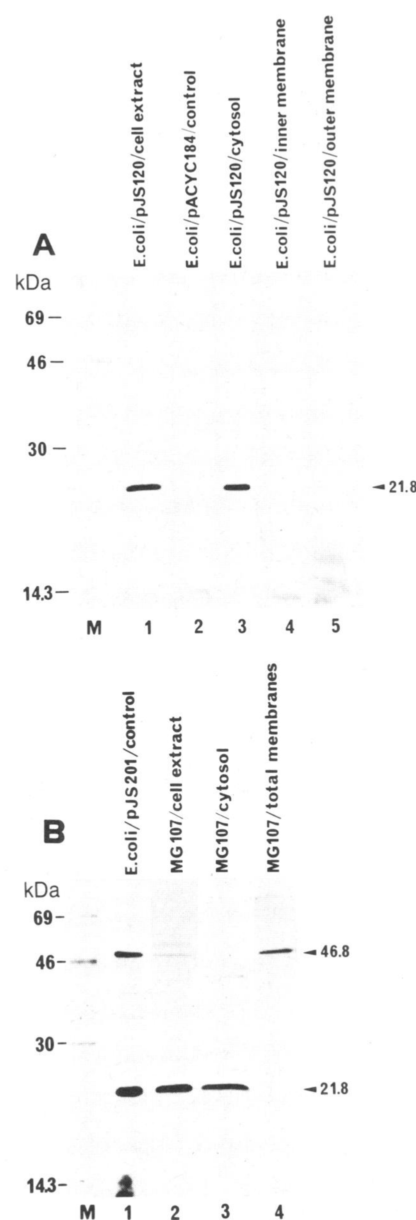

FIG. 2. Cellular location of the nodA protein in *E. coli* HB101 harboring pJS120 (*A*) and the gene products of *nodA* and *nodC* in *R. meliloti* MG107 (*B*). Cytoplasmic and membrane fractions were prepared as described in the text. Proteins were analyzed by $NaDodSO_4$/PAGE, transferred to nitrocellulose, and labeled with antibodies. (*A*) Labeled with anti-nodA antibodies; (*B*) labeled with antibodies directed against nodA and nodC proteins. The autoradiograms of the resulting immunoblots were prepared by using radioiodinated second antibodies. They were exposed for 3 days at −70°C. The position of the 21.8-kDa nodA protein and of the 46.8-kDa nodC protein is indicated by an arrowhead. Lanes M, molecular size markers.

Induction of nodA Protein in *R. meliloti*. Using *nod–lacZ* translational fusions it has been shown that the expression of the *nodABC* operon is induced by components present in plant exudates (9, 10, 42). We studied the synthesis of the nodA protein in various mutants of *R. meliloti* carrying Tn*5* insertions in the *nodA*, *-B*, and *-C* genes by immunoblot analysis. The data presented in Fig. 3 confirm the previous observation that the expression of the *nodA* gene is stimulated by the presence of plant exudate and that a Tn*5* insertion in the *nodA* gene (lane 4) prevents synthesis of the nodA protein (43). Moreover, transposon insertions in the *nodC* gene, which encodes a transmembrane protein (12), did not

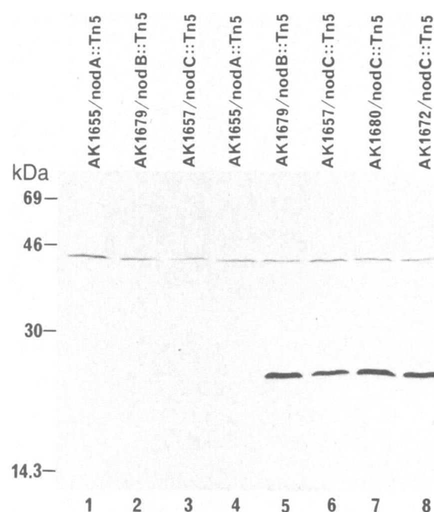

FIG. 3. Synthesis of the nodA protein in mutants of *R. meliloti* carrying Tn*5* insertions in the *nod* genes as indicated. Bacterial cell extracts were analyzed by $NaDodSO_4$/PAGE, immunoblotting, and autoradiography. Lanes 1–3, mutants grown without plant exudate; lanes 4–8, *R. meliloti* mutant strains grown for 16 hr in the presence of plant exudate. Arrowhead marks position of the nodA protein.

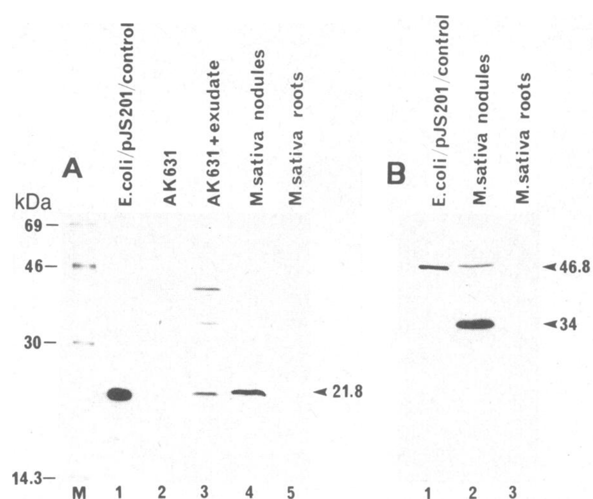

FIG. 4. Induction of *nodA* gene expression by plant exudate and detection of the gene products of *nodA* (*A*) and *nodC* (*B*) in mature nodules of *M. sativa*. Proteins were analyzed as described in Fig. 2. (*A*) Labeled with anti-nodA antibodies; (*B*) labeled with antibodies directed against the nodC protein.

affect the synthesis of the nodA protein (lanes 6–8). Thus, we can exclude the fact that the *nodC* protein is involved in the uptake of the plant factors that induce the *nodABC* operon.

Various UV-absorbing compounds isolated from peas and sweet peas (29, 30) were tested for their ability to induce the expression of the *nodA* gene. Of all compounds assayed, only the hormone trigonelline (44), which is present in many plants (45), showed some inducing activity (Table 1). The inducing activity of trigonelline was compared to the activity of the plant flavone luteolin (31), the flavanone naringenin (32), and total plant exudate. Of all compounds tested, luteolin showed the highest inducing effect (Table 1). This flavone, however, inhibits bacterial growth (≈37% at 10 μM and ≈72% with 100 μM), which may explain the reduced inducing activity at higher concentrations. Trigonelline was not toxic to the cells and showed increasing activity at relatively high concentrations.

Mature nodules from *M. sativa*, which were induced by the wild-type *R. meliloti* strain AK631, were analyzed for the presence of the nodA and nodC proteins using the appropriate antibodies (Fig. 4). The autoradiogram of the immunoblot shows a strong 21.8-kDa protein band (Fig. 4*A*, lane 4), indicating that the nodA protein is present in nodules. Immunoblot analysis of *M. sativa* nodules using antibodies against nodC shows the 46.8-kDa protein band and a strong 34-kDa polypeptide band (Fig. 4*B*, lane 2). The 34-kDa protein band may result from the processing of the nodC protein. This protein, which was localized on the cell surface (12), may be modified when the rhizobia differentiate into the morphologically altered bacteroids.

Table 1. Induction of *nodA* gene expression in *R. meliloti* AK 631

Inducer	Concentration, μM	% relative activity*
Luteolin	10	100
	100	34
Plant exudate	—	69
Naringenin	10	17
Trigonelline	10	6
	500	11
No addition	—	1
Others†	500	1

Cell extracts of *R. meliloti* were analyzed by $NaDodSO_4$/PAGE, immunoblotting, and autoradiography. Autoradiograms were scanned and peak areas corresponding to the nodA protein band were determined with an integrator.

*Based on a value of 100 for luteolin.

†The heterocyclic exudate components β-(isoxazolin-5-on-2-yl)alanine, β-(uracil-3-yl)alanine, 2-(3-amino-3-carboxypropyl)-isoxazolin-5-one, and acetosyringone, the inducer of the *vir* region of *Agrobacterium tumefaciens* (33) did not induce the expression of *nodA*.

DISCUSSION

We overproduced the nodA protein fused to a portion of the λ cI repressor in *E. coli*. This fusion protein was purified from inclusion bodies and was used to raise antibodies against nodA in rabbits. Monospecific polyclonal antibodies were prepared by affinity chromatography and made it possible to detect and localize the nodA protein in *E. coli* and *R. meliloti* by immunoblotting.

In *R. meliloti*, the *nodA* gene expresses a 21.8-kDa protein, which represents only a very small fraction of the total cellular protein. The nodA protein is involved in very early steps of nodule formation (3, 5) but has also been detected in mature nodules (Fig. 4*A*). This result could indicate that the nodA protein may also play a role during later stages of the symbiosis. It remains to be elucidated whether this protein is still synthesized during nodule formation or is accumulated in the bacteroids during early steps of nodulation. Although the nodA protein contains hydrophobic regions (40), it was found not to be associated with the bacterial membranes. In cell fractionation experiments, we localized the nodA protein in the cytosol of *E. coli* and *R. meliloti* (Fig. 2). Indirect evidence that the nodA protein is in the cytosol and thus not accessible to antibodies was obtained by plant nodulation experiments in which *R. meliloti* was inoculated onto its host plant together with anti-nodA antibodies. No inhibition of nodulation was observed. In a previous study, we localized a cI–nodC fusion protein in the outer membrane of *E. coli* (12), but until now the nodC protein had not been detected in *Rhizobium*. Using monospecific antibodies against nodC, we could clearly detect the nodC protein in the membrane fraction of *R. meliloti* (Fig. 2*B*, lane 4) and in mature nodules of *M. sativa* (Fig. 4*B*). Furthermore, a 34-kDa protein, which probably represents a modified form of the nodC protein was immunologically detected in nodules. Whether this putative

processed nodC protein has some biological function requires further investigation.

The expression of the *nod* genes can be induced by plant exudates (9, 10). It has been reported recently that the flavone luteolin, which was isolated from seed exudates of alfalfa, can activate the synthesis of the nod proteins (31). We independently tested various compounds isolated from root exudates of leguminous plants for their ability to induce *nod* gene expression. Gene-inducing activity was found with the plant hormone trigonelline (Table 1). The mitotic cycle hormone trigonelline (44) is widespread in plants (45) and is present in the exudate of seeds of leguminous plants in high concentrations (30).

Our results indicate that the nodC transmembrane protein is not necessary for the uptake of the inducers of the nodA protein (Fig. 3). We assume that the nodC membrane protein may be a receptor transducing a signal molecule from the bacterial to the plant cell. Functional assays are necessary to elucidate whether the nodA protein is involved in generating such a signal molecule within the bacterial cell.

We thank Adam and Eva Kondorosi (Biological Research Center, Szeged, Hungary) for providing Tn*5* mutants of *R. meliloti* and for helpful discussions, Fernand Lambein (State University of Ghent, Ghent, Belgium) for providing trigonelline and other compounds from root exudates of leguminous plants, and Sharon Long (Stanford University, Stanford, CA) for information prior to publication. We also thank Frans de Bruijn for discussions and critical reading of the manuscript and Maret Kalda and Dietrich Bock for photographic work. This work was supported by a grant from Bundesministerium für Forschung und Technologie (BCT 03652/project 13).

1. Banfalvi, Z., Sakanyan, V., Koncz, C., Kiss, A., Dusha, I. & Kondorosi, A. (1981) *Mol. Gen. Genet.* **184**, 318–325.
2. Rosenberg, C., Boistard, P., Denarie, J. & Casse-Delbart, F. (1981) *Mol. Gen. Genet.* **184**, 326–333.
3. Kondorosi, E., Banfalvi, Z. & Kondorosi, A. (1984) *Mol. Gen. Genet.* **193**, 445–452.
4. Schmidt, J., John, M., Kondorosi, E., Kondorosi, A., Wieneke, U., Schröder, G., Schröder, J. & Schell, J. (1984) *EMBO J.* **3**, 1705–1711.
5. Egelhoff, T. T., Fischer, R. F., Jacobs, T. W., Mulligan, J. T. & Long, S. R. (1985) *DNA* **4**, 241–248.
6. Downie, J. A., Knight, C. D., Johnston, A. W. B. & Rossen, L. (1985) *Mol. Gen. Genet.* **198**, 255–262.
7. Fischer, R. F., Tu, J. K. & Long, S. R. (1985) *Appl. Environ. Microbiol.* **49**, 1432–1435.
8. Djordjevic, M. A., Schofield, P. R., Ridge, R. W., Morrison, N. A., Bassam, B. J., Plazinski, J., Watson, J. M. & Rolfe, B. G. (1985) *Plant Mol. Biol.* **4**, 147–160.
9. Mulligan, J. T. & Long, S. R. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6609–6613.
10. Rossen, L., Shearman, C. A., Johnston, A. W. B. & Downie, J. A. (1985) *EMBO J.* **4**, 3369–3373.
11. Rostas, K., Kondorosi, E., Horvath, B., Simoncsits, A. & Kondorosi, A. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 1757–1761.
12. John, M., Schmidt, J., Wieneke, U., Kondorosi, E., Kondorosi, A. & Schell, J. (1985) *EMBO J.* **4**, 2425–2430.
13. Dougan, G. & Sherratt, D. (1977) *Mol. Gen. Genet.* **15**, 151–160.
14. Brent, R. & Ptashne, M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4204–4208.
15. Boyer, H. W. & Roulland-Dussoix, D. (1969) *J. Mol. Biol.* **41**, 459–472.
16. Kondorosi, A., Kondorosi, E., Banfalvi, Z., Putnoky, P., Török, I., Stepkowski, T., Schmidt, J. & John, M. (1985) in *Natural Products Chemistry 1984*, eds. Zalewski, R. I. & Skolik, J. J. (Elsevier, Amsterdam), pp. 643–654.
17. Kondorosi, A., Kondorosi, E., Banfalvi, Z., Dusha, I., Putnoky, P., Toth, J., Bachem, C. (1984) in *Proceedings of the Fifteenth International Congress of Genetics* (Oxford/IBH, New Delhi), Vol. 2, pp. 205–216.
18. Göttfert, M., Horvath, B., Kondorosi, E., Putnoky, P., Rodriguez-Quinones, F. & Kondorosi, A. (1986) *J. Mol. Biol.* **191**, 411–420.
19. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
20. Amann, E., Brosius, J. & Ptashne, M. (1983) *Gene* **25**, 167–176.
21. Chang, A. C. Y. & Cohen, S. N. (1978) *J. Bacteriol.* **134**, 1141–1156.
22. Nakamura, K. & Inouye, M. (1982) *EMBO J.* **1**, 771–775.
23. Kondorosi, A., Svab, Z., Kiss, G. B. & Dixon, R. A. (1977) *Mol. Gen. Genet.* **151**, 221–226.
24. Beringer, J. E. (1974) *J. Gen. Microbiol.* **84**, 188–198.
25. Miller, T. T. & Stone, H. O. (1978) *J. Immunol. Methods* **24**, 111–125.
26. De Mey, J. R. (1983) in *Immunohistochemistry*, ed. Cuello, A. C. (Wiley, Chichester), pp. 347–372.
27. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
28. Towbin, H., Staehelin, T. & Gordon, T. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
29. Kuo, Y.-H., Lambein, F., Ikegami, F. & Van Parijo, R. (1982) *Plant Physiol.* **70**, 1283–1289.
30. Ikegami, F., Kuo, Y.-H. & Lambein, F. (1982) *Arch. Int. Physiol. Biochim.* **90**, B35–B36.
31. Peters, K., Frost, J. W. & Long, S. R. (1986) *Science* **233**, 977–980.
32. Zaat, B., van Brussel, A., Wijffelman, C., Spaink, H., Okker, R., Pees, E. & Lugtenberg, B. (1986) in *Third International Symposium on the Molecular Genetics of Plant-Microbe Interactions*, Montreal, Canada, p. 134 (abstr.).
33. Stachel, S. E., Messens, E., Van Montagu, M. & Zambryski, P. (1985) *Nature (London)* **318**, 624–629.
34. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
35. Schröder, J., Hillebrand, A., Klipp, W. & Pühler, A. (1981) *Nucleic Acids Res.* **9**, 5187–5202.
36. Ito, K., Sato, T. & Yura, T. (1977) *Cell* **11**, 551–559.
37. Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254.
38. Brosius, J., Dull, T. J., Sleeter, D. D. & Noller, H. F. (1981) *J. Mol. Biol.* **148**, 107–127.
39. Ouchterlony, Ö. (1958) *Prog. Allergy* **5**, 1–78.
40. Török, I., Kondorosi, E., Stepkowski, T., Posfai, J. & Kondorosi, A. (1984) *Nucleic Acids Res.* **12**, 9509–9524.
41. Berg, D. E., Wein, A. & Crossland, L. (1980) *J. Bacteriol.* **142**, 439–446.
42. Innes, R. W., Kuempel, P. L., Plazinski, J., Canter-Cremers, H., Rolfe, B. G. & Djordjevic, M. A. (1985) *Mol. Gen. Genet.* **201**, 426–432.
43. Egelhoff, T. T. & Long, S. R. (1985) *J. Bacteriol.* **164**, 591–599.
44. Evans, L. S. & Tramontano, W. A. (1981) *Am. J. Bot.* **68**, 1282–1289.
45. Willeke, U., Heeger, V., Meise, M., Neuhann, H., Schindelmeiser, I., Vordemfelde, K. & Barz, W. (1979) *Phytochemistry* **18**, 105–110.