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Suppression of Nodulation Gene Expression in Bacteroids of *Rhizobium leguminosarum* Biovar viciae

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The expression of *nod* genes of *Rhizobium leguminosarum* bv. viciae in nodules of *Pisum sativum* was investigated at both the translational and transcriptional levels. By using immunoblots, it was found that the levels of NodA, NodI, NodE, and NodO proteins were reduced at least 14-fold in bacteroids compared with cultured cells, whereas NodD protein was reduced only 3-fold. Northern (RNA) blot hybridization, RNase protection assays, and in situ RNA hybridization together showed that, except for the *nodD* transcript, none of the other *nod* gene transcripts were present in bacteroids. The amount of *nodD* transcript in bacteroids was reduced only two- to threefold compared with that in cultured cells. Identical results were found with a *Rhizobium* strain harboring multicopies of *nodD* and with a strain containing a NodD protein (NodD604) which is activated independently of flavonoids. Furthermore, it was found that mature pea nodules contain inhibitors of induced *nod* gene transcription but that NodD604 was insensitive to these compounds. In situ RNA hybridization on sections from *P. sativum* and *Vicia hirsuta* nodules showed that transcription of inducible *nod* genes is switched off before the bacteria differentiate into bacteroids. This is unlikely to be due to limiting amounts of NodD, the absence of inducing compounds, or the presence of anti-inducers. The observed switch off of transcription during the development of symbiosis is a general phenomenon and is apparently caused by a yet unknown negative regulation mechanism.

Bacteria of the genus *Rhizobium* are able to establish a symbiosis with leguminous plants, resulting in formation of root nodules in which the bacteria, in an altered form designated as bacteroids, reduce atmospheric nitrogen to ammonia. Successful nodulation is a host-specific process in the sense that *Pisum* and *Vicia* species are host plants for *Rhizobium leguminosarum* biovar (bv.) viciae, alfalfa is a host for *R. meliloti*, and *Trifolium* sp. is a host for *R. leguminosarum* bv. trifolii.

Bacterial *nod* (for nodulation) genes localized on a Sym (for symbiosis) plasmid code for proteins involved in early steps in nodulation. The *nodD* gene is the only constitutively transcribed *nod* gene in free-living cells. In *R. leguminosarum* bv. viciae and *R. leguminosarum* bv. trifolii, *nodD* is present as a single copy whereas in *R. meliloti* four allelic forms, designated *nodD1*, *nodD2*, *nodD3*, and *syrM*, have been identified. The NodD protein binds specifically to *nod* boxes (18, 19, 22, 28), conserved DNA sequences in the upstream untranslated region of other *nod* genes (11, 40, 46, 49), and induces transcription of the other *nod* genes, provided that NodD protein is activated by an inducer of plant origin. These inducers have been identified as flavones and flavanones (17, 34, 37, 65), while isoflavones and coumarins act as anti-inducers for these species (13, 17). It is very likely that the NodD protein interacts directly with the inducer molecules (2, 5, 21, 24, 32, 52, 53), although binding of flavonoids to NodD protein has not yet been demonstrated.

The inducible *nodABC* and *nodFEL* genes are involved in early steps of nodulation, as reflected by the Nod$^-$ phenotype of *nodABC* mutants and the strongly reduced nodulation of *nodFEL* mutants. The products of these genes function in root hair curling, infection thread formation, and initiation of cortical cell division (6, 9, 14, 45, 56, 61). The common *nodABC* genes are involved in the synthesis of extracellular factors (48), one of which has recently been identified in *R. meliloti* (29). This factor is modified by host-specific *nod* gene products, resulting in effective nodules on a limited range of host plants (1, 16, 38, 48). Other *nod* genes identified in *R. leguminosarum* bv. viciae are *nodIJ*, *nodMNT* (6, 54, 55), and *nodO* (11, 15). Mutations in these genes have more or less severe effects on nodulation, depending on the host plant.

Induction of expression of *nod* genes and their functioning in early steps in nodulation have firmly been established for all rhizobia, but whether the *nod* genes are also expressed in later stages of symbiosis has been reported for *R. meliloti* only (47). By using fusions of the appropriate genes with *gusA*, it was found that the inducible *nod* genes are not expressed at all and that expression of *nodD1* and *nodD3* is decreased dramatically in older zones of alfalfa nodules (47). Since β-glucuronidase is a stable reporter enzyme, the picture of the temporal expression of *nod* genes might be obscured. In this report, we describe *nod* gene expression in nodules of *R. leguminosarum* bv. viciae by using a direct approach by analyzing the products and the transcripts. It was found that *nodD* transcription is reduced two to threefold in bacteroids. The inducible *nod* genes are not transcribed in bacteroids, and their expression stops before release of the bacteria from the infection thread. This result is in agreement with that found for *R. meliloti*. We investigated several possible explanations for the switch off of the *nod* genes. Neither the absence of inducers nor the presence

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TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Relevant characteristics	Source or reference
R. leguminosarum		
248	*R. leguminosarum* bv. viciae wild type	26
RBL1532	248 Rif^r Spc^r cured of Sym plasmid pRL1JI	9
RBL1402	248 pRL1JI *nodD2*::Tn*5*	61
LPR5045	*R. leguminosarum* bv. trifolii Rif^r cured of Sym plasmid	23
RBL5560	LPR5045 with Sym plasmid pRL1JI	65
RBL5561	LPR5045 with pRL1JI *nodD2*::Tn*5*	65
E. coli		
KMBL1164	Δ(*lac-pro*) *supE thi* F$^-$	van der Putte
JM101	Δ(*lac-pro*) *supE thi* (F'*traD36 proAB lacI^{q}ZΔ*M15)	63
DH5αF'	Δ(*lacZYA-arg*) *supE thi recA1 lacZ*ΔM15	Promega
Plasmids		
pRK2013	IncColE1, helper plasmid for tripartite mating	12
pBS1KS+	Bluescript vector, cloning vector	Stratagene
pMP107	IncColE1 carrying *nodABC*	This study
pMP154	IncQ carrying the *pr. nodA-lacZ*	49
pMP280	IncP carrying the *pr. nodD-nodD*	53
pMP604	IncP carrying FITA-type *nodD604*	52
pMP1210	IncColE1 carrying *nod'FE*	51
pMP2010	IncColE1 carrying *nodDnodF'*	This study
pMP2020	Bluescript vector carrying *nodDnodF'*	This study
pMP2023	Bluescript vector carrying 5' part of *nodD*	This study
pMP2024	Bluescript vector carrying *nodA* sequences	This study
pT7.BB	IncColE1 carrying *fixC'XnifAB'* of pSymPRE	39

[a] All *nod* sequences originated from pRL1JI, except *nodD604*, which is coded by pMP604. *pr.*, promoter.

of anti-inducers or limitation for NodD protein was found to be responsible for the switch off of the inducible *nod* genes.

MATERIALS AND METHODS

Bacterial strains and crosses. The *R. leguminosarum* strains used are listed in Table 1. Strains RBL1402 and RBL5561 were used as hosts for plasmids pMP604, containing FITA-type *nodD604* (52), and pMP280 (53). *Escherichia coli* JM101 and KMBL1164 were both used as hosts for plasmids during cloning procedures, except for transcription vectors, which were kept in strain DH5αF'. Plasmids were crossed from *E. coli* to *R. leguminosarum* by using tripartite mating as described previously (12).

Nodulation assay and isolation of bacteroids. Seeds of *Pisum sativum* cv. Finale and *Vicia hirsuta* were surface sterilized, inoculated with appropriate rhizobia, and cultured on gravel by published procedures (35). *P. sativum* was inoculated with *R. leguminosarum* bv. viciae 248, RBL1402(pMP280), or RBL1402(pMP604), and *V. hirsuta* was inoculated with strain RBL5560 or RBL5561(pMP604).

Sprout dry weights of 40 pea plants were determined 21 days after inoculation by cutting the stem right above the seed, freezing the sprouts in liquid nitrogen, and lyophilizing them for 48 h. This determination was performed three times.

Bacteroids were isolated from pea root nodules of 50 plants 21 days after inoculation. The method used was that of Katinakis et al. (27), except that the isolation buffer was 0.6 M sucrose-50 mM morpholine propanesulfonic acid (MOPS) (pH 7.5)–2.5 mM $MgCl_2$–10 mM KCl–1 mM dithiothreitol–4% (wt/vol) polyvinylpyrrolidone–5 mM *p*-aminobenzamidine. The purity of the bacteroid preparation was determined in two ways. Cells were counted by microscopy, in which the large Y-shaped bacteroids can easily be discriminated from free-living bacteria, and by determination of the number of CFU on selective media consisting of TY agar (3) supplemented with antibiotics.

Protein analyses. Rhizobia were grown in TY medium (3) supplemented with 20% (vol/vol) B$^-$ medium (57) to an A_{620} of 0.6. For induction of *nod* genes, the medium was supplemented with 1 μM naringenin. After harvesting by centrifugation, cells were suspended in 20% (wt/vol) sucrose–50 mM Tris-HCl (pH 8.5)–0.1 mM dithiothreitol–200 μg of DNase I ml^{-1}–200 μg of RNase A ml^{-1}–500 μM phenylmethylsulfonyl fluoride–50 μg of soybean trypsin inhibitor ml^{-1}–10 μg of leupeptin ml^{-1} and lysed by three passages through a French press at 15,500 lb · in^{-2}. Subsequently, the sucrose was diluted to 7% (wt/vol), the debris was removed by centrifugation for 20 min at 1,000 × *g*, and the cleared lysate obtained was used for protein analysis. Protein preparations of bacteroids were obtained by lysis of the cells in sodium dodecyl sulfate (SDS) sample buffer (30). Soluble proteins present in the growth medium or in the peribacteroid space were recovered by centrifugation after precipitation in 5% trichloroacetic acid and dissolved in SDS sample buffer (30).

Proteins were separated by SDS-polyacrylamide gel electrophoresis (30) and transferred to nitrocellulose by using a semidry blot apparatus (LKB Biotechnology, Uppsala, Sweden). Immunoreactions were performed by published procedures (43). Polyclonal antibodies against *R. leguminosarum* NodD, NodE, NodI, and NodO proteins and against elongation factors Tu and Ts of *E. coli* have already been described (references 43, 51, 42, 10, and 58, respectively). Affinity-purified antibodies against NodA (44) were a kind gift of M. John and J. Schmidt of the Max Planck Institute for Plant Breeding (Cologne, Germany).

The amount of protein present in cleared lysates of cultured cells or in bacteroid preparations was estimated as described by Markwell et al. (31), with bovine serum albumin as the standard, and was related to the number of cells. Maximally 110 μg of total cell protein of bacteroids could be analyzed on immunoblots without overloading the gels.

Immunoblots were scanned in one dimension to determine the levels of Nod protein in protein preparations, and the peak values obtained were corrected for varying lane width. In the quantification of NodD protein, the amount of a stable degradation product from NodD with an apparent molecular mass of 23 kDa (43) was included. During the preparation of protein samples, this product is rapidly formed and it is stable but the amount in which it is present in protein samples differs from one preparation to another.

RNA isolation. To obtain RNA from cultured cells, bacteria were grown in TY medium (3) supplemented with 20% (vol/vol) B$^-$ medium (57) and, if appropriate, also supple-

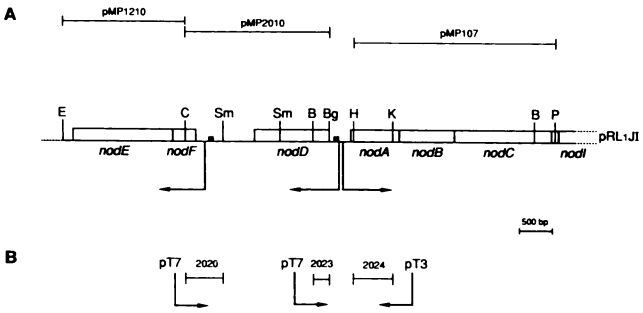

FIG. 1. Sequences that yielded *nod* probes for RNA analysis. The *nod* probes were obtained as subclones from part of the *nod* region of *R. leguminosarum* bv. viciae Sym plasmid pRL1JI, which is indicated in the center. Open reading frames are represented by open boxes, and directions of transcription are shown by arrows. Small black boxes represent *nod* boxes. (A) *nod* probes used in Northern blot hybridization. (B) fragments used for synthesis of the antisense RNAs used as *nod* probes in RNase protection assays. The directions of in vitro transcription and the vector promoters used are indicated. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sm, *Sma*I.

mented with 1 μM naringenin to an A_{620} of 0.5 to 0.8. The bacteria were collected by centrifugation and stored at −80°C for at least 30 min, and RNA was isolated by the hot phenol method as described earlier (60).

Nodule RNA was isolated from pea root nodules 21 days after inoculation of 25 plants. The nodules were kept constantly frozen in liquid nitrogen during collection. After the nodules were ground in a mortar, the frozen powder was extracted with hot phenol and the RNA was precipitated with LiCl as described previously (60).

Bacteroid RNA was obtained from bacteroids isolated by using the procedure of Katinakis et al. (27) with the following modifications. Nodules kept constantly frozen in liquid nitrogen were ground in sterile isolation buffer consisting of 0.4 M sucrose–50 mM MOPS (pH 7.5)–2.5 mM $MgCl_2$–10 mM KCl–1 mM dithiothreitol–4% (wt/vol) polyvinylpyrrolidone–1,000 U of RNase inhibitor ml^{-1}. The procedure was terminated after the step in which bacteroids still containing the peribacteroid membrane are obtained. Subsequently, the RNA was isolated as described above.

RNA concentrations were measured spectrophotometrically, and their quality was judged after gel electrophoresis and staining with ethidium bromide or 0.01% toluidine blue.

Northern (RNA) blot analysis. RNAs were electrophoretically separated by using denaturing 2% agarose-formamide gels in MOPS buffer and transferred to GeneScreen filters (New England Nuclear Corp., Boston, Mass.) by standard methods (41). Hybridization was performed at 45°C in 50% formamide–5× SSPE (1× SSPE is 150 mM NaCl–10 mM sodium phosphate–1 mM EDTA)–5% SDS–100 μg of denatured herring sperm DNA ml^{-1} for 68 h. Isolated restriction fragments containing *nod* sequences (Fig. 1A) or *nifA* sequences from pT7.BB (39) were nick translated and used as probes. The blots were washed at 65°C with 1× SSPE–0.1% SDS and subsequently with 0.5× SSPE–0.1% SDS. The filters were exposed to Fuji X-ray film at −80°C with intensifying screens. The signals were quantified by scanning the autoradiograms in two dimensions.

RNase protection assay. Transcription vectors pMP2020, pMP2023, and pMP2024 were constructed by cloning restriction fragments containing *nodF*, *nodD*, and *nodA* sequences, respectively, in Bluescript vector pBS1KS+. Transcripts were synthesized by using a TransProbe T kit (Pharmacia LKB Biotechnology, Uppsala, Sweden). Incomplete antisense transcripts of *nodF* and *nodD* were obtained from pMP2020 linearized with *Sma*I and from pMP2023 linearized with *Bgl*II, respectively, by using T7 RNA polymerase. By using T3 RNA polymerase and pMP2024 linearized with *Hin*dIII, incomplete antisense transcripts of *nodA* were synthesized. These antisense transcripts (Fig. 1B) were used as probes for detection of specific RNAs in total RNA preparations. To obtain highly labeled probes, transcription was performed with 250 ng of template DNA and 125 μCi of [α-^{32}P]UTP (3,000 Ci · $mmol^{-1}$) with no addition of unlabeled UTP. Because of the limiting amount of UTP, shorter transcripts are formed as well. After incubation for 15 min at 37°C, probes were treated with DNase I and precipitated three times as previously described (20). Hybridization occurred at 45°C in 80% formamide–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)-400 mM NaCl–1 mM EDTA–0.5 × 10^6 to 1 × 10^6 cpm of probe and the amounts of RNA indicated. Further treatments, including those with RNase and proteinase K, were performed as previously described (20). Samples were analyzed on 6% polyacrylamide–7 M urea sequencing gels (41), and RNase-resistant complexes were visualized by autoradiography with intensifying screens.

RNA in situ hybridization. Nodules of *V. hirsuta* or *P. sativum* were picked 15 days after inoculation and subsequently fixed, embedded, and sectioned as described by Van de Wiel et al. (59). Seven-micrometer-thick sections were hybridized with partly degraded ^{35}S-labeled RNA probes essentially as described by Cox and Goldberg (8), with previously described modifications (59). To obtain probes, the entire *nodC*, *nodE*, and *nifA* genes were separately cloned in Bluescript vector pBS1KS+. Antisense *nodC* RNA was synthesized after digestion of the vector with *Hin*dIII, within the *nodC* sequence, and using T7 RNA polymerase through which two-thirds of the gene was transcribed. As a control, sense *nodC* RNA was made of the same construct linearized with *Bam*HI by using T3 RNA polymerase. Antisense *nodE* RNA was synthesized from the T7 promoter, while sense *nodE* RNA was transcribed by T3 RNA polymerase after digestion of the vector with *Xho*I and *Eco*RI, respectively, both in the polylinker of the vector. The *nifA* probe was made by T7 RNA polymerase of the *Xba*I-linearized vector. After hybridization, slides were coated with Kodak NTB2 nuclear emulsion and exposed for 1 to 4 weeks at 4°C. Afterwards, the sections were stained with 0.25% toluidine blue, mounted with DPX, and photographed by using dark-field and epipolarization optics.

Extraction of nodules. Pea nodules were picked 21 days after inoculation with *R. leguminosarum* bv. viciae 248, frozen in liquid nitrogen, grounded in a mortar, and extracted with methanol and subsequently with butanol as previously described (36). The extract was dried by evaporation and dissolved in methanol and is further referred to as nodule methanol extract. Such an extract from *V. sativa* has been shown to contain flavonoids (36).

Induction assay. Induced transcription from the *nodA* promoter was measured as units of β-galactosidase activity by using strains LPR5045(pMP280, pMP154) and LPR 5045(pMP604, pMP154). Assays were performed as previously described, using 90 nM naringenin for induction (64, 65). Inhibition of *nodA* transcription was determined by growing the cells in medium supplemented with 90 nM naringenin, which has been shown to be suboptimal (65), and different amounts of nodule methanol extract, as indicated.

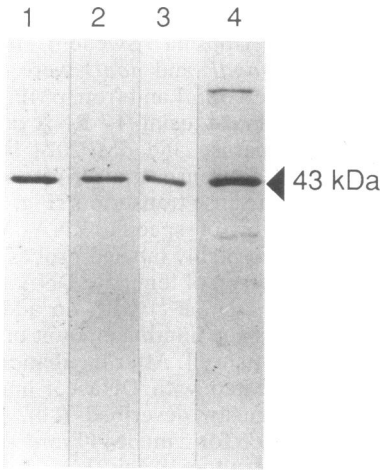

FIG. 2. Occurrence of EF-Tu in protein preparations of cultured cells and bacteroids of wild-type *R. leguminosarum* bv. viciae 248. Lanes: 1, cultured cells induced with naringenin; 2, noninduced cultured cells; 3, bacteroids; 4, cleared *E. coli* lysate. The position of *E. coli* EF-Tu is indicated by an arrowhead. Lanes 1 to 3 each contained 1.25 μg of total cell protein, whereas lane 4 was loaded with 2.0 μg of protein.

Miscellaneous. Cloning, transformation, nick translation, and gel electrophoresis of nucleic acids were performed by standard methods (41). Scanning was performed with a laser scanner from LKB (Uppsala, Sweden).

Materials. Restriction enzymes, RNase inhibitor, and RNA molecular weight markers were purchased from Boehringer (Mannheim, Germany). Radioactive nucleotides were obtained from Amersham International plc (Amersham, United Kingdom), DPX mountant was from BDH (Poole, United Kingdom), and other chemicals and enzymes were from Sigma (St. Louis, Mo.).

RESULTS

Quantification of protein levels in cultured bacteria and bacteroids. To compare the levels of Nod proteins of free-living cells and bacteroids, the total amount of cell protein was chosen as a criterion. This choice was based on experiments in which the amount of protein per cell and the concentration of a protein with an essential function in the cell had been determined. The amount of protein present per 10^9 cultured cells was found to be 0.21 mg. Bacteroids harvested 21 days after inoculation of *P. sativum* contain 1.6 mg of protein per 10^9 cells (4). Thus, bacteroids contain approximately 7.5-fold more protein per cell than do free-living bacteria.

The level of elongation factor Tu (EF-Tu), an essential protein, was determined on immunoblots containing equal amounts of protein derived from cultured cells and from bacteroids of *R. leguminosarum* bv. viciae 248. The two cell types contained comparable levels of EF-Tu per milligram of total protein (Fig. 2). The specificity of the reaction was confirmed by the following observations. (i) The cross-reacting protein in material from *R. leguminosarum* bv. viciae had the same migration as *E. coli* EF-Tu, (ii) an antiserum raised against the isolated GTP-binding domain of EF-Tu reacted with a protein with identical migration on immunoblots (data not shown), and (iii) no other cross-reacting proteins were detected. In conclusion, the concentration of EF-Tu per cell, either free living or bacteroid, is constant. This same result was found when antibodies against elongation factor Ts (EF-Ts) were used (data not shown). On the basis of these results, total cell protein was used as the standard in comparison of the levels of Nod protein of cultured bacteria with those of bacteroids.

Comparison of levels of Nod proteins in bacteroids and free-living bacteria. To investigate whether the *nod* genes are expressed in bacteroids, the occurrence of different Nod proteins was tested by using immunoblots containing material from cultured cells and bacteroids of wild-type *R. leguminosarum* bv. viciae 248 isolated 21 days after inoculation of peas. The NodD protein was present in both induced and uninduced free-living cells (Fig. 3A, lanes 1 and 2), in agreement with a constitutively transcribed *nodD* gene. Also, a NodD signal was detected in protein preparations of bacteroids (Fig. 3A, lane 3). Quantification of the amounts of NodD (see Materials and Methods) in bacteroids and cultured cells by scanning of several different immunoblots showed that the level of NodD protein in bacteroids was reduced to 25 to 35% of the level present in cultured bacteria.

On immunoblots containing material from cultured bacteria, all of the Nod proteins, NodA, NodI, NodE, and NodO, gave strong signals provided that the bacteria were induced (Fig. 3B, lanes 1 and 4). However, in protein preparations from bacteroids neither NodA, NodI, nor NodO could be detected whereas NodE protein gave a weak signal (Fig. 3). Because NodO protein is excreted in the medium by cultured bacteria (10), isolated peribacteroid membrane and peribacteroid space material were also analyzed for the occurrence of NodO protein. In neither fraction was the protein detected (Fig. 3B, lanes 5 to 8). The inability to detect NodA and NodI proteins in a sample of 110 μg of protein from bacteroids indicates that their levels are reduced at least 18-fold compared with those of cultured cells, since 6 μg of total cell protein was enough to detect both proteins. To determine the levels of NodE proteins in bacteroids and free-living bacteria, signals on immunoblots were compared by scanning. As shown in Fig. 4 for cultured bacteria, the peak values from the signals have a linear relationship with the amount of protein used. When different preparations of 85 μg of protein from bacteroids were analyzed, a peak value of 0.24 ± 0.023 was found, corresponding with the peak value found with 6 μg of protein from cultured cells. This result indicates that at least 14-fold less NodE protein is present in bacteroids.

Since the bacteroid preparations were found to be contaminated with only 5% free-living cells, it is concluded that in bacteroids the *nodD* expression level is lowered and the inducible *nod* genes are expressed at a very low level, if at all.

Comparison of transcription levels in bacteroids and free-living bacteria. To determine whether the low levels of NodD protein and the absence of other Nod proteins in nodules were due to control at the transcriptional level, RNA analyses were performed. Steady-state levels of RNA were examined by three different approaches, namely, Northern blot hybridization, an RNase protection assay, and RNA in situ hybridization. In both Northern blot and in situ hybridizations, a *nifA* probe derived from the Sym plasmid of *R. leguminosarum* bv. viciae PRE was used as a positive control. The rationale for choosing this gene was as follows. (i) It codes for a transcriptional regulator protein, which probably means that it is transcribed at a low level comparable to that of the *nod* genes, (ii) the size of the transcript is

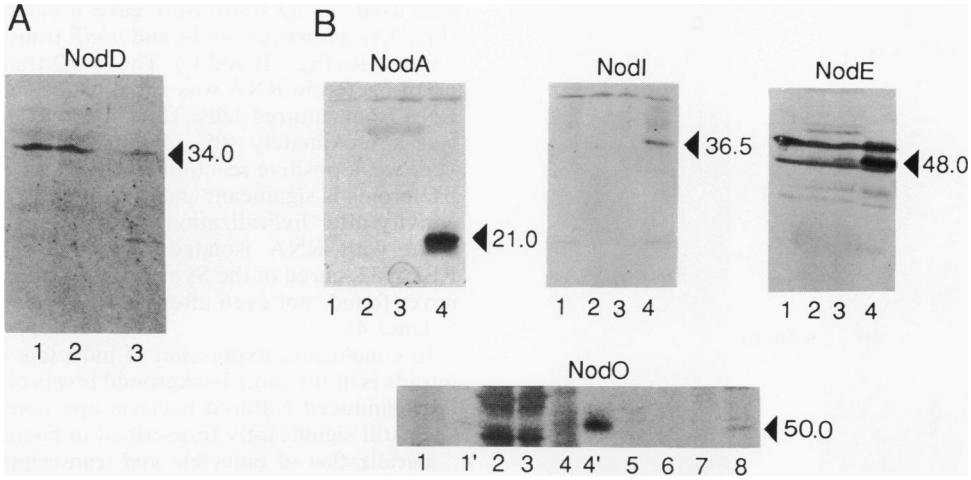

FIG. 3. Immunological detection of Nod proteins in cultured bacteria and bacteroids of *R. leguminosarum* bv. viciae. (A) Occurrence of NodD protein in wild-type strain 248. Lanes: 1 and 2, induced and noninduced cultured bacteria, respectively; 3, bacteroids. The protein band visible under the indicated NodD protein is its stable degradation product, with a relative mobility of 23 kDa. With bacteroid preparations of strains RBL1402(pMP280) and RBL1402(pMP604), the same results were found. (B) Occurrence of inducible *nod* gene products in cultured bacteria of wild-type strain 248, grown in the absence (lane 1) or presence (lane 4) of naringenin, and in bacteroids of strains RBL1402(pMP280) (lane 2) and RBL1402(pMP604) (lane 3). Lanes 1′ and 4′ contained proteins from the growth medium of cultured bacteria used to test for the presence of NodO protein. Fractions of nodules harboring strain RBL1402(pMP604) represent nodule supernatants containing symplast (lane 5), peribacteroid membrane (lane 6), proteins of peribacteroid space (lane 7), and bacteroids surrounded by the peribacteroid membrane (lane 8). Bacteroid preparations of wild-type strain 248 showed indistinguishable results. The apparent molecular masses of the Nod proteins as estimated by their migration rates in SDS-polyacrylamide gel electrophoresis are indicated in kilodaltons. The amounts of total cell protein loaded per lane were 20 μg for cultured bacteria and 85 μg for bacteroids. Nonspecific bands were also made visible by using preimmune serum.

of the same order as those of the *nod* genes, and (iii) the gene is probably transcribed only in bacteroids (25, 39).

In Northern blot hybridization experiments, a strong signal was obtained, indeed, with a *nifA* probe in RNA preparations from pea nodules while no signal was obtained with RNA isolated from cultured bacteria (Table 2, line 1). In contrast, with *nodABC* and *nodFE* probes no reaction or only a very weak one was found with pea nodule RNA while strong reactions were found in RNA preparations from induced cultured bacteria (Table 2, lines 2 and 3). Only low amounts of these transcripts were found in noninduced cultured cells, presumably reflecting background promoter activity. With a *nodD* probe, a much weaker signal was found in induced cultured wild-type bacteria than when *nodABC* and *nodFE* probes were used, indicating the presence of lower *nodD* transcript levels (Table 2, line 4). When nodule RNA was analyzed with the *nodD* probe, a very weak positive reaction was found (Table 2). These results indicate that none of the *nod* genes tested is significantly transcribed in nodules.

To check whether the apparent absence of *nod* transcripts could be due to the detection limits of Northern blot hybridization, the more sensitive RNase protection assay, which

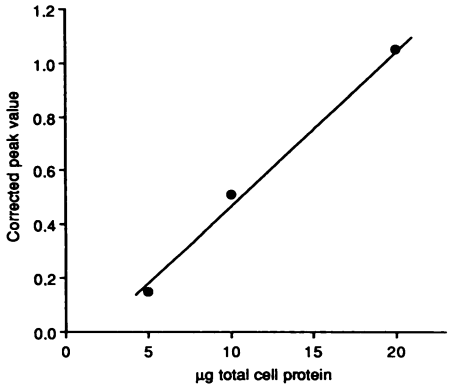

FIG. 4. Determination of the amount of NodE protein in bacteroids. Peak values of the signal on immunoblots containing material derived from cultured cells appeared to be linearly related to the amount of total cell protein, provided that these values were corrected for lane width.

TABLE 2. Quantification of signals on Northern blots[a]

Probe	Amt of total RNA[b] from:		
	Cultured cells[c]		Nodules[d]
	Noninduced	Induced with 1 μM naringenin	
nifA	0.24	0.20	23.52
nodABC	16.34	190*	7.06
nodFE	7.68	195*	2.07
nodD	ND[e]	37.54	1.98

[a] The numbers represent integrals of signals determined by scanning of the autoradiograms in two dimensions.
[b] Wild-type *R. leguminosarum* bv. viciae 248 was used.
[c] Five-microgram samples were used.
[d] Gels could be maximally loaded with 16 μg of RNA without overloading. Quantitative comparison of the numbers in column 3 with those in columns 1 and 2 may not be appropriate, since in samples of total nodule RNA, RNAs of bacteroid and plant origins were present. Values are corrected for background absorbance, and those marked with an asterisk are not absolute (too low) because in these cases the strength of the signal is not linear with exposure time.
[e] ND, not done.

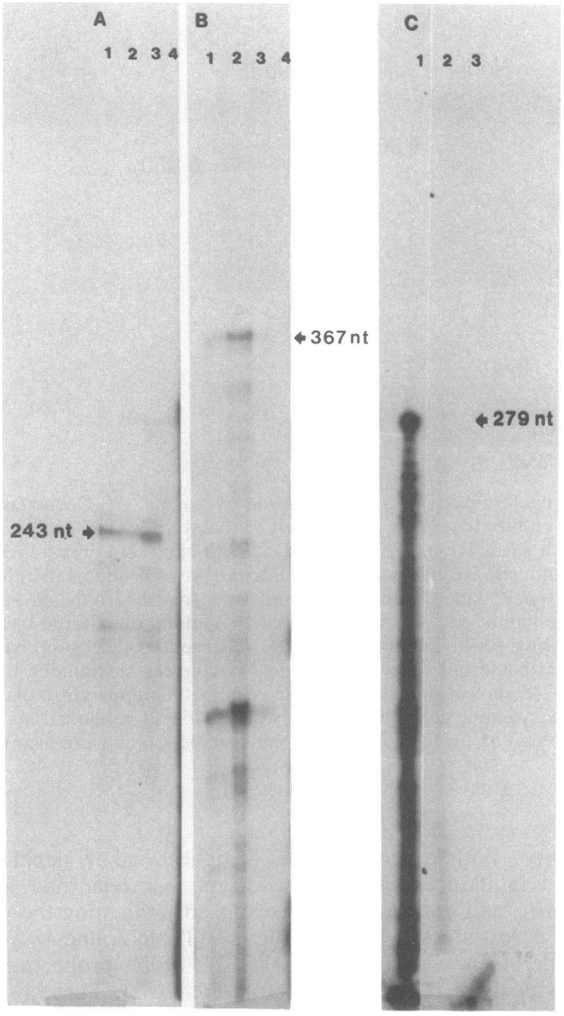

FIG. 5. Detection of *nod* transcripts in wild-type *R. leguminosarum* bv. viciae 248 by using the RNase protection assay with *nodD* (A) and *nodA* (B) probes. Lanes: 1, bacteroid RNA (35 μg); 2, RNA of induced cultured bacteria (3 μg); 3, nodule RNA (75 μg); 4, RNA of induced cultured bacteria of strain RBL1532, cured of the Sym plasmid (5 μg). (C) The *nodF* probe. Lanes were loaded with RNA isolated from induced cultured bacteria (lane 1, 3 μg), from nodules (lane 2, 75 μg), and from induced cultured bacteria of strain RBL1532 (lane 3, 5 μg). This autoradiogram was overexposed for lane 2 to visualize the weak signal of lane 2. The positions of the incomplete *nod* transcripts complementary to the in vitro-synthesized antisense RNAs are indicated. RNAs isolated from nodules or bacteroids of strains RBL1402(pMP604) and RBL1402(pMP280) gave identical results. nt, nucleotides.

allows detection of one to five RNA copies per cell (41), was used. To maximize sensitivity, very highly labeled small probes were used and RNA isolated from either pea nodules or isolated bacteroids of wild-type *R. leguminosarum* bv. viciae 248 was analyzed. Both preparations were compared with RNA isolated from induced cultured bacteria. The occurrence of *nodA*, *nodD*, and *nodF* transcripts was tested by using antisense RNAs containing 100% homology over 367, 243, and 279 nucleotides, respectively. The three *nod* transcripts gave a strong signal with 3 μg of RNA derived from induced cultured bacteria (Fig. 5). When a 25-fold excess of nodule RNA or a 12-fold excess of bacteroid RNA was used, *nodD* transcripts gave a clear positive reaction (Fig. 5A). However, *nodA* and *nodF* transcripts were hardly detectable (Fig. 5B and C). The *nodD* transcript signal in 35 μg of bacteroid RNA was equal to the signal from 15 μg of RNA from cultured cells. Thus, the level of *nodD* expression was approximately 40% of that in cultured bacteria. The very weak positive reaction of *nodA* and *nodF* transcripts in bacteroids is significant and is not due to incomplete RNase activity after hybridization, because in the control experiment with RNA isolated from cultured cells of strain RBL1532, cured of the Sym plasmid, a positive reaction was never found, not even after prolonged exposure times (Fig. 5, lanes 4).

In conclusion, expression of inducible *nod* genes in bacteroids is at the same background levels observed with RNA of noninduced cultured bacteria and *nodD* is the only *nod* gene still significantly transcribed in bacteroids.

Localization of inducible *nod* transcripts in nodules of *V. hirsuta*. To investigate where within the nodule switch off of the inducible *nod* genes occurs, in situ RNA hybridizations were performed. By using antisense *nodC* and *nodE* RNA probes on a section of *V. hirusuta* nodules harboring wild-type strain RBL5560, it was found that both *nodABCIJ* and *nodFEL* transcripts were relatively abundant in the invasion zone. The amount of these transcripts declined very rapidly in the early symbiotic zone, where the bacteria are released from the infection thread, and they were not visible in the late symbiotic zone, not even after 4-weeks of exposure (Fig. 6). With sense *nodC* and *nodE* RNA probes, no signal was found (data not shown), indicating that the signal observed was not due to hybridization with the DNA of the bacteria. Identical results were obtained with sections of *P. sativum* nodules containing strain 248 (data not shown). The *nifA* transcript was easily detectable in infected cells of the late symbiotic zone but not in the invasion zone of both *V. hirsuta* and *P. sativum* nodules (62). Since infection threads are present in the invasion zone only (Fig. 6A), the data indicate that *nodABCIJ* and *nodFEL* are still transcribed in the infection thread and that switch off of the inducible *nod* gene occurs before the bacteria differentiate into bacteroids.

By what mechanism are *nod* genes switched off? To test whether the reduced *nod* gene expression in nodules is due to the absence of inducer molecules or the inaccessibility of inducers for NodD protein, proteins of bacteroids of *R. leguminosarum* RBL1402(pMP604) were analyzed, since it has been reported that the NodD protein encoded by pMP604 activates inducible *nod* gene expression, even in the absence of flavonoid inducers (52). Pea plants inoculated with RBL1402(pMP604) or control strain RBL1402(pMP280) were nodulated as efficiently as when they were inoculated with wild-type strain 248 (data not shown). Additionally, and in agreement with the observation of improved nitrogen fixation on *V. sativa* plants (50), it was found that the sprout dry weight per plant was significantly 5 to 10% higher for those infected with RBL1402(pMP604) than those infected with RBL1402(pMP280). The results of the analyses of Nod proteins of bacteroids of strain RBL1402(pMP604), low *nodD* expression and no expression of the inducible *nod* genes, were indistinguishable from those found for wild-type strain 248 and strain RBL1402(pMP280) (Fig. 3B, lanes 2 and 3). In strain RBL1402(pMP280), which harbors more than one copy of *nodD* per cell, a fivefold higher NodD protein concentration was measured (data not shown). In conclusion, neither a constitutively activated NodD protein nor a higher copy number of wild-type *nodD* resulted in increased levels of inducible *nod* gene products in bacteroids. Addi-

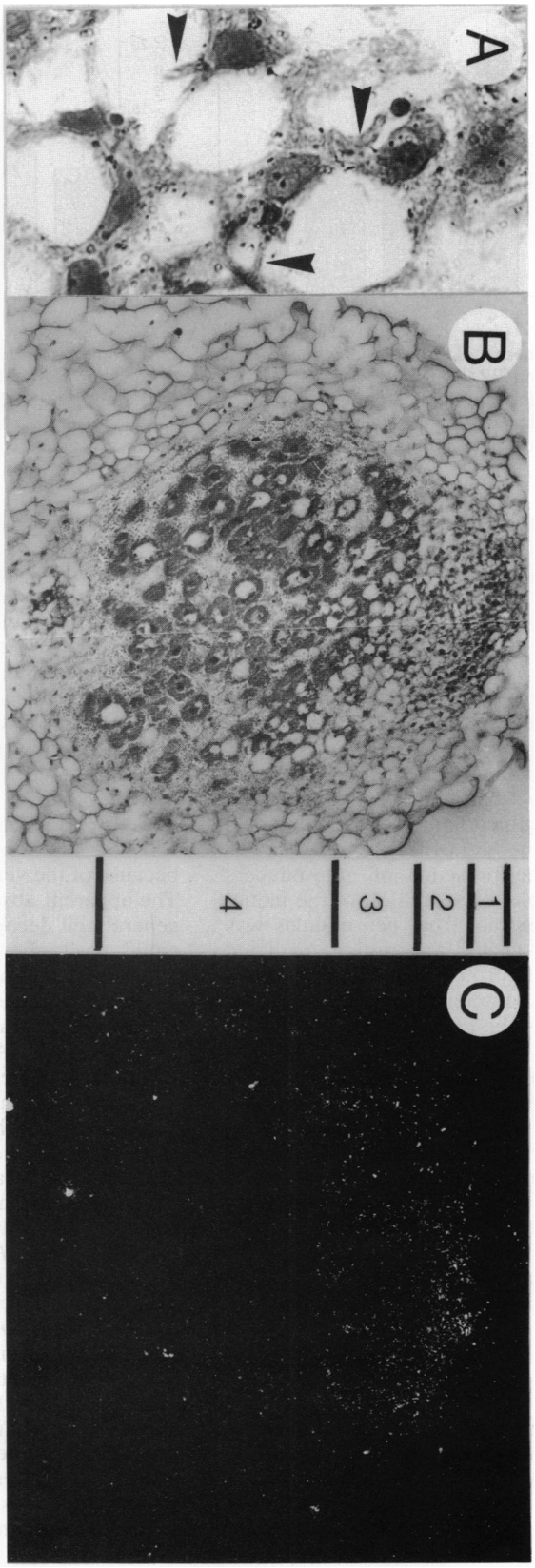

FIG. 6. Localization of the *nodABCIJ* transcript in a 15-day-old nodule of *V. hirsuta* containing *R. leguminosarum* RBL5561(pMP604) by RNA in situ hybridization. (A and B) Bright-field micrographs. Indicated are: 1, the meristem; 2, the invasion zone; 3, the early symbiotic zone; 4, the late symbiotic zone. Panel A shows an enlargement of a part of the invasion zone in which the infection threads are indicated by arrowheads. (C) Dark-field micrograph of a section hybridized with antisense *nodC* RNA in which the silver grains are visible as white dots. Sections hybridized with the *nodE* probe were indistinguishable. Nodules containing wild-type strain RBL5560 as well as hybridization on pea nodules gave identical results.

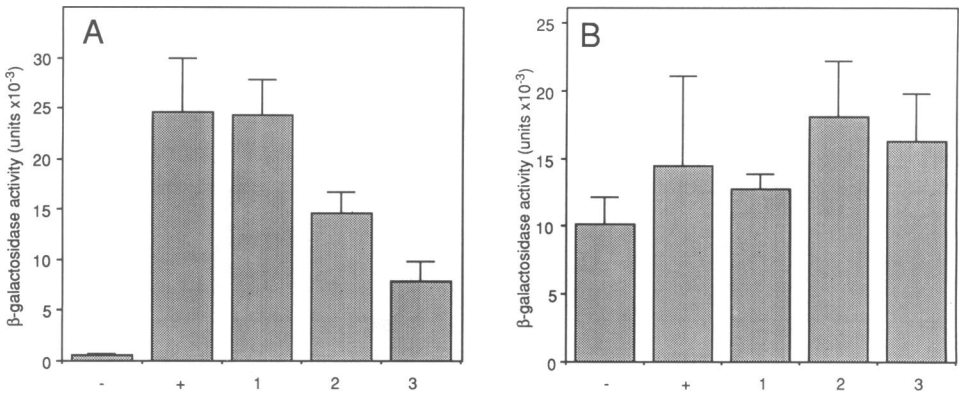

FIG. 7. Influence of nodule methanol extract on induced transcription from the *nodA* promoter in *R. leguminosarum* measured as β-galactosidase activity. (A) Strain LPR5045(pMP280, pMP154) containing wild-type *nodD*. (B) Strain LPR5045(pMP604, pMP154) containing FITA *nodD604*. Supplements to the growth medium: −, nothing; +, 90 nM naringenin; 1, 2, and 3, 0.006, 0.03, and 0.15% (vol/vol) nodule methanol extract, respectively, in addition to 90 nM naringenin. The β-galactosidase units are averages of at least three independent experiments in which values were measured in duplicate. Standard deviations are indicated only in the positive direction.

tionally, by using the RNase protection assay with bacteroid RNA from strain RBL1402(pMP604) it was found that the inducible *nod* genes *nodABC* and *nodFE* were not transcribed in bacteroids isolated from pea nodules (data not shown). This result was confirmed by in situ RNA hybridization on sections of nodules from another host plant as well. Analysis of *V. hirsuta* nodules containing strain RBL5561(pMP604) showed the presence of both *nodABCIJ* and *nodFEL* transcripts in the invasion zone, but no signal was detected in infected cells harboring bacteroids (Fig. 6). Therefore, FITA NodD604 behaves like wild-type NodD with respect to transcription activation of the inducible *nod* genes within the nodule, indicating that absence of inducers does not cause switch off of the *nod* genes.

To investigate whether the presence of anti-inducers within the nodule is responsible for switch off of the inducible *nod* genes, a methanol extract from pea nodules was tested for inhibitors of *nod* gene transcription mediated through either wild-type NodD protein or FITA NodD604. Transcription from the *nodA* promoter in a strain containing wild-type *nodD* is inhibited by the nodule methanol extract in a concentration-dependent way (Fig. 7A). Addition of a 0.15% (vol/vol) concentration of the extract to the growth medium resulted in only 30% induction. In a *Rhizobium* strain harboring *nodD604*, no inhibition of transcription from the *nodA* promoter was observed, however (Fig. 7B). This latter result is consistent with previous data which showed that *Rhizobium* strains containing FITA *nodD604* were insensitive to all tested commercial anti-inducers for positive activation of the inducible *nod* genes (50). Our present results, therefore, indicate that switch off of the inducible *nod* genes within nodules is not due to the presence of anti-inducing compounds.

DISCUSSION

The inducible *nod* genes are switched off in bacteroids. Many Sym plasmid-localized *nod* genes are essential in the early stages of symbiosis, but it is still unknown whether they also play a role in later stages of this process. As a direct approach, we tested the presence of Nod proteins and *nod* transcripts in bacteroids. It was found that the levels of the inducible Nod proteins NodA, NodI, NodE, and NodO were reduced at least 14- to 18-fold in bacteroids. In contrast, NodD protein was reduced only two- to threefold (Fig. 3). Although bacteroids have approximately a sevenfold larger volume than bacteria, the protein concentrations in the two types of cells appeared to be comparable. The concentrations of the essential proteins EF-Tu and EF-Ts, measured as controls, were found to be equal in free-living bacteria and in bacteroids (Fig. 2). Therefore, the observed decrease in levels of Nod proteins in bacteroids represents a true decline of expression.

Transcription of inducible *nod* genes was determined on the RNA level by using *nodABC* and *nodF* probes, and neither of these genes was found to be expressed above background levels in bacteroids. Although steady-state levels of RNA were measured, this conclusion is justified because of the very short half-life of prokaryotic transcripts. The apparent absence of these *nod* transcripts is not due to general cell decay because both *nifA* and *nodD* transcripts could easily be detected by Northern blot and in situ hybridizations and in an RNase protection assay, respectively. It is unlikely that the very weak positive signals of *nodA* and *nodF* are caused by contaminating chromosomal DNA in the RNA preparation, because in the RNase protection assays the hybridization conditions were so stringent that DNA-RNA hybrids could hardly stabilize (7). Thus, it is more likely that the weak positive signals of *nodA* and *nodF* are due to either contaminating bacteria, i.e., ca. 5% of the bacteroid preparation, or background transcription. This conclusion is confirmed by the results of the in situ RNA hybridization. Although the other inducible *nod* operons, *nodMNT* and *nodO*, have not been tested on the RNA level, the absence of NodO protein in nodules, as well as former data (11, 13, 49), indicates that these genes are regulated in the same way as *nodABCIJ* and *nodFEL*. In conclusion, the inducible *nod* genes of *R. leguminosarum* bv. viciae are not transcribed in later stages of symbiosis and consequently have been switched off. Data obtained by in situ RNA hybridization indicate that switch off of the inducible *nod* genes occurs after the formation of infection threads but before the bacteria differentiate into bacteroids (Fig. 6). Since only a weak, diffuse *nodABCIJ* and *nodFEL* transcript signal is visible in the early symbiotic zone, it is likely that expression of the inducible *nod* genes terminates just before the bacteria are released from the infection threads. A similar result has been reported recently for *R. meliloti*,

although the alfalfa nodules were divided only in a meristematic and a central zone (47).

Our data from the protein analyses and from the RNase protection experiments are in agreement, since they both indicate that *nodD* expression in bacteroids is reduced two- to threefold. This result was not confirmed by the data from the Northern hybridization. However, since the total nodule RNA preparation also contains plant RNA besides RNA of bacteroid origin, it may not be appropriate to compare the data in the last column of Table 2 with those of the first two columns, for which RNA from cultured bacteria was used. Apparently, the situation for *nodD* transcription in nodules of *R. leguminosarum* bv. viciae differs from the situation in *R. meliloti*, in which transcription of *nodD1* and *nodD3* is decreased manyfold in older alfalfa nodules (47).

Possible mechanisms for switch off of the inducible *nod* genes include activities of inducers or anti-inducers and the role of NodD protein.

The inducible *nod* genes are not switched off because of lack of inducers or the presence of anti-inducers. The NodD604 protein encoded by FITA-type *nodD* is, in its activation of inducible *nod* genes, insensitive to the presence or absence of inducing flavonoids or anti-inducers (50). Since expression of inducible *nod* genes was also not found in bacteroids of a *Rhizobium* strain containing *nodD604* (Fig. 3B), it is very likely that these genes are not switched off because of the absence of inducing flavonoids. It was found that pea nodules contain inhibitors of *nod* gene transcription (Fig. 7A). Since transcription from the *nodA* promoter was not inhibited in the presence of NodD604 by a methanol extract from nodules which contains the inhibitors, it is very likely that these anti-inducers are not responsible for switch off of the inducible *nod* genes.

The inducible *nod* genes are likely not switched off because of limiting levels of NodD protein. Because NodD protein is the transcriptional activator of the inducible *nod* genes, it is feasible that in bacteroids concentrations of NodD protein too low to induce transcription of the other *nod* genes are present. By raising the copy number of *nodD*, it has been shown for *nodO* of *R. leguminosarum* bv. viciae (11) and *nodC* of *R. meliloti* (33) that the rate of *nod* gene expression increases. No data are available, however, about inducing capacity under conditions of decreased levels of NodD protein. This question makes sense, since our data presented in this report, as well as previous observations (47), indicate that the constitutively expressed *nodD* gene is also negatively regulated in bacteroids. In cultured cells of wild-type *R. leguminosarum* bv. viciae 248, only low amounts of NodD protein are present (43) and in bacteroids the amount is reduced approximately 65% further (Fig. 3A). This is in agreement with the two- to threefold reduction in the level of *nodD* transcripts (Fig. 5A). However, in bacteroids of a strain harboring *nodD* on a plasmid with a copy number of about five, the NodD protein concentration is approximately as high as in a free-living wild-type cell. In these bacteroids, the inducible Nod proteins and transcripts were also absent. Therefore, it is unlikely that limitation of NodD protein in bacteroids is the cause of switch off of the inducible *nod* genes.

The mechanism by which the inducible *nod* genes are switched off during development of symbiosis, therefore, is still not well understood. It may involve either factors of a physiological nature or a repressing transcriptional regulator. Moreover, negative regulation of *nodD* transcription in bacteroids is an intriguing phenomenon, since this gene has always been viewed as the only *nod* gene transcribed constitutively. Whether the same mechanism is responsible for reduced expression of *nodD* and the inducible *nod* genes in bacteroids is unknown.

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