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 nodII, 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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. leguminosarum 248</td>
<td>R. leguminosarum bv. vicieae wild type</td>
<td>26</td>
</tr>
<tr>
<td>RBL1532</td>
<td>248 RIf’ Spc’ cured of Sym plasmid pRLJ1</td>
<td>9</td>
</tr>
<tr>
<td>RBL1402</td>
<td>248 pRLJ1 nodD2: Tn5</td>
<td>61</td>
</tr>
<tr>
<td>LPR5045</td>
<td>R. leguminosarum bv. trifoli</td>
<td>23</td>
</tr>
<tr>
<td>RBL5560</td>
<td>LPR5045 with Sym plasmid pRLJ1</td>
<td>65</td>
</tr>
<tr>
<td>RBL5561</td>
<td>LPR5045 with pRLJ1 nodD2: Tn5</td>
<td>65</td>
</tr>
<tr>
<td>E. coli KMBL1164</td>
<td>Δ(lac-pro) supE thi F’</td>
<td>van der Putte</td>
</tr>
<tr>
<td>JM101</td>
<td>Δ(lac-pro) supE thi (F’traD6 proAB lacPZΔM15)</td>
<td>63</td>
</tr>
<tr>
<td>DH5αF’</td>
<td>Δ(lacZΔgα1) supE thi recA1 lacPZΔM15</td>
<td>Promega</td>
</tr>
</tbody>
</table>

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 nodFE | 51 |
| pMP2010 | IncColE1 carrying nodDn0dF | This study |
| pMP2020 | Bluescript vector carrying nodDn0dF | This study |
| pMP2023 | Bluescript vector carrying 5’ part of nodD | This study |
| pMP2024 | Bluescript vector carrying nodA sequences | This study |
| pT7BB | IncColE1 carrying fixC”NifAB’ of pSymPRE | 39 |

* All nod sequences originated from pRLJ1, 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 RBL5560 were used as hosts for plasmids pMP604, containing FITA-type nodD604 (52), and pMP280 (53). Escherichia coli JM101 and KMRL164 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 rhizobial, and cultured on gravel by published procedures (35). P. sativum was inoculated with R. leguminosarum bv. vicieae 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 Katanakis et al. (27), except that the isolation buffer was 0.6 M sucrose-50 mM morpholine propane sulfonic acid (MOPS) (pH 7.5)-2.5 mM MgCl2-10 mM KCl-1 mM dithiothreitol-4% (wt/vol) polyvinylpyrrolidone-5 mM p-aminobenzamide. 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 A620 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-1-300 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 per in2. Subsequently, the sucore was diluted to 7% (wt/vol), the debris was removed by centrifugation for 20 min at 1,000 x g, and the cleared extract 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 smedey 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. Maximaly 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 Nod 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-
ment with 1 μM naringenin to an \( \Delta \text{G}_{\text{m}} \) 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 Katnakis 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 MgCl₂–10 mM KCl–1 mM dithiothreitol–4% (wt/vol) polyvinylpyrroli- done–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 p17 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). Complete antisense transcripts of nodF and nodD were obtained from pMP2020 linearized with SmaI and from pMP2023 linearized with BglII, respectively, by using T7 RNA polymerase. By using T3 RNA polymerase and pMP2024 linearized with HindIII, 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-32P]UTP (3,000 Ci mmol⁻¹) with no addition of unla- beled 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 × 10⁶ to 1 × 10⁶ 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 Velde et al. (59). Seven-micrometer-thick sections were hybridized with partly degraded 35S-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 HindIII, 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 BamHI 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 XhoI and EcoRI, respectively, both being the polycloning vectors. The nifA probe was made by T7 RNA polymerase of the Xbal-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.
immunoblots reacting proteins of bacteroids confirmed protein, had the amounts of living approximately of chosen living E. coli were 4280 SCHLAMAN United (Poole, Kingdom), were RNA and gel with Boehringer (Mannheim, Germany). Restricted enzymes, RNase inhibitor, and RNA molecular weight markers were purchased from Boehringer (Mannheim, Germany). Radioactive nucleotides were obtained from Amersham International plc (Amer-
sham, 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 concentra-
tion 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 cul-
tured 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, corre-
sponding 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 con-
taminated 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 anal-
yses 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 compa-
parable to that of the nod genes, (ii) the size of the transcript is
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 nodule.

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

<table>
<thead>
<tr>
<th>Probe</th>
<th>Noninduced</th>
<th>Induced with 1 μM naringenin</th>
<th>Nodules</th>
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</thead>
<tbody>
<tr>
<td>nifA</td>
<td>0.24</td>
<td>0.20</td>
<td>23.52</td>
</tr>
<tr>
<td>nodABC</td>
<td>16.34</td>
<td>190*</td>
<td>7.06</td>
</tr>
<tr>
<td>nodFE</td>
<td>7.68</td>
<td>195*</td>
<td>2.07</td>
</tr>
<tr>
<td>nodD</td>
<td>ND*</td>
<td>37.54</td>
<td>1.98</td>
</tr>
</tbody>
</table>

* The numbers represent integrals of signals determined by scanning of the autoradiograms in two dimensions.
* Wild-type *R. leguminosarum* bv. viciae 248 was used.
* Five-microgram samples were used.
* 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.
* ND, not done.
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. hirsuta* 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 of bacteroids or *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 expression of nod protein was increased in 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-
modules gave identical results. Sections hybridized with the mock probe were indistinguishable. Nuclei containing signal hybridized with mock RNA in which the T7 promoter are visible as white dots. Sections hybridized with the T7 probe were indistinguishable. Nuclei containing signal hybridized with T7 RNA in which the T7 promoter are indicated by arrowheads. (A) Dark-field micrograph of a section hybridized with antisense T7 RNA. Panel A shows an enlargement of a part of the invasion zone in which the infection threads are indicated by arrowheads. (B) Bright-field micrograph. Indicated are: 1. The attachment zone; 2. the invasion zone; 3. the early symbiotic zone; 4. the late symbiotic zone. Panel C shows in hybridization of a sample containing signal RNA in which the T7 promoter are indicated by arrowheads.
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 nodABCJ 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 Syn 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 nipA 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 nodD 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 nodABCJ and nodFEL. In conclusion, the inducible nod genes of R. leguminosarum bv. vicieae 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 nodABCJ 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 mer-
istematically 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-
tothreefold. 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 expres-
sion 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
are 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 nega-
tively 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 approxi-
mately 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 regula-
tor. 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 con-
stitutively. Whether the same mechanism is responsible for
reduced expression of nodD and the inducible nod genes in
bacteroids is unknown.

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SUPPRESSION OF NODULATION GENE EXPRESSION


