JOURNAL OF BACTERIOLOGY, July 1989, p. 4045–4053
0021-9193/89/074045-09$02.00/0

Vol. 171, No. 7

Symbiotic Properties of Rhizobia Containing a Flavonoid-Independent Hybrid *nodD* Product

HERMAN P. SPAINK, ROB J. H. OKKER,* CAREL A. WIJFFELMAN, TEUN TAK, LEENTJE GOOSEN-DE ROO, ELLY PEES, ANTON A. N. VAN BRUSSEL, AND BEN J. J. LUGTENBERG

Department of Plant Molecular Biology, Leiden University, Nonnensteeg 3, 2311 VJ Leiden, The Netherlands

Received 23 December 1988/Accepted 24 April 1989

A hybrid *nodD* gene consisting of 75% of the *nodD1* gene of *Rhizobium meliloti* at the 5′ end and 27% of the *nodD* gene of *Rhizobium trifolii* at the 3′ end activates the six tested inducible *nod* promoters of *Rhizobium leguminosarum*, *R. trifolii*, or *R. meliloti* to maximal levels, even in the absence of flavonoids. In strains containing such a constitutive activating *nodD* gene, transcription of *nod* genes started at the same site as in flavonoid-induced strains containing a wild-type *nodD* gene. In contrast to heterologous wild-type *nodD* products, the constitutive activating *nodD* gene does not cause a limitation of the host range. Furthermore, *R. leguminosarum*, *R. trifolii*, and *R. meliloti* strains containing the constitutive activating *nodD* gene induce (pseudo) nodules on tropical leguminous plants. Comparison of the symbiotic properties of rhizobia containing the constitutive *nodD* hybrid gene with those of rhizobia containing various wild-type *nodD* genes indicates that the activation of the *nodD* product by flavonoids is of crucial importance during the process of infection thread formation and, surprisingly, also during nitrogen fixation.

Bacteria of the genera *Rhizobium* and *Bradyrhizobium* are able to establish a symbiosis with leguminous plants, resulting in root nodules. In these root nodules a differentiated form of the bacterium, the bacteroid, is present which is able to fix atmospheric nitrogen. The processes of nodulation and nitrogen fixation are highly host specific in that a particular bacterial species can only form nitrogen-fixing nodules on a limited number of host species. The symbiosis (Sym) plasmid-localized nodulation (*nod*) genes of *Rhizobium* spp., which are crucial for nodulation and which determine the host specificity of nodulation (3, 5, 6, 15, 38), are expressed only in the presence of the constitutively expressed positive regulatory *nodD* gene and of an inducer exuded by the host roots (16, 21, 25, 29, 30). The inducing compounds have been identified as flavones, flavanones, or closely related compounds (8, 22, 23, 40). Also, substances have been identified which inhibit induction of *nod* genes (4, 8).

The *nodD* gene is highly conserved within *Rhizobium* spp. Several *Rhizobium* species harbor more than one *nodD* gene (1, 11, 12, 24). Recently it has been shown that the *nodD* gene can be a determinant of host specificity, presumably as a result of its interaction with specific sets of flavonoids in a species-specific way (14, 32, 39). A class of hybrid *nodD* genes, constructed by in vivo homologous recombination and consisting of a 5′ part of the *nodD1* gene of *Rhizobium meliloti* and a 3′ part of the *nodD* gene of *Rhizobium trifolii*, appeared to activate the inducible *nodABCIJ* promoter of *Rhizobium leguminosarum* to the maximal level, even in the absence of a flavonoid inducer (31). In this paper we describe the properties of *nodD604*, a representative of this class of hybrids. The possession of the *nodD604* gene instead of the appropriate wild-type *nodD* gene appeared to confer different symbiotic properties to *Rhizobium* species in that (i) in contrast to heterologous wild-type *nodD* genes, it does not cause a limitation of the host range and (ii) it causes a substantial improvement in the nitrogen-fixing abilities of *Rhizobium* species of two tested cross-inoculation groups.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1. Plasmids were mobilized from *Escherichia coli* KMBL1164 to *Rhizobium* spp. as described previously (30), using pRK2013 as a helper plasmid. Selection against the *E. coli* donor strains was performed using 20 mg of piperacillin (Pipcil; DG Lederle, Etten-Leur, The Netherlands) per liter, an antibiotic to which *Rhizobium* bacteria are naturally insensitive.

Construction of transcription indicator strains. Upstream regions of inducible *nod* genes of *R. leguminosarum*, *R. trifolii*, and *R. meliloti* were cloned in the transcriptional indicator IncQ plasmid pMP190 (30), which allows detection of promoter activity as β-galactosidase activity. The cloned regions and the restriction sites used are indicated in Fig. 1A. With the resulting plasmids pMP154, pMP168, pMP250, pMP194, pMP193, and pMP295, 24 *Rhizobium* strains, representing the combinations of the six inducible *nod* promoters with each of the three wild-type *nodD* genes and the hybrid *nodD604* gene, were constructed. These wild-type *nodD* genes are present on the IncP plasmids pMP280, pMP283 (32), and pMP261 for *R. leguminosarum*, *R. trifolii*, and *R. meliloti* (the *nodD1* gene), respectively (Fig. 1A). The hybrid *nodD604* gene is present in the IncP plasmid pMP604 (31). The bacterial induction test strain used as the acceptor was *R. trifolii* LPR5045 (Table 1).

Induction assays. Assays for β-galactosidase activity were performed as described previously (30). Each test was performed in triplicate, and variation of the expression levels was within 10%. To test inhibition of induction, the isoflavones daidzein and genistein (obtained from Sarsyntex, Merignac, France) and the coumarin umbelliferone (obtained from Sigma Chemical Co., St. Louis, Mo.) were added in a concentration of 400 nM. At this concentration, these compounds inhibited *nod* gene expression by 50 to

TABLE 1. Bacterial strains

Strain	Relevant characteristics	Source or reference
E. coli KMBL1164	Δ(*lac-pro*) *thi* F⁻	P. van der Putte
Rhizobium spp.		
LPR5045	*R. trifolii* RCR5, cured of Sym plasmid, Rif^r	13
RBL5561	LPR5045 with *R. leguminosarum* Sym plasmid pRL1JI, *nodD*::Tn*5*	38
ANU851	*R. trifolii* ANU843 with *nodD*::Tn*5*	5
RmD1D2D3-1	*R. meliloti* Rm1021 with *nodD1*::Tn*5*, *nodD2*::Tm^a, *nodD3*::Sp^b	12
Bradyrhizobium sp. strain 32H1	Wild-type *Bradyrhizobium* sp. isolated from cowpea	34

^{a} Gene encoding trimethoprim resistance.
^{b} Gene encoding spectinomycin resistance.

80% with the wild-type *nodD* products in the presence of 400 nM luteolin (obtained from Carl Roth, Karlsruhe, Federal Republic of Germany). Plant extracts were prepared from 7-day-old *Vicia sativa*, *Trifolium repens*, and *Trifolium pratense* plants using boiling methanol. These extracts, which contain a variety of phenolic compounds present in the plant, were tested for their influence on the *nod* gene expression in the presence of the *nodD604* gene. The extracts were tested at a concentration at which they were able to induce the *nodABCIJ* promoter of *R. leguminosarum* to high levels in the presence of the *R. trifolii nodD* gene and in a fivefold-higher concentration.

Determination of transcriptional start sites. DNA fragments containing the upstream regions of the *R. leguminosarum nodABCIJ* and *nodFE* operons are present in the M13 phages MPM7 and MPM11. The *Bgl*II restriction fragments (Fig. 1B) containing the separate *nodABCIJ* and *nodFE* promoters linked to the M13 primer sequence were cloned in the IncQ vector pMP190, resulting in the plasmids pMP266 and pMP274, respectively (Fig. 1B). These broad-host-range plasmids contain the M13 system primer sequence located at the 3′ end of the cloned inserts, and therefore primer extension experiments with produced fusion mRNA can be performed using the 15-mer M13 sequencing primer (obtained from Boehringer, Mannheim, Federal Republic of Germany). The plasmids pMP266 and pMP274 were mobilized into *R. trifolii* LPR5045 containing either the *R. leguminosarum* wild-type *nodD* gene or the hybrid *nodD604* gene. These *nodD* genes were present on the IncP plasmids pMP280 and pMP604, respectively (Fig. 1A). The resulting *Rhizobium* strains were grown for 8 h in the presence or absence of a 1 μM concentration of the flavone luteolin, a good activator of the wild-type *nodD* product. Subsequently mRNA was isolated according to methods described previously (36). Primer extension experiments were performed according to published methods (18) using ^{32}P-end-labeled DNA primers with the modification that the reverse transcriptase reaction was performed at 25°C, considering the small size of the primer. Polynucleotide kinase and reverse transcriptase were obtained from Promega Biotech (Leiden, The Netherlands), and [γ-^{32}P]dATP was from Amersham International plc (Amersham, U.K.). The sequence ladders shown were obtained using the M13 phage MPM7 or MPM11 (Fig. 1B), which was sequenced with the dideoxy-chain termination method (27) using a ^{32}P-end-labeled primer.

Infection thread and nodulation assays. The formation of infection threads was studied using the methods described by Vasse and Truchet (37). Plant tests were performed as described previously (35). For each *Rhizobium*-plant species combination at least 10 plants were used and nodulation was scored 3 weeks after infection unless indicated otherwise. *Bradyrhizobium* sp. strain 32H1 was used as a positive control for nodulation tests on tropical legumes. This strain nodulated 100% of the tested tropical legumes within 3 weeks after infection. Bacteria were reisolated from root nodules after external sterilization with 96% ethanol and with 0.1% $HgCl_2$. Reisolated bacteria were tested for nodulation ability on *T. repens*, *V. sativa*, and *Melilotus alba* plants. Plant seeds were gifts of the botanical gardens of Leiden (The Netherlands), Caen (France), and Leningrad (USSR), the Zentral Institut für Genetik und Kulturpflanzenforschung (Gutersleben, German Democratic Republic), and the Estaçao Agronomica Nacional (Oeiras, Portugal). Seeds from *V. sativa* subsp. *nigra* and *Trifolium leucanthum* were harvested in this laboratory.

Acetylene reduction tests. At least 30 plants were infected per strain, and acetylene reduction tests were performed on nodulated plants as described previously (35) at various times after infection. As a control on the influence of the test on subsequent nitrogen fixation, acetylene reduction tests were only performed 32 days after infection. Since similar data were obtained, this result indicates that the acetylene treatments did not influence the nitrogen-fixing ability of the test plants.

Electron microscopy. Nodules and nodulelike structures were excised from seedlings 28 days after inoculation. They were fixed as a whole in 2.5% glutaraldehyde–2% paraformaldehyde–0.1 M sodium cacodylate buffer (pH 7.2) at room temperature. The specimens were postfixed in buffered 1% osmium tetroxide, dehydrated in ethanol, and embedded in Epon. Ultrathin sections were poststained with uranyl acetate and lead citrate and examined in a Philips EM300 electron microscope at 60 kV.

RESULTS

Analysis of the constitutive activating phenotype. The *nodD604* gene was tested for its ability to activate six inducible *nod* promoters derived from *R. leguminosarum*, *R. trifolii*, and *R. meliloti*. The levels of activation by *nodD604* were compared with the levels observed in the presence of the homologous *nodD(1)* genes and the flavone luteolin, which is an excellent activator of each of these *nodD* genes (32). *nodD604* was able to activate each tested *nod* promoter in the absence of inducer (Table 2). This level of activation was at least as high as, and in most cases much higher than, the levels reached in the presence of the homologous *nodD* gene and luteolin. In several cases the heterologous wild-type *nodD* genes gave induced activities which were also much higher than with the homologous *nodD* gene. With respect to the level of activation, the *nodD604* gene resembles the activated parental *R. trifolii nodD* gene. The level of transcription caused by *nodD604* was not influenced by the addition of the isoflavonoids daidzein and genistein or the coumarin umbelliferone. These compounds are known as inhibitors of induction of *nod* genes in *R. leguminosarum* and *R. trifolii* (4, 8). Furthermore, methanol extracts containing flavonoids from *V. sativa*, *T. repens*, or *T. pratense* seedlings did not affect *nod* gene transcription in the presence of the *nodD604* gene.

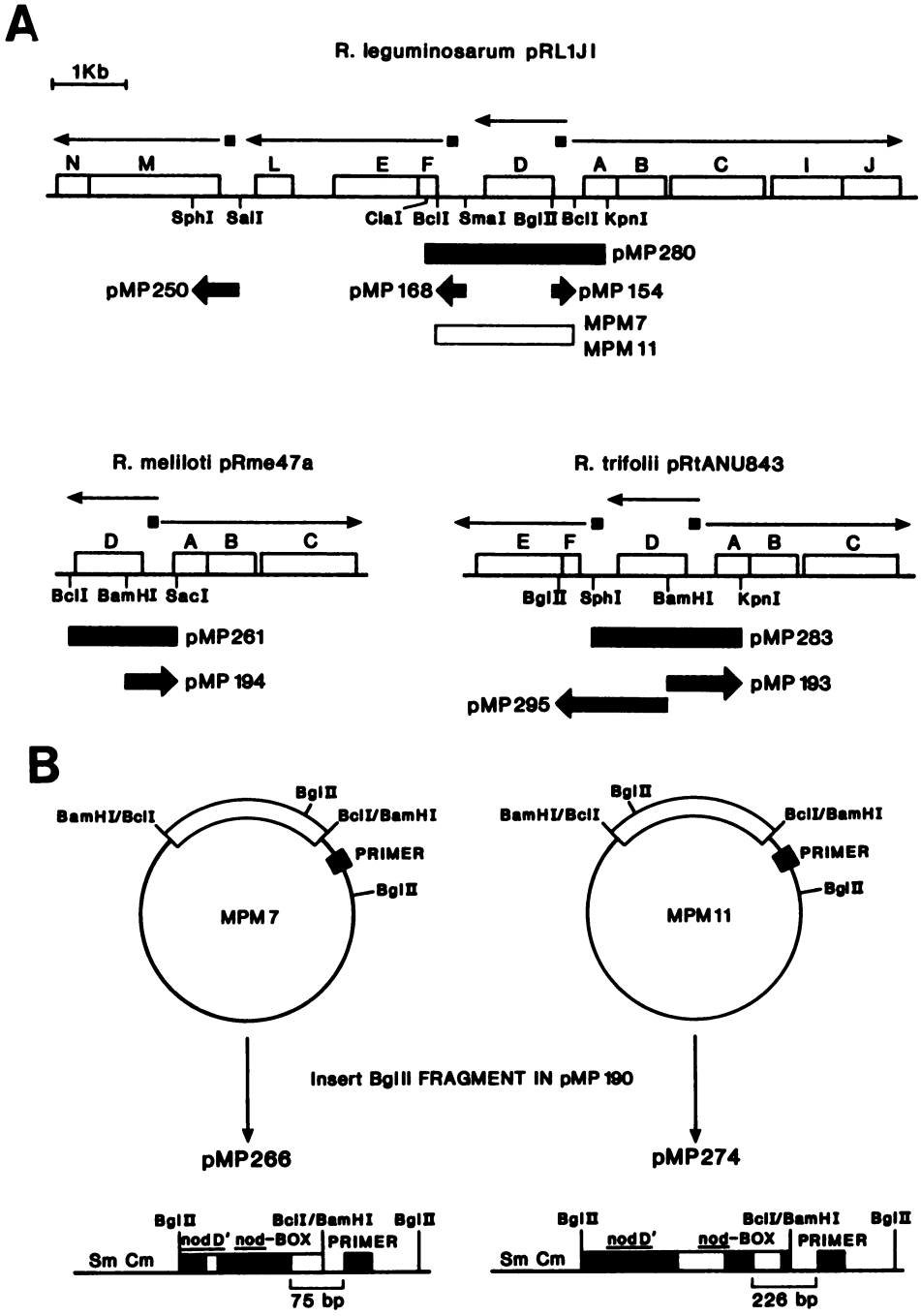

FIG. 1. Construction of plasmids. (A) Restriction fragments derived from the nodulation regions of *R. leguminosarum*, *R. trifolii*, and *R. meliloti* are indicated as black boxes when cloned in the IncP plasmid pMP92 (30), as arrows when cloned in the transcription indicator plasmid pMP190 (30), or as open boxes when cloned in the M13 phage mp8 (20), as indicated in panel B. The size and position of the indicated *nod* genes are according to published data (7, 10, 28, 32, 33) except for the size of the *nodE* gene of *R. trifolii*, which is according to unpublished results. Also indicated, by thin arrows, is the direction of the transcription of these genes (9, 10, 16, 30, 33). *nod* boxes, strongly conserved sequences which precede every inducible *nod* operon (3, 26, 28–30), are indicated by black boxes. (B) Construction of pMP266 and pMP274, used for the determination of transcriptional start sites of *nodABCIJ* and *nodFE* operons, respectively. The open box in MPM7 and MPM11 is the *Bcl*I fragment indicated in panel A, cloned in the *Bam*HI site of mp8. The indicated *Bgl*II fragments containing the *nodABCIJ* and *nodFE* promoters were cloned in the IncQ vector pMP190, resulting in pMP266 and pMP274, respectively.

To study the transcription of inducible *nod* promoters in the presence of a *nodD604* product on a molecular level, primer extension experiments with isolated mRNA were performed. The results (Fig. 2) with the *nodABCIJ* and *nodFE* promoter of *R. leguminosarum* show that *nodD604* indeed was able to activate transcription in the absence of an inducer. The transcriptional start sites used were identical to those with the control wild-type *R. leguminosarum nodD* in

TABLE 2. Expression of inducible *nod* promoters of various *Rhizobium* species in the presence or absence of luteolin with hybrid or wild-type *nodD* genes

Cloned *nod* promoter (*Rhizobium* species)	Relative expression level[a] with various *nodD* genes							
	R. leguminosarum nodD		*R. trifolii nodD*		*R. meliloti nodD1*		Hybrid *nodD604*	
	−	+	−	+	−	+	−	+
nodABCIJ (*R. leguminosarum*)	1	100	3	110	1.5	43	142	130
nodABC (*R. trifolii*)	4	73	5	100	3	23	105	108
nodABC (*R. meliloti*)	19	200	16	286	11	100	287	271
nodFE (*R. leguminosarum*)	9	100	11	227	11	27	253	227
nodFE (*R. trifolii*)	3	30	5	100	2	14	102	98
nodM (*R. leguminosarum*)	4	100	10	180	6	30	180	170

[a] In absence (−) or presence (+) of 400 nM luteolin. β-Galactosidase levels with the tested promoters are presented relative to the expression level in the presence of the homologous *nodD* gene and the inducer luteolin (underlined, 100%). The 100% levels for the promoters, indicated as units of β-galactosidase, were: 20,000 U for *nodABCIJ* of *R. leguminosarum*, 11,000 U for *nodABC* of *R. trifolii*, 7,000 U for *nodABC* of *R. meliloti*, 7,500 U for *nodFE* of *R. leguminosarum*, 10,000 U for *nodFE* of *R. trifolii*, and 5,000 U for *nodM* of *R. leguminosarum*.

the presence of the inducer luteolin. No other transcriptional start sites were present between those visible in the figure and the primer sites of pMP274 (Fig. 2C) and pMP266 (not shown). For the *nodFE* operon, therefore, we can conclude that no other transcriptional start sites are present between the *nod* box and the translational start site of *nodF*. The two transcriptional start sites of the *R. leguminosarum nodFE* operon correspond with two of the four reported transcriptional start sites of the *R. meliloti nodFE* operon (10).

Nodulation properties of rhizobia containing various wild-type *nodD* genes or *nodD604*. The *nodD604* and the *nodD(1)* genes of *R. meliloti*, *R. trifolii*, and *R. leguminosarum* were each mobilized into *R. leguminosarum* and *R. trifolii* strains in which the wild-type *nodD* had been inactivated by a Tn*5* insertion. Each of the resulting two sets of four strains was subsequently inoculated on several plant species belonging to the cross-inoculation group of the particular bacterial species. The results with five representative plant species of each cross-inoculation group (Table 3) show that replacement of the original *nodD* gene of *R. leguminosarum* or *R. trifolii* by a wild-type *nodD* gene of another species narrows the host range. Apparently these wild-type *nodD* genes have evolutionarily adapted to plants of their own cross-inoculation group. One group of *Trifolium* species was further characterized with respect to root hair curling response and infection thread formation (Table 4). The results show that the limitation of the host range by heterologous *nodD* genes is not caused at the level of root hair curling but rather at the level of the formation of the infection thread.

In contrast to the studied wild-type *nodD* genes, the *nodD604* gene can function in the nodulation of every tested nontropical legume host (Table 3). The number of nodules on each plant with *nodD604* was the same as with the homolo-

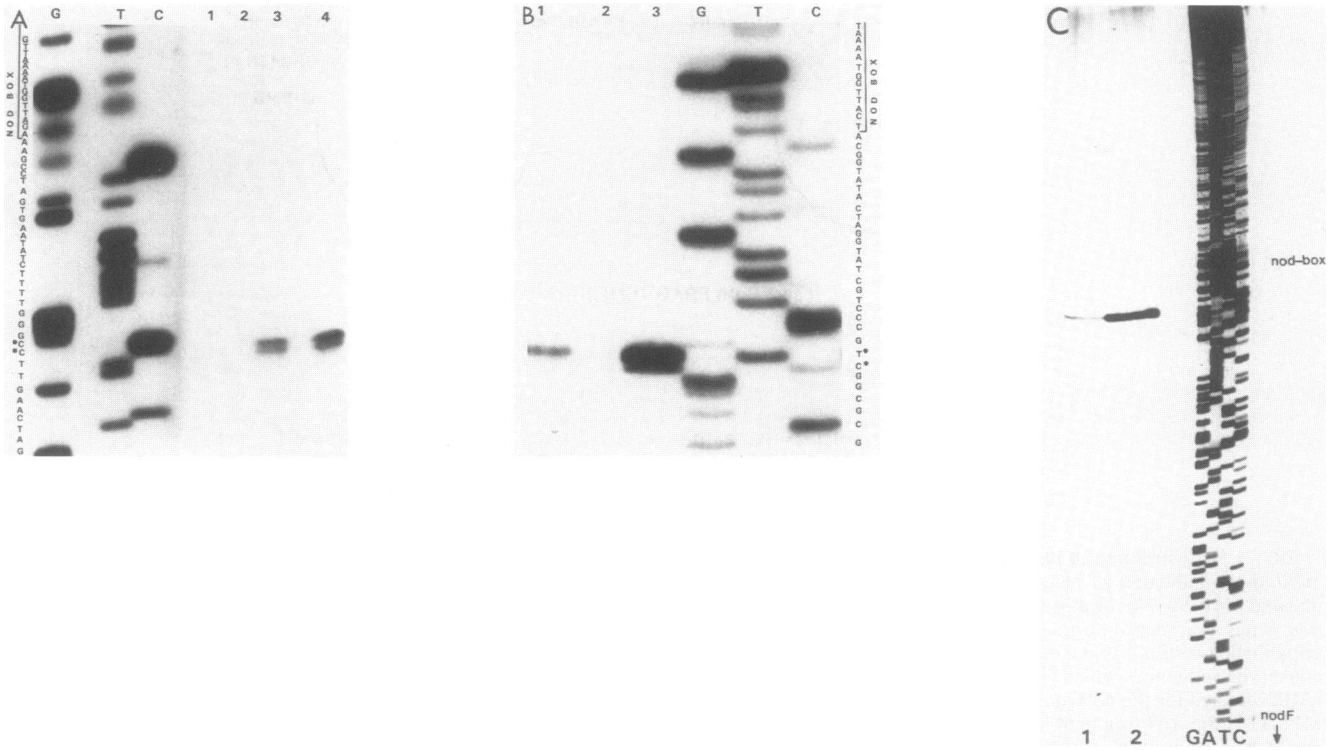

FIG. 2. Comparison of transcriptional start sites of the *R. leguminosarum nodABCIJ* (A) and *nodFE* (B and C) operons in the presence of *nodD604* and the *R. leguminosarum* wild-type *nodD* gene. (A) Lane 1, no *nodD* present, with luteolin; lane 2, *nodD R. leguminosarum* present, no inducer; lane 3, *nodD R. leguminosarum* present, with luteolin; lane 4, *nodD604* present, no inducer. (B) Lane 1, *R. leguminosarum nodD* present, with luteolin; lane 2, *R. leguminosarum nodD* present, no inducer; lane 3, *nodD604* present, no inducer. (C) Lane 1, *R. leguminosarum nodD* present with luteolin; lane 2, *nodD604* present, no inducer. The surrounding sequences of the transcriptional start sites of the *nodABCIJ* and *nodFE* operons, marked by dots, are indicated at the edges of the lanes. Also indicated are the *nod* boxes (see legend to Fig. 1).

TABLE 3. Nodulation of plants of several cross-inoculation groups by *Rhizobium* strains containing hybrid or wild-type *nodD* genes

Rhizobium strain and test plant	Nodulation characteristics[a] (avg no. of nodules per nodulated plant) with each *nodD* gene			
	R. leguminosarum nodD	*R. trifolii nodD*	*R. meliloti nodD1*	Hybrid *nodD604*
R. leguminosarum RBL5561				
Vicia sativa	+ (2)	+ (2)	+ (2)	+ (2)
V. angustifolia	+ (38)	+ (40)	+ (45)	+ (40)
V. lathyroides	+ (5)	+ (4)	−	+ (5)
Lathyrus nissolia	+ (3)	−	+ (8)	+ (5)
Vicia vicoides	+ (8)	+/− (2)	+/− (1)	+ (7)
R. trifolii ANU851				
Trifolium repens	+ (4)	+ (4)	+ (2)	+ (4)
T. lappaceum	+ (3)	+ (3)	+ (3)	+ (3)
T. leucanthum	−	+ (4)	+ (2)	+ (5)
T. scabrum	+ (4)	+ (2)	−	+ (3)
T. arvense[b]	−	+ (12)	−	+ (12)
R. leguminosarum RBL5561[c]				
Macroptilium atropurpureum	−	−	NT	+/−
Lablab purpureus	−	−	NT	−
Leucaena leucocephalum	−	−	NT	−
R. trifolii ANU851[c]				
M. atropurpureum	NT	−	−	+ (6)
L. purpureus	NT	−	−	+ (4)
L. leucocephalum	NT	−	−	+/− (1)
R. meliloti RmD1D2D3-1[c]				
M. atropurpureum	NT	−	−	+ (3)
L. purpureus	NT	−	−	+ (1)
L. leucocephalum	NT	−	−	+ (1)

[a] +, More than 80% of plants nodulated; +/−, 10 to 30% of plants nodulated; −, no plants nodulated. NT, Not tested.

[b] Identical nodulation frequencies were observed with *T. pratense*, *T. campestre*, *T. angustifolium*, *T. pannonicum*, and *T. squamosum*.

[c] Nodulation was scored 6 weeks after infection; nodulated plants did not fix nitrogen.

gous wild-type *nodD* gene (Table 3), and normal nodulation kinetics were observed (results not shown).

The *nodD604* gene even extended the host range of nodulation of *R. trifolii* ANU851 and *R. meliloti* RmD1D2 D3-1 to the tropical legumes *Macroptilium atropurpureum* (siratro), *Lablab purpureus*, and *Leucaena leucocephalum*. *R. leguminosarum* RBL5561 containing the *nodD604* gene was able to nodulate siratro plants but not the other tested tropical legumes (Table 3). *Rhizobium* bacteria could be isolated from the root nodules of these tropical legumes, and these bacteria appeared to have nodulation characteristics identical to those of the original inoculated *Rhizobium* strains. The root nodules on siratro induced by the strains containing *nodD604* were further characterized by electron microscopy. The nodules consisted of irregularly shaped and loosely packed tissue that could be damaged easily. The general anatomy of the nodules was similar to that of the nodules induced by the control strain 32H1: vascular tissue in the cortex, a cambial zone, meristematic activity, and a zone consisting of cells containing plastids with large starch grains. However, in contrast to the control with 32H1, the nodules contained neither infection threads nor bacteroids.

Comparison of nitrogen fixation ability. To study the nitrogen-fixing ability of *Rhizobium* species containing *nodD604*, *V. sativa* and *T. repens*, representatives of two different cross-inoculation groups, were inoculated with the *Rhizobium* strains, containing either the *nodD604* gene or the wild-type *nodD* genes, which had been used for the experiments described in Table 3. Nitrogen-fixing ability of nodulated plants was measured at various times during a period of 6 weeks. *R. leguminosarum* RBL5561, when harboring a wild-type *R. leguminosarum nodD* gene, fixed nitrogen more efficiently on *V. sativa* than when harboring the wild-type *R. trifolii* or *R. meliloti nodD(1)* gene (Fig. 3), whereas all the wild-type *nodD* genes conferred the same efficiency of nitrogen fixation on *T. repens*. *Rhizobium* strains containing the *nodD604* gene fixed significantly more nitrogen than strains containing wild-type *nodD* genes during the first 14 (with *V. sativa*) or even 45 (with *T. repens*) days after inoculation. These differences in nitrogen fixation are apparently not related to differences in nodule number or nodulation kinetics (Table 5). In conclusion, the *nodD* gene is an important factor for determination of the level of nitrogen fixation.

Two nodules induced on *T. repens* by *R. trifolii* ANU851, containing the hybrid *nodD604* gene, and two nodules induced by strain ANU851, containing the wild-type *R. trifolii nodD* as a control, were compared in more detail. The average efficiency of nitrogen fixation by these nodules on the plant after 24 days of inoculation was 12.8 nmol · h^{-1} and 6.2 nmol · h^{-1} for strains containing *nodD604* and wild-type *nodD*, respectively. The nodules induced by the *nodD604*-containing strain were similar to those induced by the control strain in their external morphology and nodule mass (0.7 ± 0.1 mg). However, electron microscopy revealed that nodules caused by strains containing *nodD604* differed from those caused by the control strain in their internal morphology (Fig. 4). (i) The infected cells in the symbiotic zone of the nodules contained only half the number of bacteroids present in the same zone of the control nodules. The nodule cells infected with the *nodD604*-containing strain (Fig. 4a) contained 1.2 bacteroids per μm^2 of cell section (2,426 μm^2, from six cells, was analyzed), and the nodule cells infected with the control strain (Fig. 4b) contained 2.2 bacteroids per μm^2 of cell section (2,495 μm^2, from six cells, was analyzed). This difference was statistically significant in a chi-squared test ($P < 0.0001$). (ii) The bacteroids were significantly larger than the control bacteroids (Fig. 4c and 4d). The bacteroids of the *nodD604*-containing strain had a mean width of 1.2 μm and mean length of 2.1 μm. The bacteroids of the wild-type

TABLE 4. Hair curling and infection thread formation on various *Trifolium* species infected with strain ANU851 containing various *nodD* genes[a]

Plant species	Phenotype[b] of *nodD* gene from source:									
	R. trifolii		*R. meliloti*		*R. leguminosarum*		*nodD604*		None (pMP92)	
	HAC	INF	HAC	INF	HAC	INF	HAC	INF	HAC	INF
T. repens	+	+	+	+	+	+	+	+	−	−
T. arvense	+	+	+	−	+	−	+	+	−	−
T. pratense	+	+	+	−	+	−	+	+	−	−
T. campestre	+	+	+	−	+	−	+	+	−	−
T. angustifolium	+	+	+	−	+	−	+	+	−	−
T. squamosum	+	+	+	−	+	−	+	+	−	−
T. leucanthum	+	+	+	+	+	−	+	+	−	−

[a] Nodulation characteristics of the *Rhizobium* strains used are given in Table 3.

[b] +, Phenotype as with wild-type *R. trifolii* ANU843; −, no hair curling (HAC) or infection thread formation (INF) detectable.

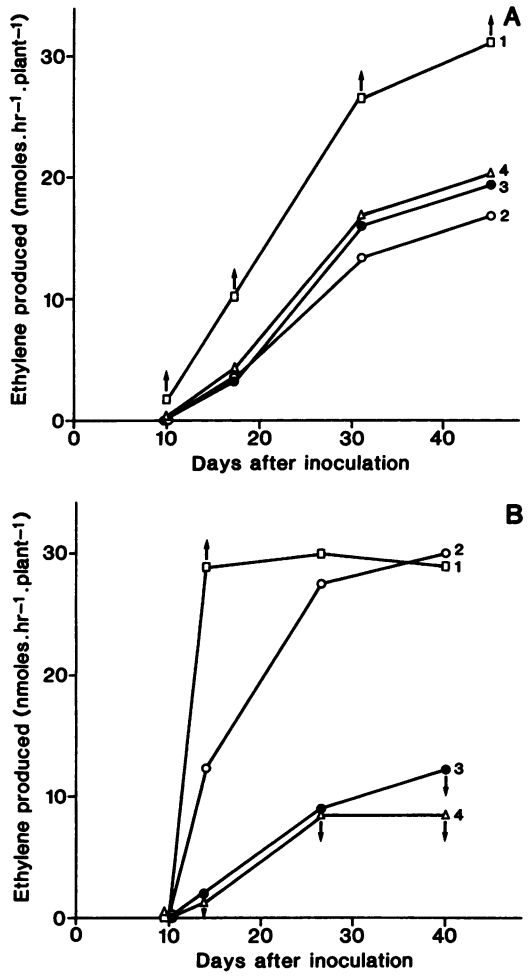

FIG. 3. Nitrogen-fixing ability of *Rhizobium* strains containing a cloned wild-type or hybrid *nodD* gene. The derivatives of *R. trifolii* ANU851 (A) and *R. leguminosarum* RBL5561 (B) used for the experiments described in Table 3 were tested for their nitrogen fixation abilities on *T. repens* (A) and *V. sativa* (B). The indicated average acetylene reduction values for each strain were statistically compared using Student's *t* test and the nonparametric test of Mann and Whitney (19). Results indicated by arrows up or down were significantly higher or lower, respectively, in both tests ($\alpha = 0.05$) than results with the *Rhizobium* strains containing the *nodD* genes of *R. trifolii* (panel A, curve 3) and *R. leguminosarum* (panel B, curve 2) for their host plants *T. repens* and *V. sativa*, respectively. Symbols: □, hybrid *nodD604*; ○, *R. leguminosarum nodD*; ●, *R. trifolii nodD*; △, *R. meliloti nodD1*.

nodD-containing strain had a mean width of 0.9 μm and a mean length of 1.6 μm. The mean widths and lengths appeared to be significantly different in a Mann-Whitney test ($\alpha = 0.005$) (19). (iii) The peribacteroid spaces were larger and contained more vesicles than did those of the control bacteroids (Fig. 4c and 4d).

DISCUSSION

We have demonstrated that the six tested inducible *nod* promoters are transcribed constitutively to a high level in the presence of the *nodD604* gene (Table 2 and Fig. 2) and that this activation is affected neither by the tested inducers and anti-inducers nor by root extracts. This constitutive activating *nodD* gene functions excellently in the nodulation of all tested host plants and even extends the host range of nodulation of *R. trifolii* and *R. meliloti* to several tropical legumes (Table 3). In contrast, the heterologous wild-type *nodD* genes tested narrow the host range of nodulation when replacing indigenous *nodD* genes. The limitation of the host range by heterologous *nodD* genes has been shown to be correlated with the low inducing capacity of the root exudate of the host (32). However, the stage of the symbiotic process in which the wild-type *R. leguminosarum* and *R. meliloti nodD* genes appear to be limiting for nodulation on several *Trifolium* species appears to be infection thread formation rather than root hair curling. An explanation could be that for root hair curling a lower level of *nod* gene expression is needed than for infection thread formation.

These observations confirm the importance of the function of the *nodD* product as a determinant contributing to host specificity during the early stages of nodulation. Moreover, the observations that (i) both the level of nitrogen fixation and the number and morphology of the bacteroids depend on the source of the *nodD* gene and that (ii) a constitutive activating *nodD* gene confers superior nitrogen fixation abilities suggest that the presence of an activated *nodD* product is also of crucial importance during later stages of symbiosis.

Assuming an important role of an activated *nodD* gene in symbiotic nitrogen fixation, there are two possible explanations for the differential behavior of *Rhizobium* strains harboring the *nodD604* gene or one of the wild-type *nodD* genes in nitrogen fixation. First, the activation of a wild-type *nodD* product could be limited by too low concentrations of inducers inside the plant roots, or, second, the activation of a wild-type *nodD* product inside the roots could be antagonized by inhibitory compounds like isoflavonoids or coumarins (4, 8). Our results show that none of these factors is relevant to the *nod* gene transcription in the presence of the *nodD604* gene. With either explanation, a major question remains of how an activated *nodD* product can influence

TABLE 5. Comparison of nodulation kinetics and average number of nodules per nodulated plant with the *nodD604* or homologous wild-type *nodD* genes in the experiment of Fig. 3[a]

Days after inoculation	Avg no. of nodules per nodulated plant				% Nodulated plants			
	T. repens		*V. sativa*		*T. repens*		*V. sativa*	
	nodD604	Wild-type *nodD*	*nodD604*	Wild-type *nodD*	*nodD604*	Wild-type *nodD*	*nodD604*	Wild-type *nodD*
6	2	3			90	80	0	0
9	4	4	2	2	100	100	70	80
17	6	7	2	2	100	100	90	90

[a] Bacterial acceptor strains used were *R. trifolii* ANU851 for *T. repens* and *R. leguminosarum* RBL5561 for *V. sativa*.

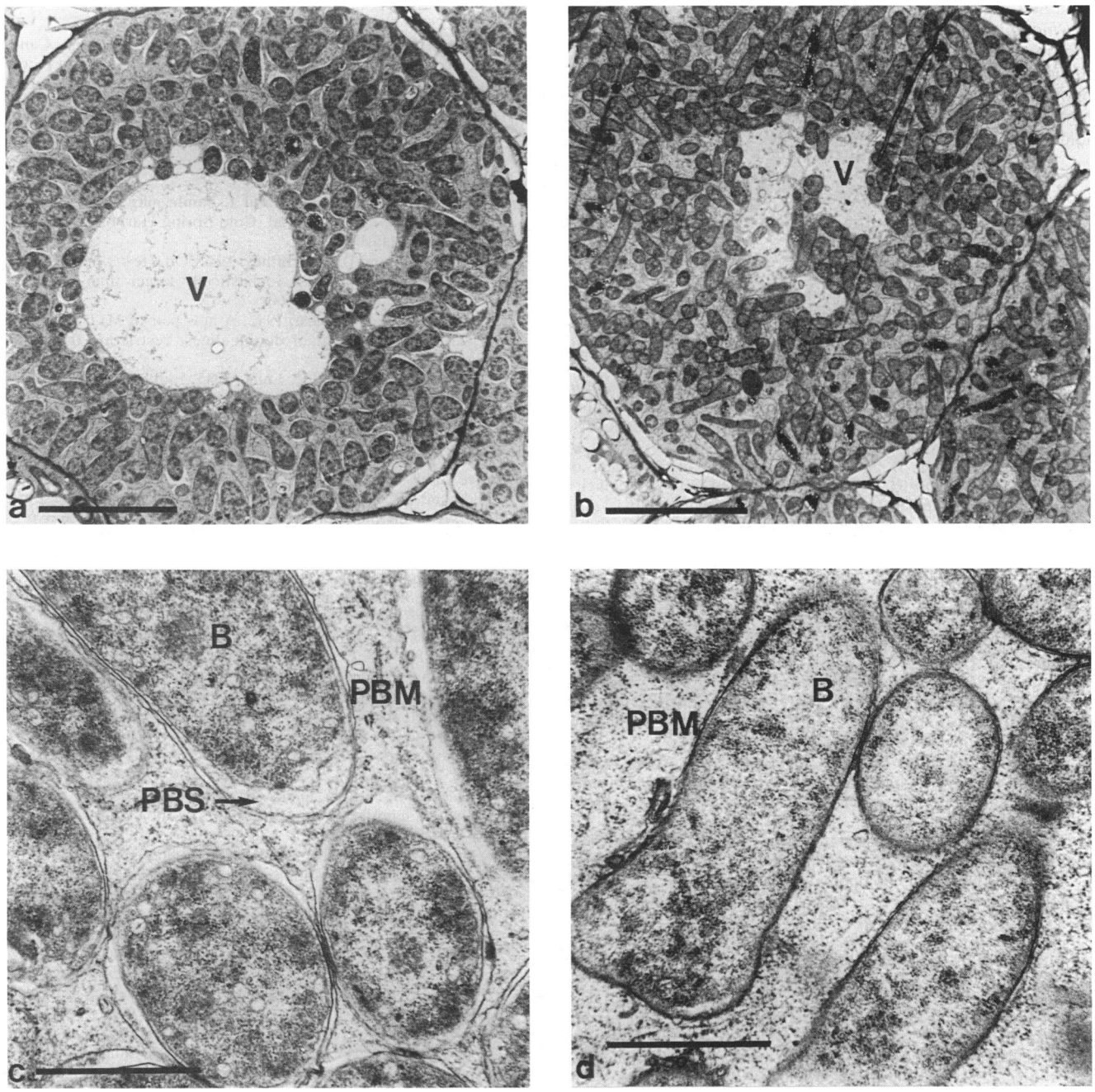

FIG. 4. Electron microscopy of *T. repens* nodules induced by *R. trifolii* ANU851 containing the hybrid *nodD604* gene (panels a and c) or the *R. trifolii* wild-type *nodD* gene (panels b and d). (a and b) Infected nodule cells of the symbiotic zone in comparable stages of symbiosis. Bacteroids fill the whole plant cytoplasm except for the vacuoles (V). Bars, 10 μm. (c and d) Details of the bacteroids (B) as present in the infected nodule cells presented in panels a and b. Note in panel c the wide peribacteroid spaces (PBS) containing vesicles and other membranous structures, bounded by a rather winding peribacteroid membrane (PBM). In panel d the peribacteroid membrane lies close to the outer membrane of the bacteroids. Bars, 1 μm.

nitrogen fixation. One possibility is that this product influences bacteroid development or nitrogen fixation or both by increasing the expression of the inducible *nod* genes. Consistent with this explanation is the recent observation that the *nodC* product is present in increased levels in bacteroids (17), indicating that this *nod* gene is also expressed in the bacteroid. An alternative explanation is that an activated *nodD* product directly influences bacteroid development or nitrogen fixation or both without involvement of the inducible *nod* genes.

The *nodD604* gene represents a novel class of *nodD* genes which are phenotypically very different from any of the known wild-type *nodD* genes and other *nodD* mutants (2, 31). Since *Rhizobium* strains containing a constitutive *nodD* hybrid gene are apparently no longer limited in host range by the flavonoids produced by their host plants, they are very

suitable for studying other factors which limit the host range of *Rhizobium* spp. or which limit bacteroid development in non-host plants. The involvement of *nodD* in symbiotic nitrogen fixation suggests a role of *nodD* in the later stages of symbiotic development. Furthermore, the demonstrated advantages of the *nodD604* gene for nitrogen fixation, if they hold under field conditions, could be of practical importance.

ACKNOWLEDGMENT

We thank Bram Wetselaar for providing us with seeds of various plant species.

LITERATURE CITED

1. **Appelbaum, E. R., D. V. Thompson, K. Idler, and N. Chartrain.** 1988. *Rhizobium japonicum* USDA191 has two *nodD* genes that differ in primary structure and function. J. Bacteriol. **170**:12–20.
2. **Burn, J., L. Rossen, and A. W. B. Johnston.** 1987. Four classes of mutations in the *nodD* gene of *Rhizobium leguminosarum* biovar. *viciae* that affect its ability to autoregulate and/or activate other *nod* genes in the presence of flavonoid inducers. Genes Dev. **1**:456–464.
3. **Debellé, F., and S. B. Sharma.** 1986. Nucleotide sequence of *Rhizobium meliloti* RCR2011 genes involved in host specificity of nodulation. Nucleic Acids Res. **14**:7453–7472.
4. **Djordjevic, M. A., J. W. Redmond, M. Batley, and B. G. Rolfe.** 1987. Clovers secrete specific phenolic compounds which either stimulate or repress *nod* gene expression in *Rhizobium trifolii*. EMBO J. **6**:1173–1179.
5. **Djordjevic, M. A., P. R. Schofield, and B. G. Rolfe.** 1985. Tn5 mutagenesis of *R. trifolii* host-specific nodulation genes results in mutants with altered host-range ability. Mol. Gen. Genet. **200**:463–471.
6. **Downie, J. A., G. Hombrecher, Q. S. Ma, C. D. Knight, B. Wells, and A. W. B. Johnston.** 1983. Cloned nodulation genes of *Rhizobium leguminosarum* determine host-range specificity. Mol. Gen. Genet. **190**:359–365.
7. **Egelhoff, T. T., R. F. Fisher, T. W. Jacobs, J. T. Mulligan, and S. R. Long.** 1985. Nucleotide sequence of *Rhizobium meliloti* 1021 nodulation genes: *nodD* is read divergently from *nodABC*. DNA **4**:241–248.
8. **Firmin, J. L., K. E. Wilson, L. Rossen, and A. W. B. Johnston.** 1986. Flavonoid activation of nodulation genes in *Rhizobium* reversed by other compounds present in plants. Nature (London) **324**:90–92.
9. **Fisher, R. F., H. L. Brierley, J. T. Mulligan, and S. R. Long.** 1987. Transcription of *Rhizobium meliloti* nodulation genes: identification of a *nodD* transcription initiation site *in vitro* and *in vivo*. J. Biol. Chem. **262**:6849–6855.
10. **Fisher, R. F., J. A. Swanson, J. T. Mulligan, and S. R. Long.** 1987. Extended region of nodulation genes in *Rhizobium meliloti* 1021. II. Nucleotide sequence, transcription start sites and protein products. Genetics **117**:191–201.
11. **Göttfert, M., B. Horvath, E. Kondorosi, P. Putnoky, F. Rodriguez-Quinones, and A. Kondorosi.** 1986. At least two different *nodD* genes are necessary for efficient nodulation on alfalfa by *Rhizobium meliloti*. J. Mol. Biol. **191**:411–420.
12. **Honma, M. A., and F. M. Ausubel.** 1987. *Rhizobium meliloti* has three functional copies of the *nodD* symbiotic regulatory protein. Proc. Natl. Acad. Sci. USA **84**:8558–8562.
13. **Hooykaas, P. J. J., F. G. M. Schnijdewindt, and R. A. Schilperoort.** 1982. Identification of the Sym plasmid of *Rhizobium leguminosarum* strain 1001 and its transfer to and expression in other Rhizobia and *Agrobacterium tumefaciens*. Plasmid **8**: 73–82.
14. **Horvath, B., C. W. Bachem, J. Schell, and A. Kondorosi.** 1987. Host-specific regulation of nodulation genes in *Rhizobium* is mediated by a plant-signal, interacting with the *nodD* gene product. EMBO J. **6**:841–848.
15. **Horvath, B., E. Kondorosi, M. John, J. Schmidt, I. Török, Z. Gyorgypal, I. Barabas, U. Wieneke, J. Schell, and A. Kondorosi.** 1986. Organization, structure and symbiotic function of *Rhizobium meliloti* nodulation genes determining host specificity for alfalfa. Cell **46**:335–343.
16. **Innes, R. W., P. L. Kuempel, J. Plazinski, H. C. J. Canter Cremers, B. G. Rolfe, and M. A. Djordjevic.** 1985. Plant factors induce expression of nodulation and host-range genes in *R. trifolii*. Mol. Gen. Genet. **201**:426–432.
17. **John, M., J. Schmidt, U. Wieneke, H. D. Krussman, and J. Schell.** 1988. Transmembrane orientation and receptor-like structure of the *Rhizobium meliloti* common nodulation protein NodC. EMBO J. **7**:583–588.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Mann, H. B., and D. R. Whitney.** 1947. On a test whether one of two random variables is stochastically larger than the other. Ann. Math. Statist. **18**:50–60.
20. **Messing, J., and J. Vieira.** 1982. A new pair of M13 vectors for selecting either strand of double-digest restriction fragments. Gene **19**:269–276.
21. **Mulligan, J. T., and S. R. Long.** 1985. Induction of *Rhizobium meliloti nodC* expression by plant root exudate requires *nodD*. Proc. Natl. Acad. Sci. USA **82**:6609–6613.
22. **Peters, N. K., J. W. Frost, and S. R. Long.** 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* genes. Science **233**:977–980.
23. **Redmond, J. W., M. Batley, M. A. Djordjevic, R. W. Innes, P. L. Kuempel, and B. G. Rolfe.** 1986. Flavones induce expression of nodulation genes in *Rhizobium*. Nature (London) **323**: 632–635.
24. **Rodriguez-Quinones, F., Z. Banfalvi, P. Murphy, and A. Kondorosi.** 1987. Interspecies homology of nodulation genes in *Rhizobium*. Plant Mol. Biol. **8**:61–75.
25. **Rossen, L., C. A. Shearman, A. W. B. Johnston, and J. A. Downie.** 1985. The *nodD* gene of *Rhizobium leguminosarum* is autoregulatory and in the presence of plant exudate induces the *nodA,B,C* genes. EMBO J. **4**:3369–3373.
26. **Rostas, K., E. Kondorosi, B. Horvath, A. Simoncsits, and A. Kondorosi.** 1986. Conservation of extended promoter regions of nodulation genes in *Rhizobium*. Proc. Natl. Acad. Sci. USA **83**:1757–1761.
27. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
28. **Schofield, P. R., and J. M. Watson.** 1986. DNA sequence of *Rhizobium trifolii* nodulation genes reveals a reiterated and potentially regulatory sequence preceding *nodABC* and *nodFE*. Nucleic Acids Res. **14**:2891–2903.
29. **Shearman, C. A., L. Rossen, A. W. B. Johnston, and J. A. Downie.** 1986. The *Rhizobium leguminosarum* nodulation gene *nodF* encodes a polypeptide similar to acyl-carrier protein and is regulated by *nodD* plus a factor in pea root exudate. EMBO J. **5**:647–652.
30. **Spaink, H. P., R. J. H. Okker, C. A. Wijffelman, E. Pees, and B. J. J. Lugtenberg.** 1987. Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. Plant Mol. Biol. **9**:27–39.
31. **Spaink, H. P., C. A. Wijffelman, R. J. H. Okker, and B. J. J. Lugtenberg.** 1989. Localization of functional regions of the *Rhizobium nodD* product using hybrid *nodD* genes. Plant Mol. Biol. **12**:59–73.
32. **Spaink, H. P., C. A. Wijffelman, E. Pees, R. J. H. Okker, and B. J. J. Lugtenberg.** 1987. *Rhizobium* nodulation gene *nodD* as a determinant of host specificity. Nature (London) **328**:337–340.
33. **Surrin, B. P., and J. A. Downie.** 1988. Characterization of the *Rhizobium leguminosarum* genes *nodLMN* involved in efficient host-specific nodulation. Mol. Microbiol. **2**:173–183.
34. **Van Brussel, A. A. N., J. W. Costerton, and J. J. Child.** 1979. Nitrogen fixation by *Rhizobium* sp. 32H1. A morphological and ultrastructural comparison of asymbiotic and symbiotic nitrogen fixing forms. Can. J. Microbiol. **25**:352–361.
35. **Van Brussel, A. A. N., T. Tak, A. Wetselaar, E. Pees, and C. A. Wijffelman.** 1982. Small leguminosae as test plants for nodulation of *Rhizobium leguminosarum* and other *Rhizobia* and

Agrobacteria harbouring a leguminosarum plasmid. Plant Sci. Lett. **27**:317–325.

36. **Van Slogteren, G. M. S., J. H. C. Hoge, P. J. J. Hooykaas, and R. A. Schilperoort.** 1983. Clonal analysis of heterogenous crown gall tumour tissues induced by wild-type and shooter mutant strains of *Agrobacterium tumefaciens*: expression of T-DNA genes. Plant Mol. Biol. **2**:321–333.
37. **Vasse, J. M., and G. L. Truchet.** 1984. The Rhizobium-legume symbiosis: observation of root infection by bright-field microscopy after staining with methylene blue. Planta **161**:487–489.
38. **Wijffelman, C. A., E. Pees, A. A. N. Van Brussel, R. J. H. Okker, and B. J. J. Lugtenberg.** 1985. Genetic and functional analysis of the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. Arch. Microbiol. **143**:225–232.
39. **Wijffelman, C. A., B. Zaat, H. Spaink, I. Mulders, A. A. N. Van Brussel, R. Okker, R. De Maagd, and B. J. J. Lugtenberg.** 1986. Induction of *Rhizobium nod* genes by flavonoids: differential adaptation of promoter, *nodD* gene and inducers for various cross-inoculation groups, p. 123–135. *In* B. Lugtenberg (ed.), Recognition in microbe-plant symbiotic and pathogenic interactions. NATO ASI Series, vol. H4. Springer Verlag, Berlin.
40. **Zaat, S. A. J., C. A. Wijffelman, H. P. Spaink, A. A. N. Van Brussel, R. J. H. Okker, and B. J. J. Lugtenberg.** 1987. Induction of the *nodA* promoter of *Rhizobium leguminosarum* Sym plasmid pRL1JI by plant flavanones and flavones. J. Bacteriol. **169**:198–204.