The *Rhizobium* sp. BR816 *nodO* Gene Can Function as a Determinant for Nodulation of *Leucaena leucocephala*, *Phaseolus vulgaris*, and *Trifolium repens* by a Diversity of *Rhizobium* spp.

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Introduction of the subcloned *nodO* gene of *Rhizobium* sp. BR816 into *R. meliloti* 1021, *R. leguminosarum* bv. *trifolii* ANU843, and *Azorhizobium caulinodans* ORS571 significantly stimulated nodulation and infection of *Leucaena leucocephala* by these transconjugants. The BR816 *nodO* gene was also shown to suppress the nodulation defect of the *nodU* mutants of *Rhizobium* sp. NGR234 and *R. tropici* CIAT899 on *L. leucocephala* and of the *nodE* mutant of *R. leguminosarum* bv. *trifolii* ANU843 on white clover. Overproduction of NodO in BR816 extended its host range to *Trifolium repens*. While a *Rhizobium* sp. BR816 strain, mutated in *nodO*, had reduced nodulation and competitiveness on *P. vulgaris*, no altered phenotype was observed for this mutant on *L. leucocephala*. Analysis of the transcriptional activation of *nodO* in BR816 revealed enhanced expression in the presence of apigenin, under both aerobic and microaerobic conditions. Moreover, *nodO* was also expressed in the bacteroids of *L. leucocephala* nodules.

Additional keywords: host-specific nodulation.

The symbiotic interaction between soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*, collectively called rhizobia, and the roots of leguminous plants is a very specific process, as each bacterial microsymbiont forms nodules only on a restricted number of hosts and each host is nodulated by a restricted number of microsymbionts. The lipochitooligosaccharide Nod factors, synthesized by the rhizobial nodulation (*nod*) genes, are the key signals in this specific process. The expression of the *nod* genes is under control of the regulatory *nodD* gene product, which is activated by components of the root exudates, mostly flavonoids, and binds to a conserved DNA sequence, the *nod*-box upstream of the *nod* genes (Rostas et al. 1986; Spaink et al. 1987). These *nod* genes code for proteins that contribute either to the synthesis of the Nod factor *N*-acetylglucosamine backbone (e.g.,

nodABC), to its decoration by different chemical groups (e.g., *nodFE*, *nodSU*, *nodHPQ*), or to the secretion of Nod factors (*nodIJ*). The Nod factor substituents are important in determining the host range of legumes nodulated by a given rhizobial strain (for an overview see van Rhijn and Vanderleyden 1995; Denarié et al. 1996).

Rhizobium sp. BR816 was isolated from *Leucaena leucocephala* and is able to nodulate a wide range of tropical leguminous plants, including *Phaseolus vulgaris* (Hernández-Lucas et al. 1995). Besides four regulatory *nodD* genes and *nodSU*, *nodPQ*, and *nodFE* genes (van Rhijn et al. 1993, 1994), we isolated a DNA region that contains an open reading frame, the putative product of which shows homology with the *R. leguminosarum* bv. *viciae* NodO protein (van Rhijn et al. 1996). The *nodO* gene was first identified in *R. leguminosarum* bv. *viciae*. In this species, *nodO* is induced in the presence of an active NodD and codes for a Ca²⁺ binding protein that is secreted into the growth medium (de Maagd et al. 1989; Economou et al. 1990; Sutton et al. 1996). The function of NodO in the *Rhizobium*-plant interaction remains obscure. Downie and Surin (1990) postulated that the *R. leguminosarum* bv. *viciae* NodO protein is a host determinant for nodulation of vetch (*Vicia hirsuta*). Whereas mutation of *nodO* does not significantly affect vetch nodulation (Economou et al. 1989), in the absence of *nodFE*, *nodO* is essential for nodulation of pea and vetch (Downie and Surin 1990; Economou et al. 1994). Also, a host range extension of an *R. leguminosarum* bv. *trifolii* *nodE* mutant to vetch has been observed after introduction of the *R. leguminosarum* bv. *viciae* *nodO* gene. NodO is not involved in Nod factor decoration (Spaink et al. 1991) and Sutton and co-workers (1994) demonstrated that NodO forms large, cation-selective channels in artificial bilayers. It was proposed that NodO contributes in initiating the signal transduction cascade that eventually leads to nodule formation. So far, *nodO* has only been reported in *R. leguminosarum* bv. *viciae* and *Rhizobium* sp. BR816.

The *Rhizobium* sp. BR816 DNA region, containing the *nodO* gene, was isolated by the capacity of the responding cosmid (pBRF2Δ110) to enable the narrow-host-range bean symbiont *R. etli* CE3 to nodulate *L. leucocephala* (van Rhijn et al. 1996). Cosmid pBRF2Δ110 could also suppress the

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nodulation defect of a *Rhizobium* sp. NGR234 *nodS* mutant on *L. leucocephala*. In this study, we report on the functional analysis of the BR816 *nodO* gene.

RESULTS

Increased nodulation on *L. leucocephala* by rhizobial species carrying the *Rhizobium* sp. BR816 *nodO* gene.

The BR816 *nodO* gene was subcloned in the broad-host-

range vector pVK100 (Knauf and Nester 1982) and the resulting construct, pFAJ1500 (Table 1), was introduced into *R. etli* CE3, *R. leguminosarum* bv. *trifolii* ANU843, *R. meliloti* 1021, and *Azorhizobium caulinodans* ORS571. These wild-type strains nodulated *L. leucocephala* with a very low efficiency and the induced nodules did not fix nitrogen (Table 2). Introduction of the BR816 *nodO* gene into these four heterologous rhizobia resulted in a significantly higher nodule number and dry weight, compared with the corresponding

Table 1. Bacterial strains and plasmids

Strain/plasmid	Characteristics	Reference
Rhizobia		
BR816	Wild-type isolate from <i>Leucaena leucocephala</i>	Hungria et al. 1993
FAJ1505	BR816 <i>nodO</i> ::Km	This study
FAJ1506	BR816 <i>nodO</i> :: <i>gusA</i> -Km	This study
FAJ1509	BR816 <i>nodA</i> :: <i>gusA</i> -Km	This study
CE3	Sm ^r derivative of <i>Rhizobium etli</i> CFN42	Noel et al. 1984
Rm1021	Sm ^r derivative of <i>R. meliloti</i> 2011	Meade et al. 1982
ORS571	<i>Azorhizobium caulinodans</i> type strain	Dreyfus et al. 1988
ANU843	Wild-type <i>R. leguminosarum</i> bv. <i>trifolii</i>	Djordjevic et al. 1985
K11	ANU843 <i>nodE</i> ::Tn5	Djordjevic et al. 1985
NGR234	<i>Rhizobium</i> sp. NGR234 wild-type isolate from <i>Lablab purpureus</i>	Lewin et al. 1987
NGR234 <i>nodU</i>	NGR234 <i>nodU</i> ::Sp	Lewin et al. 1990
CIAT899	<i>R. tropici</i> IIB type strain	Martinez-Romero et al. 1991
CIAT899 <i>nodU</i>	CIAT899 <i>nodU</i> ::Nm	Waelkens et al. 1995
Plasmids		
pVK100	Cosmid derivative of pRK290 (incP-1), Km ^r , Tc ^r	Knauf and Nester 1982
pBRF2Δ110	pVK100 containing pSym region of <i>Rhizobium</i> sp. BR816, Km ^r , Tc ^r	van Rhijn et al. 1996
pBRD28	pUC19 containing <i>nodABCS</i> genes of <i>Rhizobium</i> sp. BR816, Ap ^r	van Rhijn et al. 1993
pJQ200SK	Suicide vector, containing <i>sacB</i> genes of <i>Bacillus subtilis</i> , Gm ^r	Quandt and Hynes 1993
pJQ200uc1	Suicide vector, containing <i>sacB</i> genes of <i>B. subtilis</i> , Gm ^r	Quandt and Hynes 1993
pHP45-Km	Vector, containing Ω Km interposon, Ap ^r , Km ^r	Prentki and Kirsch 1984
pWM6	Vector, containing Ω <i>gusA</i> -Km interposon, Ap ^r , Km ^r	Metcalf and Wanner 1993
pBlueScript	Ap ^r	Stratagene, La Jolla, CA
pFAJ1500	pVK100 derivative containing a 2-kb <i>HindIII/BamHI</i> fragment of pBRF2Δ110, Tc ^r	This study
pFAJ1504	pVK100 derivative containing 2-kb <i>SmaI/EcoRI</i> fragment of pBRF2Δ110, Tc ^r	This study
pFAJ1505	pJQ200SK derivative containing 3.5-kb fragment with <i>nodO</i> ::Km	This study
pFAJ1506	pJQ200SK derivative containing 5.2-kb fragment with <i>nodO</i> :: <i>gusA</i> -Km	This study
pFAJ1507	pBlueScript containing a 0.26-kb <i>PstI/SmaI</i> fragment of pFAJ1500	This study
pFAJ1509	pJQ200uc1 derivative containing 5-kb fragment with <i>nodA</i> :: <i>gusA</i> -Km	This study
pFAJ1515	pBluescript containing 2-kb <i>Sall</i> fragment of pBRD28	This study
pRK2013	ColE1 helper plasmid for triparental mating, Km ^r	Figurski and Helinski 1979
pRK2073	ColE1 helper plasmid for triparental mating, Sm ^r	Figurski and Helinski 1979

Table 2. Nodule number, nodule dry weight, and acetylene reduction activity (ARA) induced on *Leucaena leucocephala* by *Rhizobium* sp. BR816, its mutant in *nodO* (FAJ1505), by *R. etli* CE3, *R. leguminosarum* bv. *trifolii* ANU843, *R. meliloti* 1021, and *Azorhizobium caulinodans* ORS571 wild-type strains (WT), and by their transconjugants carrying the entire *Rhizobium* sp. BR816 *nodO* gene (+pFAJ1500) or only the *nodO* promoter region (pFAJ1504)

Strain	Nodule number ^x (no./plant)			Nodule dry weight ^x (mg/plant)			ARA ^x (μmoles/h*plant)		
	WT	+pFAJ1500	+pFAJ1504	WT	+pFAJ1500	+pFAJ1504	WT	+pFAJ1500	+pFAJ1504
BR816	28.4 (9.6)	ND ^y	ND	17.9 (8.0)	ND	ND	1.213 (0.572)	ND	ND
FAJ1500	36.6 (17.5)	ND	ND	25.3 (10.4)	ND	ND	1.031 (0.306)	ND	ND
CE3	2.5 (5.0)	33.8 ^z (5.1)	8.5 (10.3)	0.2 (0.4)	4.5 ^z (1.5)	0.2 (0.5)	0.000	0.030 ^z (0.067)	0.000
ANU843	8.2 (17.2)	71.6 ^z (46.3)	ND	1.9 (4.2)	17.0 ^z (9.3)	ND	0.000	0.178 ^z (0.227)	ND
Rm1021	9.0 (10.5)	89.4 ^z (79.6)	ND	7.0 (4.3)	19.6 ^z (14.1)	ND	0.000	0.632 ^z (0.862)	ND
ORS571	6.0 (10.4)	46.0 ^z (21.5)	ND	1.0 (1.5)	16.7 ^z (4.6)	ND	0.000	0.574 ^z (0.336)	ND

^x Values are the means of four replicate plants. Standard deviations are indicated between parentheses.

^y Not determined.

^z Value obtained for transconjugant differs significantly from the value obtained for the corresponding WT strain ($P \leq 0.05$).

wild-type strains, and the nodules showed nitrogen-fixing ability. An *R. etli* CE3 transconjugant, carrying only the *nodO* promoter region (pFAJ1504), did not show significantly increased nodule number, nodule dry weight, or acetylene reduction activity (ARA) on *L. leucocephala*. Microscopical examination of sections of nodules induced by *A. caulinodans* ORS571 did not show infected cells (Fig. 1A). However, in nodules induced by ORS571 carrying pFAJ1500, infection of cells had occurred (Fig. 1B). Nevertheless, the number of infected cells in these nodules was lower than in nodules induced by *Rhizobium* sp. BR816, for which *L. leucocephala* is a natural host (Fig. 1C).

The *Rhizobium* sp. BR816 *nodO* gene suppresses the nodulation defect of mutants in certain host-specific nodulation genes.

The BR816 *nodO* gene was transferred on pFAJ1500 into the *nodU* mutants of *Rhizobium* sp. NGR234 (NGR234Q26; Lewin et al. 1990) and *R. tropici* CIAT899 (CIAT899*nodU*; Waelkens et al. 1995) and the resulting transconjugants were tested for their phenotypes on *L. leucocephala* (Table 3). On *L. leucocephala*, NGR234Q26 and CIAT899*nodU* form smaller, round-shaped nodules (Fix⁺) with a significantly lower nodule dry weight than that of the cylindrical nodules induced by the wild-type parents (Lewin et al. 1990; Waelkens et al. 1995). Introduction of the BR816 *nodO* gene into these mutants significantly increased their nodule dry weight on *L. leucocephala*, to a value that is comparable to the nodule dry weight induced by the parental strain. The presence of the BR816 *nodO* gene also restored the decreased nitrogen-fixing capacity observed with the NGR234 *nodU* mutant, but did not restore nitrogen fixation capacity in the CIAT899 *nodU* mutant.

pFAJ1500 was also introduced into a *nodE* mutant of *R. leguminosarum* bv. *trifolii* ANU843 (K11; Djordjevic et al. 1985). K11 nodulated white clover poorly, with a 4-day delay, compared with the parental strain ANU843 (Fig. 2). Nodule formation by the K11 transconjugant carrying the BR816 *nodO* gene had a slight delay (about 1 day) compared with ANU843, but nodulation was accelerated by 3 days, compared with K11. From 17 to 20 days after inoculation a significantly higher nodule number was observed for this transconjugant.

The absence of nodulation by an *R. meliloti* strain mutated in the *nodH* gene (resulting in the absence of a sulfate group at the reducing end of the Nod factor; Roche et al. 1991) could not be suppressed by the *Rhizobium* sp. BR816 *nodO* gene (data not shown).

Increased copy number of the *Rhizobium* sp. BR816 *nodO* gene enables BR816 to form an effective symbiosis with *T. repens*.

Rhizobium sp. BR816 is not able to induce nodules on clover plants. Introduction of the BR816 *nodO* gene on a multi-copy plasmid (pFAJ1500) into BR816 conferred on this strain the ability to nodulate white clover (*T. repens*). Nodules induced by this transconjugant, however, appeared with a delay, compared with the nodules induced by the *R. leguminosarum* bv. *trifolii* wild-type strain ANU843. Once nodule formation had started, however, the number of nodules induced by the BR816 transconjugant increased rapidly and at harvest (51 days after inoculation) about the same number of nodules

were obtained for this transconjugant as for ANU843 (26 nodules per plant). Although some of these nodules were Fix⁺, they were smaller and displayed a lower ARA than the nodules induced by ANU843.

Role of *nodO* in the interaction of *Rhizobium* sp. BR816 with *L. leucocephala* and *P. vulgaris*.

A *nodO* mutant of BR816 (FAJ1505) was constructed and tested for its phenotype on *L. leucocephala* and *P. vulgaris*. On *L. leucocephala* this mutant did not show a significantly different nodule number, nodule dry weight, or ARA (Table 2). When this mutant was inoculated onto bean plants, however, nodulation by FAJ1505 was slightly delayed, compared with the wild-type BR816 strain. Then, days after inoculation, only 25% of the plants inoculated with FAJ1505 were nodulated while 60% of the plants inoculated with the wild-type strain were nodulated. Moreover, nodules induced by this mutant, 21 days after inoculation, were smaller (with a significantly lower nodule dry weight) and showed a significantly lower ARA (Table 4). The competitiveness of the *nodO* mutant, FAJ1505, relative to the BR816 parental strain was evaluated by applying various ratios of BR816 to FAJ1505 onto *L. leucocephala* and bean seedlings and determination of the nodule occupancies by each strain at harvest. A competitiveness index (CI) was calculated with a mathematical model derived by Beattie et al. (1989) that relates the ratio of competing strains in a mixed inoculum ($I_{BR816}/I_{FAJ1505}$) to their ratio in the nodules ($P_{BR816}/P_{FAJ1505}$). A CI of zero is obtained when BR816 and FAJ1505 occupy an equal proportion of the nodules at an inoculum ratio 1:1 and is characteristic for a mutant that is equal in competitiveness to BR816. A CI greater than zero indicates that the mutant is less competitive than BR816 and a CI less than zero indicates that the mutant is more competitive than BR816 (see Materials and Methods). In mixed inoculation of BR816 and FAJ1505 on *P. vulgaris* a positive CI of 0.223 was obtained, indicating a decreased competitiveness for FAJ1505 on beans. On *L. leucocephala*, a negative CI of -0.070 indicated a slightly higher nodule occupancy for FAJ1505. However, in this latter coinoculation study, the CI was almost zero, and the slightly higher nodule occupancy observed for FAJ1505 might have been due to the double infections (from 0 to 40% in sterilized matrixes; Beattie et al. 1989) in which only FAJ1505 (resistance to kanamycin; see Materials and Methods) was detected.

Analysis of the transcriptional regulation of *nodO* in *Rhizobium* sp. BR816.

A *nod*-box-like sequence has been identified 108 bp upstream of the putative *nodO* start codon. Comparison of this *nod*-box with the BR816 *nodA* promoter sequence and with the *nod*-box consensus sequence presented by Spaink et al. (1987) nevertheless revealed various mismatches (nine with the consensus sequence). The observed homology with the *nod*-box consensus sequence suggests that *nodO* is induced by flavonoids. However, the fact that the *nod*-box is poorly conserved could indicate that, similar to the *nodO* gene expression in *R. leguminosarum* bv. *viciae*, BR816 *nodO* expression might differ from the expression of other *nod* genes. The induction of *nodO* and *nodA* in the presence of apigenin (strongest *nod* gene inducer in *Rhizobium* sp. BR816; van Rhijn et al. 1994) was compared in an aerobic as well as in a

microaerobic (1% O₂) environment by use of Northern (RNA) hybridization and reporter genes. Hybridization with *nodO* antisense RNA on total RNA isolated from BR816 cells consistently showed three transcripts of different sizes (1.2, 1.0, and 0.8 kb) with RNA from BR816 cells grown in the presence of apigenin (Fig. 3; lane 2). With RNA from BR816 grown in the absence of apigenin, a weak signal could be detected (lane 1), indicating some basal expression. Also, with BR816 cells grown under low oxygen pressure, strong signals were detected when cells were grown in the presence of apigenin (lane 4). RNA obtained from the BR816 *nodO* mutant, FAJ1505, did not contain these transcripts (lane 5). In view of the large size of the *nodA* transcript (containing various other *nod* genes as well; van Rhijn et al. 1993), hybridization with *nodA* antisense RNA on total RNA isolated from BR816 did not show a single band, but rather a smear. Positive signals, however, were only obtained with RNA isolated from BR816 or FAJ1505 grown in the presence of apigenin. In contrast to *nodO*, *nodA* was not expressed under microaerobic conditions.

A more quantitative expression pattern of the BR816 *nodO* and *nodA* genes was obtained by the use of the *gusA* reporter gene. *nodO-gusA* and *nodA-gusA* integrated fusions were constructed by insertion of the promoterless *gusA* gene into the genomic *nodO* and *nodA* genes, in the same orientation as the respective genes (see Materials and Methods). The BR816 *nodA* gene was not expressed in the absence of an inducer and/or under low oxygen pressure (Table 5). Its expression increased about 35 times in the presence of apigenin. The BR816 *nodO* gene showed a basal expression that increased 6 to 7 times in the presence of apigenin. In accordance with our observation in the Northern hybridization experiments and in clear contrast to the BR816 *nodA* gene, the BR816 *nodO* gene also showed expression in microaerobic conditions and this expression increased in the presence of apigenin. As the BR816 *nodO* gene is also expressed under low oxygen pressure, it could be possible that *nodO* is active in the later stages of the symbiotic interaction as well. *L. leucocephala* nodules induced by FAJ1506 were stained with 5-bromo-4-chloro-3-indoyl- β -D-glucuronide. The presence of the blue precipitate ClBr-indigo in the infected cells indicates that *nodO* is also expressed in the bacteroids (Fig. 4).

DISCUSSION

The *Rhizobium* sp. BR816 *nodO* gene has been isolated by its ability to extend the host range of *R. etli* CE3 to *L. leucocephala* (van Rhijn et al. 1996). Here we demonstrated that this *nodO* gene can function as a host determinant for nodulation of *L. leucocephala* and *T. repens* and increases the nodulation and infection on these hosts of various heterologous wild-type strains and of certain mutants in host-specific *nod* genes. The exact action of NodO, however, is not yet clear. Sutton and co-workers (1994) demonstrated that the *R. leguminosarum* bv. *viciae* NodO protein forms cation-selective channels in lipid bilayers, allowing the movement of cations across the membrane. It was proposed that NodO might facilitate the uptake of Nod factors. Alternatively, NodO could contribute to the membrane depolarization of root hairs induced by Nod factors, one of the initial steps in the signal transduction pathway that leads to root hair deformation and cortical cell division (Ehrhardt et al. 1992). Aberrant Nod factors, produced by heterologous strains or mutants, are less active or inactive for nodule induction and elicit only a weak depolarization of the root hair membrane or no response at all (Felle et al. 1995). NodO might amplify this weak root hair depolarization and therefore initiate the signal transduction cascade (Sutton et al. 1994).

In view of the above proposed mechanism for the action of NodO, it is an intriguing observation that all defects in host-specific *nod* genes that are suppressed by *nodO* code for decorations at the nonreducing end of the Nod factor backbone: *nodS* is involved in the N-methylation of the nonreducing *N*-acetylglucosamine (Geelen et al. 1993), *nodU* in the carbamoylation of the nonreducing terminal sugar residue (Holsters et al. 1994), and *nodE* codes for a β -keto acyl synthase that contributes to the synthesis of Nod factors with poly-unsaturated fatty acid groups (Spaink et al. 1991; Demont et al. 1993). *nodO* could not suppress the defect in a host-specific *nod* gene at the reducing end (i.e., *nodH* of *R. meliloti*). It should be mentioned that not all possible mutations that affect decoration of Nod factors have been tested for suppression by *nodO*. It has been proposed earlier that there might exist more than one mechanism of Nod factor recogni-

Table 3. Nodule number, nodule dry weight, and acetylene reduction activity (ARA) induced on *Leucaena leucocephala* by *Rhizobium* sp. NGR234 and *R. tropici* CIAT899 wild-type strains (WT), by their mutants in *nodU*, and by transconjugants of these mutants carrying the *Rhizobium* sp. BR816 *nodO* gene (+pFAJ1500)

Strain	Nodule number ^z (number/plant)			Nodule dry weight ^z (mg/plant)			ARA ^z (μ moles/h*plant)		
	WT	Mutant in <i>nodU</i>		WT	Mutant in <i>nodU</i>		WT	Mutant in <i>nodU</i>	
		–	+pFAJ1500		–	+pFAJ1500		–	+pFAJ1500
NGR234	15.8 a (7.6)	13.5 a (22.4)	23.3 a (5.0)	15.9 a (6.2)	3.7 b (4.8)	13.6 a (2.4)	0.837 a (0.177)	0.162 b (0.162)	0.835 a (0.317)
CIAT899	20.8 a (12.6)	15.8 a (18.4)	28.5 a (9.8)	19.0 a (6.3)	6.2 b (5.5)	14.2 a (3.0)	1.592 a (0.486)	0.376 b (0.113)	0.386 b (0.430)

^z Values are the means of four replicate plants. Standard deviations are indicated between parentheses. For each rhizobial strain values are compared between the wild type parent, its mutant in *nodU*, and its *nodU* transconjugant carrying pFAJ1500. Values followed by the same letter are not significantly different ($P \leq 0.05$).

Fig. 1. Light micrograph of sections of *Leucaena leucocephala* nodules induced by (A) *Azorhizobium caulinodans* ORS571, (B) ORS571 carrying the BR816 *nodO* gene, and (C) *Rhizobium* sp. BR816.

tion, resulting in more than one signal transduction pathway, and different Nod factor modifications appear to be required for the elicitation of different plant developmental responses (Ardourel et al. 1994; Mylona et al. 1995). In the *R. meliloti*-alfalfa symbiosis, for example, sulfation of the reducing end of the Nod factor backbone is essential and sufficient for root hair deformation and cortical cell division while *nodE* is required to initiate the infection process (Ardourel et al. 1994).

While the *Rhizobium* sp. BR816 *nodO* gene could increase the nodulation on *L. leucocephala* of all heterologous rhizobia tested, no host range extension to *T. repens* was observed for *R. etli* CE3, *R. meliloti*, or *A. caulinodans* ORS571 carrying this *nodO* gene. The flavonoid composition of the exudates excreted by *L. leucocephala* and *T. repens* roots may differ, and it is possible that no or only a weak *nod* gene induction occurs in these rhizobial species in the presence of *T. repens* root exudates. Yet, an increased *nodO* copy number enabled BR816 to nodulate *T. repens*. BR816 contains a *nodD* product, NodD₄, that is independent of flavonoid activation (van Rhijn

et al. 1994), and this NodD might be responsible for the *nod* gene induction of this strain on *T. repens* roots.

Another possible explanation for the absence of or only poorly improved nodulation observed for some transconjugants with the *Rhizobium* sp. BR816 *nodO* gene could be the instability of the *nodO*-containing plasmid (pFAJ1500) in some rhizobial backgrounds. It has been reported earlier that strains carrying pRK290 derivatives can lose these plasmids during the infection process because of the inability to apply antibiotic selection in planta (Weinstein et al. 1992). This instability might explain why, for the CIAT899*nodU* transconjugant containing *nodO*, no restored nitrogen fixation was observed. Apparently, pFAJ1500 is not very stably maintained in the CIAT899 background. This is further substantiated by the fact that the nodulation defect of a CIAT899 *nodS* mutant could not be suppressed by *nodO* (data not shown). The only partial suppression of the nodulation defect of an *R. leguminosarum* bv. *trifolii* *nodE* mutant by the *Rhizobium* sp. BR816 *nodO* gene also may be explained by the loss of pFAJ1500 later in the symbiotic interaction.

Fig. 2. Number of nodules formed on *Trifolium repens* by *Rhizobium leguminosarum* bv. *trifolii* ANU843, its *nodE* mutant (K11), and K11 carrying the BR816 *nodO* gene (pFAJ1500). Values are the means of 20 replicate plants.

Table 4. Nodule number, nodule dry weight, and acetylene reduction activity (ARA) induced on *Phaseolus vulgaris* cv. Carioca by the *Rhizobium* sp. BR816 wild-type strain and its mutant in *nodO* (FAJ1505)

Strain	Nodule number ^z (number/plant)	Nodule dry weight ^z (mg/plant)	ARA ^z (μmoles/h*plant)
BR816	25.2 a (21.5)	8.3 a (4.4)	0.975 a (0.921)
FAJ1505	28.4 a (23.8)	3.5 b (2.8)	0.200 b (0.199)

^z Values are the means of eight replicate plants. Standard deviations are indicated between parentheses. Values followed by the same letter are not significantly different ($P \leq 0.05$).

Fig. 3. Northern (RNA) blot of total RNA isolated from *Rhizobium* sp. BR816 (lanes 1, 2, 3, and 4) and its *nodO* mutant, FAJ1505 (lane 5), grown in the absence (lanes 1 and 3) or presence (lanes 2, 4, and 5) of apigenin (500 mM) under aerobic (lanes 1, 2, and 5) or microaerobic (lanes 3 and 4) conditions. RNA was hybridized with *nodO* antisense RNA, *nodA* antisense RNA, *nodA* sense RNA, and a 16S rRNA oligonucleotide probe (see Materials and Methods); 4 μg of RNA was loaded for each sample.

As stated by Economou et al. (1994), nodulation tests may not necessarily be a good measure for the efficiency of nodule formation by a given rhizobial strain. The final number of nodules formed is controlled by the host (Caetano-Anolles and Gresshoff 1991) and only a small proportion of infections eventually lead to nodule formation. The absence of a certain *nod* gene might therefore not necessarily lead to reduced nodule number or dry weight. This could explain why, although the BR816 *nodO* gene is a host determinant for *L. leucocephala* nodulation, the *nodO* mutant, FAJ1505, induced the same nodule number and nodule dry weight on this host. To further elucidate an eventually reduced efficiency for nodule formation after mutation of *nodO*, the competitiveness of FAJ1505 was determined. Strains with a high nodule-forming efficiency, initiating and sustaining an infection rapidly and efficiently, are often found to have a competitive advantage over those that are slower and less efficient (Sargent et al. 1987; Hahn and Hennecke 1988; Stephens and Cooper 1988; de Oliveira and Graham 1990; McDermott and Graham 1990; Onishchuk et al. 1994). Coinoculation of two strains onto the same root and subsequent determination of the nodule occupancies by these strains therefore appears to be a good method to evaluate subtle differences between strains that may not be detected by comparison of separate inoculations of these strains onto different plants. The reduced competitiveness of FAJ1505 on beans confirms the earlier observed lower nodulation on beans exhibited by this mutant strain (Table 4). However, an eventually reduced nodulation on *L. leucocephala*, due to mutation in *nodO*, was not reflected in a reduced competitiveness by FAJ1505 on this host. Apparently, the mutation in *nodO* has a more severe effect on bean nodulation, while its effect on nodulation of *L. leucocephala* is possibly masked by one or more other host-specific *nod* genes. It has been postulated that the *nodSU* and *nodPQ* genes might also be important for nodulation of *L. leucocephala* (Krishnan et al. 1992; Waelkens et al. 1995; Folch-Mallol et al. 1996). It is possible that the reduced nodulation due to the absence of *nodO* becomes visible on *L. leucocephala* only after inactivation of these genes as well (with double or triple mutants).

The presence of a *nod*-box-like sequence in the promoter region of the BR816 *nodO* gene indicates that transcription of *nodO* requires the presence of a (flavonoid) activated NodD protein. However, similar to the *nod*-box preceding the *R. leguminosarum* bv. *viciae* *nodO* gene, this *nod*-box homologous sequence is poorly conserved, and it has been reported that the activation of the *nodO* promoter in *R. leguminosarum* bv. *viciae* has regulation features that differ from those observed for other *nod* promoters. Isogenic strains of *R. leguminosarum* bv.

viciae, carrying a Tn3*HoHo1* (*lacZ* transcriptional fusion) in *nodB*, *nodE*, or *nodO*, showed a higher flavonoid-dependent *nodO* induction, compared with the induction of the other *nod* genes (Economou et al. 1990). When the promoters of the *R. leguminosarum* bv. *viciae* *nodA* and *nodO* genes were cloned in front of the promoterless *lacZ* gene on pMP220, the *R. leguminosarum* *nodO* promoter showed, similar to the BR816 *nodO* promoter, a higher basal β -galactosidase activity and less induction, compared with the *nodA* promoter activity. In the presence of naringenin, the *nodA* promoter activity increased drastically (up to 34 times) while this flavonoid induced the *nodO* expression only twofold. However, higher induction could be obtained by providing extra copies of *nodD* (de Maagd et al. 1989).

The BR816 NodO protein influences the nodulation process under certain conditions and in some bacterial backgrounds. Nodules induced by certain strains carrying *nodO* contain more infected cells and do fix nitrogen. This implicates a possible role of NodO in bacterial infection. Furthermore, it is striking to observe that expression of *nodO*, in contrast to *nodA* expression, is not switched off under microaerobic conditions, but is also present in the infected cells. This implicates a role of *nodO* also in later stages of the symbiotic interaction. Further microscopic analysis, including in situ hybridization and immunolabeling experiments, of *L. leucocephala* nodules induced by BR816 and by its *nodO* mutant should add more information on the spatial and temporal expression of *nodO* and on the presence and function of the NodO product in the nodule, and will hopefully provide us with more information on the specific role of NodO during the different stages of the symbiotic interaction.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were maintained on LB agar and grown in LB broth supplemented with the appropriate antibiotics (Miller 1972). *Rhizobium* strains were maintained on yeast extract-mannitol (YEM) medium (Vincent 1970) or on tryptone-yeast (TY) medium (Beringer 1974) supplemented with the appropriate antibiotics. The concentrations of antibiotics used were as follows: 10 $\mu\text{g ml}^{-1}$ of tetracycline, 50 $\mu\text{g ml}^{-1}$ of kanamycin, 100 $\mu\text{g ml}^{-1}$ of streptomycin, 30 $\mu\text{g ml}^{-1}$ of nalidixic acid, 50 $\mu\text{g ml}^{-1}$ of spectinomycin, 50 $\mu\text{g ml}^{-1}$ of neomycin, and 25 $\mu\text{g ml}^{-1}$ of gentamycin.

DNA manipulations and plasmid constructs.

Isolation of plasmid DNA, restriction digestion, ligation, and transformation were carried out according to Sambrook et al. (1989). Restriction endonucleases and T4 DNA ligase were purchased from Boehringer Mannheim (Brussels) and used according to the supplier's instructions. For the construction of pFAJ1500, a 2-kb *HindIII*/*BamHI* fragment of pBRF2 Δ 110 (van Rhijn et al. 1996), containing the entire *nodO* gene and its promoter region, was ligated into the *HindIII*/*BglII* site of pVK100 (Knauf and Nester 1982). pFAJ1504 was made by blunt ligation of the 2-kb *SmaI*/*EcoRI* fragment of pBRF2 Δ 110, containing the *nodO* promoter region and part of the 5' sequence of *nodO*, into the *HindIII*/*BglII* site of pVK100. Matings of mobilizable plasmids from

Table 5. β -Glucuronidase activity obtained for BR816 containing an integrated transcriptional *nodA-gusA* (FAJ1509) or *nodO-gusA* (FAJ1506) fusion

Strain	β -glucuronidase activity (Miller units) ^z			
	Aerobic		Microaerobic	
	No inducer	Apigenin	No inducer	Apigenin
FAJ1509	-0.4 (0.1)	35.5 (6.4)	-0.9 (0.1)	-1.1 (0.3)
FAJ1506	11.3 (2.5)	73.3 (17.7)	3.2 (0.8)	48.8 (13.2)

^z Values are the means of 12 separate measurements. Standard deviations are indicated between parentheses.

E. coli to *Rhizobium* were done in the presence of a helper *E. coli* strain, as described by Sambrook et al. (1989).

Construction of a BR816 *nodO* mutant and of integrated, transcriptional *nodO-gusA* and *nodA-gusA* fusions.

For the construction of a BR816 *nodO* mutant and of an integrated *nodO-gusA* fusion, the 1.5-kb *PstI/BamHI* fragment of pBRF2Δ110 containing the N-terminal part of the BR816 *nodO* gene was subcloned in the suicide vector pJQ200SK (Quandt and Hynes 1993). Subsequently, *nodO* was inactivated by introduction of a Km (Prentki and Kirsch 1984) or a *gusA*-Km (Metcalf and Wanner 1993) interposon in the *SmaI* site, resulting in pFAJ1505 and pFAJ1506, respectively. For the construction of the integrated *nodA-gusA* fusion, the 1.3-kb *EcoRI/BamHI* fragment containing the BR816 *nodAB* genes (van Rhijn et al. 1993) was subcloned in pJQ200uc1 (Quandt and Hynes 1993). *nodA* was inactivated by introduction of a *gusA*-Km interposon (Metcalf and Wanner 1993) in the *SalI* site, resulting in pFAJ1509. pJQ200SK and pJQ200uc1 contain the *sacR/B* genes that code for levansucrase, an enzyme that is lethal to gram-negative bacteria when plated on sucrose. pFAJ1505, pFAJ1506, and pFAJ1509 were introduced into BR816 by triparental mating, with pRK2073 as helper. Homologous recombinants resulting from a double cross-over were selected by their resistance to 5% sucrose and to kanamycin. Mutants were verified by Southern hybridization with *nodO* (FAJ1505 and FAJ1506) or *nodA* (FAJ1509) and with DNA fragments of the kanamycin resistance cassette.

Northern hybridization.

Total RNA from overnight induced cells (500 mM apigenin under atmospheric O₂ or under 1% O₂) was isolated by the method of Ausubel et al. (1987). RNA was quantified spectrophotometrically and 4-μg RNA samples were loaded on a formaldehyde agarose gel and blotted onto a positively charged nylon membrane (Boehringer Mannheim) via capillary transfer (Sambrook et al. 1989). To verify equal loading and transfer of RNA, the loading buffer was supplemented with 50 μg of ethidium bromide per ml, allowing visualization of RNA in the gels and on the blots when illuminated with UV light. Blots were prehybridized, hybridized with a digoxigenin-labeled RNA probe (*nodO* antisense, *nodA* antisense, *nodA* sense) or oligonucleotide probe (16S rRNA of *R. meliloti*; H. V. Tichy, TÜV, Freiberg, Germany), and developed by immunochemiluminescence. The digoxigenin-labeled *nodO* and *nodA* RNA probes were obtained by cloning, respectively, the 260-bp *PstI/SmaI* fragment of pFAJ1500 or the 2-kb *SalI* fragment of pBRD28 (van Rhijn et al. 1993) into pBlueScript (Stratagene, La Jolla, CA), resulting in pFAJ1507 or pFAJ1515, and subsequent run-off transcription with T3 or T7 polymerase and the Dig RNA labeling kit of Boehringer Mannheim.

Plant tests.

Pre-germinated seeds of *L. leucocephala* or *P. vulgaris* cv. Carioca were planted in polypropylene cups containing a vermiculite:sand mixture (2:1) watered with a diluted Norris medium (beans and *L. leucocephala*; Waelkens et al. 1995) or

Fig. 4. 5-bromo-4-chloro-3-indoyl-β-D-glucuronide staining of a longitudinal section of a *Leucaena leucocephala* nodule induced by FAJ1506 (*nodO::gusA*).

containing a Jenssen medium agar slant (beans; Vincent 1970). The seedlings were inoculated with 200 µl of an over-night rhizobial culture. For competition experiments, mixtures of the diluted cultures were prepared by combining the single-inoculum suspensions at three ratios. Exact ratios of the mixed inocula were calculated following enumeration of the cell density of the single-inoculum suspension. The plants were maintained in a growth chamber at 28°C day and 24°C night temperature and a 12-h photoperiod. After 3 weeks (beans) or 8 weeks (*L. leucocephala*), plants were harvested for determination of nodule number, nodule dry weight, and ARA. In the competition experiments, strains were isolated from the nodules according to Vincent (1970) and identified by the ability to grow on TY medium containing naledixic acid and/or kanamycin. Clover (*T. repens*) plants were grown on Jenssen medium agar slants as described by van Brussel et al. (1982). In all experiments, uninoculated control plants did not show any nodules or nodulelike structures.

Microscopy.

Nodules were fixed in a 0.1 M phosphate buffer (pH 7) containing 4% p-formaldehyde and 0.25% glutaraldehyde. After dehydration through an ethanol series, specimens were infiltrated in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany) according to the supplier's instructions. Sections 5 µm thick were cut with a Micron HM360 microtome and stained with toluidine blue.

Assay for *nod-gusA* expression.

Overnight TY cultures of the *Rhizobium* sp. BR816 constructs with integrated *nodO-gusA* or *nodA-gusA* fusions were diluted 10 times in induction medium (TY containing 500 mM apigenin under atmospheric O₂ or under 1% O₂) and grown overnight. The β-glucuronidase activity was measured spectrophotometrically with the substrate *p*-nitrophenyl-β-D-glucuronide (Jefferson 1987). Units were calculated as defined by Miller (1972). To evaluate the *nodO-gusA* expression during symbiosis, nodules were cut longitudinally and stained for 2 h with a 0.1 M phosphate buffer (pH 7) solution containing EDTA (10 mM), TritonX-100 (0.1%), K₃[Fe(CN)₆] (0.5 mM), K₄[Fe(CN)₆] (0.5 mM), and 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (0.1%). Nodules were cleared with 0.5% hypochlorite prior to photography.

Data analysis.

Nodule numbers, dry weights, and ARAs were analyzed with the "means" and "general linear model (GLM)" procedure (SAS Institute, Cary, NC). Comparison among the mean values obtained for each strain was made by Duncan's multiple-range test with a 95% confidence limit. For quantitative measurement of competitiveness, a competitiveness index (CI) was calculated by the linear regression (REG) procedure with the mathematical model of Beattie et al. (1989), which relates the ratio of the competing strains in the inoculum to their ratio in the nodules ($\log[P_{BR816}/P_{FAJ1505}] = CI + k \cdot \log[I_{BR816}/I_{FAJ1505}]$). In this equation P_{BR816} and $P_{FAJ1505}$ are the proportions of nodules occupied by wild-type BR816 and its *nodO* mutant FAJ1505, respectively, $I_{BR816}/I_{FAJ1505}$ is the ratio of BR816 and FAJ1505 in the inoculum, and k is the slope, indicating the rate at which the nodule occupancy ratio changes as the inoculum ratio changes.

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LITERATURE CITED

- Ardoürel, M., Demont, N., Debellé, F., Maillet, F., de Billy, F., Promé, J.-C., Dénarié, J., and Truchet, G. 1994. *Rhizobium meliloti* lipooligosaccharide nodulation factors: Different structural requirements for bacterial entry into target root hair cells and induction of plant symbiotic developmental responses. *Plant Cell* 6:1357-1374.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1987. *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley-Interscience, New York.
- Beattie, G. A., Clayton, M. K., and Handelsman, J. 1989. Quantitative comparison of the laboratory and field competitiveness of *Rhizobium leguminosarum* biovar *phaseoli*. *Appl. Environ. Microbiol.* 55:2755-2761.
- Beringer, J. E. 1974. R-factor transfer in *Rhizobium leguminosarum*. *J. Gen. Microbiol.* 120:421-429.
- Caetano-Anolles, G., and Gresshoff, P. M. 1991. Plant genetic control of nodulation. *Annu. Rev. Microbiol.* 45:345-382.
- de Maagd, R. A., Wijffjes, A. H. M., Spaink, H. P., Ruiz-Sainz, J. E., Wijffelman, C. A., Okker, R. J. H., and Lugtenberg, B. J. J. 1989. *nodO*, a new *nod* gene of the *Rhizobium leguminosarum* biovar *viciae* sym plasmid pRL1J1, encodes a secreted protein. *J. Bacteriol.* 171:6764-6770.
- de Oliveira, L. A., and Graham, P. H. 1990. Speed of nodulation and competitive ability among strains of *Rhizobium leguminosarum* bv. *phaseoli*. *Arch. Microbiol.* 153:311-315.
- Demont, N., Debellé, R., Aurelle, H., Dénarié, J., and Promé, J. C. 1993. Role of the *Rhizobium meliloti nodF* and *nodE* genes in the biosynthesis of lipo-oligosaccharidic nodulation factors. *J. Biol. Chem.* 268:20134-20142.
- Dénarié, J., Debellé, F., and Promé, J. C. 1996. *Rhizobium* lipochitoooligosaccharide nodulation factors: Signaling molecules mediating recognition and morphogenesis. *Annu. Rev. Biochem.* 65:503-535.
- Djordjevic, M. A., Schofield, P. R., and Rolfe, B. G. 1985. Tn5 mutagenesis of *Rhizobium trifolii* host specific nodulation genes result in mutants with altered host range ability. *Mol. Gen. Genet.* 200:463-471.
- Downie, J. A., and Surin, B. P. 1990. Either of two *nod* gene loci can complement the nodulation defect of a *nod* deletion mutant of *Rhizobium leguminosarum* bv. *viciae*. *Mol. Gen. Genet.* 222:81-86.
- Dreyfus, B., Garcia, J. L., and Gillis, M. 1988. Characterization of *Azorhizobium caulinodans* gen. nov. sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. *Int. J. Syst. Bacteriol.* 38:89-98.
- Economou, A., Davies, A. E., Johnston, A. W. B., and Downie, J. A. 1994. The *Rhizobium leguminosarum* biovar *viciae nodO* gene can enable a *nodE* mutant of *Rhizobium leguminosarum* biovar *trifolii* to nodulate vetch. *Microbiology* 140:2341-2347.
- Economou, A., Hamilton, W. D. O., Johnston, A. W. B., and Downie, J. A. 1990. The *Rhizobium* nodulation gene *nodO* encodes a Ca²⁺ binding protein that is exported without N-terminal cleavage and is homologous to haemolysin and related proteins. *EMBO J.* 9:349-354.
- Economou, A., Hawkins, F. K. L., Downie, J. A., and Johnston, A. W. B. 1989. Transcription of *rhiA*, a gene on a *Rhizobium leguminosarum* bv. *viciae* Sym plasmid, requires *rhiR* and is repressed by flavonoids that induce *nod* genes. *Mol. Microbiol.* 3:87-93.
- Ehrhardt, D. W., Atkinson, E. M., and Long, S. R. 1992. Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. *Science* 256:998-1000.

- Felle, H. H., Kondorosi, E., Kondorosi, A., and Schultze, M. 1995. Nod signal-induced plasma membrane potential changes in alfalfa root hairs are differentially sensitive to structural modifications of the lipochitooligosaccharide. *Plant J.* 7:939-947.
- Figurski, D. H., and Helinski, D. R. 1979. Replication of an origin-derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. *Proc. Natl. Acad. Sci. USA* 76:1648-1652.
- Folch-Mallol, J. L., Marroqui, S., Sousa, C., Manyani, H., López-Lara, I. M., van der Drift, K. M. G. M., Haverkamp, J., Quinto, C., Gil-Serrano, A., Thomas-Oates, J., Spaink, H. P., and Megías, M. 1996. Characterization of *Rhizobium tropici* CIAT899 nodulation factors: the role of *nodH* and *nodPQ* genes in their sulfation. *Mol. Plant-Microbe Interact.* 9:151-163.
- Geelen, D., Mergaert, P., Geremia, R. A., Goormachtig, S., Van Montagu, M., and Holsters, M. 1993. Identification of *nodSUIJ* genes in Nod locus 1 of *Azorhizobium caulinodans*: evidence that *nodS* encodes a methyltransferase involved in Nod factor modification. *Mol. Microbiol.* 9:145-154.
- Hahn, M., and Hennecke, H. 1988. Cloning and mapping of a novel nodulation region from *Bradyrhizobium japonicum* by genetic complementation of a deletion mutant. *Appl. Environ. Microbiol.* 54:55-61.
- Hernández-Lucas, I., Segovia, L., Martínez-Romero, E., and Pueppke, S. G. 1995. Phylogenetic relationships and host range of *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. *Appl. Environ. Microbiol.* 61:2775-2779.
- Holsters, M., Geremia, R., Geelen, D., Fernández-López, M., Leyman, B., Van Montagu, M., Promé, J.-C., Goethals, K., Goormachtig, S., and Mergaert, P. 1994. Biosynthesis and secretion of Nod factors from *Azorhizobium caulinodans*. Pages 64-68 in: *Proc. Eur. Nitrogen Fixation Conf.*, 1st, Szeged, Hungary. G. B. Kiss and G. Endre, eds. Oficina Press, Szeged, Hungary.
- Hungria, M., Franco, A. A., and Sprent, J. I. 1993. New sources of high-temperature tolerant rhizobia for *Phaseolus vulgaris* L. *Plant Soil* 149:103-109.
- Jefferson, R. A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Mol. Biol. Rep.* 5:387-405.
- Knauf, V. C., and Nester, E. W. 1982. Wide host range cloning vectors: A cosmid clone bank of an *Agrobacterium* Ti plasmid. *Plasmid* 8:45-54.
- Krishnan, H. B., Lewin, A., Fellay, R., Broughton, W. J., and Pueppke, S. G. 1992. Differential expression of *nodS* accounts for the varied abilities of *Rhizobium fredii* USDA257 and *Rhizobium* sp. strain NGR234 to nodulate *Leucaena* spp. *Mol. Microbiol.* 6:3321-3330.
- Lewin, A., Cervantès, E., Wong, C.-H., and Broughton, W. J. 1990. *nodSU*, two new *nod* genes of the broad host range *Rhizobium* strain NGR234 encode host-specific nodulation of the tropical tree *Leucaena leucocephala*. *Mol. Plant-Microbe Interact.* 3:317-326.
- Lewin, A., Rosenberg, C., Meyer, Z. A. H., Wong, C.-H., Nelson, L., Manen, J.-F., Stanley, J., Dowling, D. N., Dénarié, J., and Broughton, W. J. 1987. Multiple host-specificity loci of the broad host-range *Rhizobium* sp. NGR234 selected using the widely compatible legume *Vigna unguiculata*. *Plant Mol. Biol.* 8:447-459.
- Martínez-Romero, E., Segovia, L., Mercante, F. M., Franco, A. A., Graham, P., and Pardo, M. A. 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* spp. trees. *Int. J. Syst. Bacteriol.* 41:417-426.
- McDermott, T. R., and Graham, P. H. 1990. Competitive ability and efficiency in nodule formation of strains of *Bradyrhizobium japonicum*. *Appl. Environ. Microbiol.* 56:3035-3039.
- Meade, H. M., Long, S. R., Ruvkun, G. B., Brown, S. E., and Ausubel, R. M. 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. *J. Bacteriol.* 149:114-122.
- Metcalfe, W. W., and Wanner, B. L. 1993. Construction of new β -glucuronidase cassettes for making transcriptional fusions and their use with new methods for allele replacement. *Gene* 129:17-25.
- Miller, J. H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Mylona, P., Pawlowski, K., and Bisseling, T. 1995. Symbiotic nitrogen fixation. *Plant Cell* 7:869-885.
- Noel, K. D., Sánchez, A., Fernández, L., Leemans, J., and Cevallos, M. A. 1984. *Rhizobium phaseoli* symbiotic mutants with Tn5 insertions. *J. Bacteriol.* 158:148-155.
- Onishchuk, O. P., Sharypova, L. A., and Simarov, B. V. 1994. Isolation and characterization of the *Rhizobium meliloti* Tn5-mutants with impaired nodulation competitiveness. *Plant Soil* 167:267-274.
- Prentki, P., and Kirsch, H. M. 1984. *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* 29:303-313.
- Quandt, J., and Hynes, M. F. 1993. Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. *Gene* 127:15-21.
- Roche, P., Debellé, F., Maillet, F., Lerouge, P., Faucher, C., Truchet, G., Dénarié, J., and Promé, J.-C. 1991. Molecular basis of symbiotic host specificity in *Rhizobium meliloti*: *nodH* and *nodPQ* genes encode the sulfation of lipo-oligosaccharide signals. *Cell* 67:1131-1143.
- Rostas, K., Kondorosi, E., Horvath, B., Simoncits, A., and Kondorosi, A. 1986. Conservation of extended promoter regions of nodulation genes in *Rhizobium*. *Proc. Natl. Acad. Sci. USA* 83:1757-1761.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sargent, L., Huang, S. Z., Rolfe, B. G., and Djordjevic, M. A. 1987. Split-root assays using *Trifolium subterraneum* show that *Rhizobium* infection induces a systemic response that can inhibit nodulation of another invasive *Rhizobium* strain. *Appl. Environ. Microbiol.* 53:1611-1619.
- Spaink, H. P., Okker, R. J. H., Wijffelman, C. A., Pees, E., and Lugtenberg, B. J. J. 1987. Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1J1. *Plant Mol. Biol.* 9:27-39.
- Spaink, H. P., Sheeley, D. M., van Brussel, A. A. N., Glushka, J., York, W. S., Tak, T., Geiger, O., Kennedy, E. P., Reinhold, V. N., and Lugtenberg, B. J. J. 1991. A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. *Nature* 354:125-131.
- Stephens, P. M., and Cooper, J. E. 1988. Variation in speed of infection of "no root hair zone" of white clover and nodulating competitiveness among strains of *Rhizobium trifolii*. *Soil Biol. Biochem.* 20:465-470.
- Sutton, J. M., Lea, E. J. A., and Downie, J. A. 1994. The nodulation-signaling protein NodO from *Rhizobium leguminosarum* biovar *viciae* forms ion channels in membranes. *Proc. Natl. Acad. Sci. USA* 91:9990-9994.
- Sutton, J. M., Peart, J., Dean, G., and Downie, J. A. 1996. Analysis of the C-terminal secretion signal of the *Rhizobium leguminosarum* nodulation protein NodO; a potential system for the secretion of heterologous proteins during nodule invasion. *Mol. Plant-Microbe Interact.* 9:671-680.
- van Brussel, A. N. N., Tak, T., Wetselaar, A., Pees, E., and Wijffelman, C. A. 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.
- van Rhijn, P., Desair, J., Vlassak, K., and Vanderleyden, J. 1994. The NodD proteins of *Rhizobium* sp. BR816 differ in their interactions with coinducers and in their activities for nodulation of different host plants. *Appl. Environ. Microbiol.* 60:3615-3623.
- van Rhijn, P. J. S., Feys, B., Verreth, C., and Vanderleyden, J. 1993. Multiple copies of *nodD* in *Rhizobium tropici* CIAT899 and BR816. *J. Bacteriol.* 175:438-447.
- van Rhijn, P., Luyten, E., Vlassak, K., and Vanderleyden, J. 1996. Isolation and characterization of a pSym locus of *Rhizobium* sp. BR816 that extends nodulation ability of narrow host range *Phaseolus vulgaris* symbionts to *Leucaena leucocephala*. *Mol. Plant-Microbe Interact.* 9:74-77.
- van Rhijn, P., and Vanderleyden, J. 1995. The *Rhizobium*-plant symbiosis. *Microbiol. Rev.* 59:124-142.
- Vincent, J. M. 1970. *A Manual for the Practical Study of the Root-Nodule Bacteria*. Blackwell Scientific Publications, Oxford.
- Waelkens, F., Voets, T., Vlassak, K., Vanderleyden, J., and van Rhijn, P. 1995. The *nodS* gene of *Rhizobium tropici* strain CIAT899 is necessary for nodulation on *Phaseolus vulgaris* and on *Leucaena leucocephala*. *Mol. Plant-Microbe Interact.* 8:147-154.
- Weinstein, M., Roberts, R. C., and Helinski, D. R. 1992. A region of the broad-host-range plasmid RK2 causes stable in planta inheritance of plasmids in *Rhizobium meliloti* cells isolated from alfalfa root nodules. *J. Bacteriol.* 174:7486-7489.