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Research Note

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*

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Introduction of a cosmid library of megaplasmid DNA of *Rhizobium* sp. BR816, a broad host range *Rhizobium* strain, into *R. etli* CE3, a narrow host range bean symbiont, resulted in the isolation of a transconjugant that could effectively nodulate *Leucaena leucocephala*. Analysis of the corresponding cosmid, pBRF2, revealed the presence of genes required for eliciting nitrogen-fixing nodules on *L. leucocephala*. Subcloning and Tn*5* tagging identified a locus responsible for the host range extension. Sequence analysis of this locus revealed an ORF that shows significant identity with NodO of *R. leguminosarum* bv. *viciae*.

Additional keywords: nodO, nodS.

Development of symbiotic nodules on leguminous plants by rhizobia is governed by sequential signal exchange between the symbiotic partners (for a review: Carlson et al. 1994; van Rhijn and Vanderleyden 1995). In this work, we describe the isolation and characterization of a pSym locus of *Rhizobium* sp. BR816, an isolate of *Leucaena leucocephala*. This locus extends the host range of several *Rhizobium* species to nodulate *L. leucocephala*. To isolate this region, we first constructed a cosmid library of the megaplasmids of *Rhizobium* sp. BR816. Megaplasmid DNA was isolated on a cesium chloride gradient according to Hirsch et al. (1980) and the partially digested DNA was ligated into the *Eco*RI site of the pVK100 (Knauf and Nester 1982) (Table 1). Then, the entire cosmid library was introduced en masse, by triparental mating (van Rhijn et al. 1993), into *Rhizobium etli* CE3. By itself, *R. etli* CE3 effectively nodulates *Phaseolus vulgaris*, but is unable to induce nodules on *L. leucocephala*. Transconjugants were selected on YM plates (Hooykaas et al. 1977) with nalidixic acid and tetracycline and were subsequently used for en masse inoculation of 40 *L. leucocephala* seedlings as described by van Rhijn et al. (1994). The seedlings were analyzed for the presence of root nodules 6 wk after inoculation. As expected, nitrogen-fixing nodules were not found on *L. leucocephala* roots inoculated with *R. etli* CE3, but small bumps were occasionally found. In contrast, fully developed effective nodules formed on *Leucaena* roots inoculated with the transconjugants. From these plants, single nodules were collected and bacteria were reisolated on YM medium supplemented with nalidixic acid and tetracycline. Plasmid DNA was isolated from 20 different bacteria and digested with *Eco*RI. A particular DNA profile, exemplified by pBRF2, was detected (Fig. 1). To confirm that this clone was responsible for the host range extension of *R. etli*, the plasmid DNA was reintroduced in CE3 and also into CNPAF512, another narrow host range bean microsymbiont. These transconjugants were then tested for nodulation of *L. leucocephala*. All *Leucaena* plants inoculated with the transconjugants containing pBRF2 formed root nodules after 4 wk. The nodules were morphologically identical to those obtained with the wild-type strain *Rhizobium* sp. BR816, and were Fix$^+$.

Comparison of the pBRF2 DNA restriction profile with that of cosmids containing the different *nodD*s of *Rhizobium* sp. BR816 (van Rhijn et al. 1993) revealed an overlap with the BR816 cosmid that carries $nodD_2$ and other DNA sequences. Previous experiments have shown that $nodD_2$ of *Rhizobium* sp. BR816 is able to complement the *nodD* mutation of NGR234 for the nodulation of *L. leucocephala* (van Rhijn et al. 1993, 1994). However, the host range was not extended to *L. leucocephala* upon inoculation of $nodD_2$ alone into *R. etli* CE3 (van Rhijn et al. 1994). Because *nodS* is an essential gene for the nodulation of *L. leucocephala* in *Rhizobium* sp. NGR234 and *R. tropici* CIAT899, as well as in *Azorhizobium caulinodans* (Lewin et al. 1990; Waelkens et al. 1995), we tested pBRF2 DNA for hybridization with pA16 (Lewin et al. 1990), which contains the *nodSU* genes of NGR234. No hybridization signal was observed (data not shown).

Four deletion clones of pBRF2 were constructed to localize more precisely the region that is responsible for host range extension of *R. etli* CE3 to *Leucaena* (Fig. 1). These subclones were transferred to CE3 and tested for their ability to nodulate *L. leucocephala*. All *R. etli* transconjugants which

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Genbank accession number U26451.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Rhizobium		
BR816	Wild-type isolate from *L. leucocephala*	EMBRAPA, Brazil
CIAT899	Wild-type isolate from *P. vulgaris*	EMBRAPA, Brazil
CFN299	Wild-type isolate from *P. vulgaris*	Martinez et al. 1987
NGR234 (Rif^r)	Rif^r derivative of *Rhizobium* sp. NGR234	Lewin et al.1990
NGR234 *Ω 25*	*nodS* mutant of NGR234, Rif^r, Km^r	Lewin et al. 1990
CE3	Str^r derivative of wild-type strain CFN42	Noel et al. 1984
CNPAF512	Wild-type isolate from *P. vulgaris*	EMBRAPA, Brazil
Rm1021	Str^r derivative of *R. meliloti* SU47	Meade et al. 1982
ANU843	Wild type *R. leguminosarum* bv. *trifolii*	Djordjevic et al. 1985
A34	*R. leguminosarum* bv. *viciae* strain 8401/pRL1JI	Downie et al. 1983
ORS571	*Azorhizobium caulinodans* wild-type strain	Dreyfus et al. 1988
Plasmids		
pVK100	Km^r,Tc^r cosmid derivative of pRK290 (Inc-P)	Knauf and Nester 1982
pBRF2	Derivative of pVK100, containing region of BR816 that extend host range to *L. leucocephala*	This study
pBRF2Δx	*Eco*RI deletion clone of pBRF2 (Figure 1)	This study
pBR7E5	pVK100 cosmid containing the *nodD2* region of BR816	This study
pGV910	Inc-P-? (unclassified) broad host range vector	Van den Eede et al. 1992
pBRGV110	pGV910 derivative containing the 6 kb *Eco*RI of pBRF2Δ110	This study
pBRGVTn5-x	Tn*5* insertions in pBRGV110 as indicated in Figure 1	This study
pA16	Subclone of the *nodSU* genes from NGR234 cloned in pRK7813, Tc^r	Lewin et al. 1990
pRK2013	ColE1, helper plasmid for tripartite mating, Km^r	Figurski and Helinski 1979

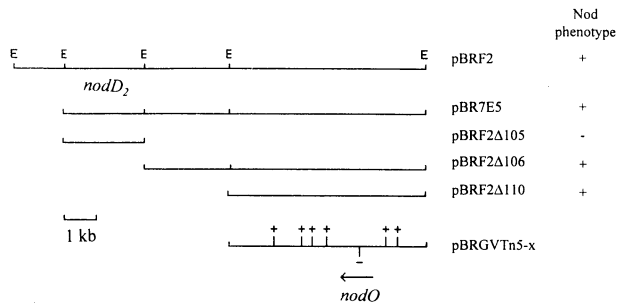

Fig. 1. Physical map of the plasmid pBRF2 and its derivatives. The nodulation phenotype on *Leucaena leucocephala* of the different constructs when introduced in *Rhizobium etli* CE3, indicated at the right by + or −. The insertion position of the different Tn*5*- derivatives of pBRGV110 (pBRGVTn5-x) are indicated by a vertical line linked with the corresponding nodulation phenotype. E: *Eco*RI.

Table 2. Nodulation phenotype[a] of different *Rhizobium* strains on *Leucaena leucocephala*

Inoculum	Nodules per plant: liquid medium	Nodules per plant: vermiculite	Nodule dry weight (mg/plant)	ARA (μmoles /h plant)	Shoot dry weight (mg/plant)
BR816	14.3 A	30.2 A	16.0 A	1.6 A	404 A
CE3 (pBRF2)	11.0 B	29.4 A	9.5 B	0.9 B	178 B
CE3 (pBRF2Δ110)	10.7 B	23.4 A	3.7 C	0.0 C	96 C
CE3	0.0 C	2.5[b] B	0.2 C	0.0 C	98 C

[a] Mean values for five plants. The plants grown in vermiculite were further analyzed on nodule dry weight, acetylene reduction activity (ARA) and shoot dry weight. Results indicated by the same letter are not significantly different (Duncan $P \leq 0.05$).

[b] In the case of CE3 no fully developed nodules could be detected.

carried clones containing the 6-kb *Eco*RI fragment induced nodules on *L. leucocephala* (Fig. 1). However, nodules elicited by CE3(pBRF2Δ110) were Fix$^-$, in contrast to those induced by CE3(pBRF2) (Table 2). This result clearly indicates that pBRF2 contains information both for nodulation and effective nitrogen fixation.

In an attempt to identify other nodulation genes located on pBRF2Δ110, the 6-kb *Eco*RI fragment of pBRF2Δ110 was cloned into pGV910 (Van den Eede et al. 1992) and random Tn*5* mutagenesis was carried out as described by Michiels et al. (1988). Several Tn*5* insertions scattered over the insert were obtained (Fig. 1). After conjugation into *R. etli* CE3, the mutants were tested for nodulation. One Tn*5* mutant completely lost the ability to nodulate *L. leucocephala* (Fig. 1). DNA sequence analysis of this region revealed the presence of an ORF of 816 nucleotides (Genbank accession number U26451) and comparison of the deduced amino acid sequence with the protein data bank revealed 43.4% identity with the deduced amino acid sequence of *R. leguminosarum* bv. *viciae nodO* (de Maagd et al. 1989) (Fig. 2). The observed similarity is further substantiated by the conservation of a Ca^{2+}-binding domain, characterized by a variable number of tandem repeats consisting of a nine amino acid motif (Economou et al. 1990) (Fig. 2).

DNA hybridizations were performed with *nodO* of BR816 as a probe against total DNA of *R. tropici* strains CIAT899 and CFN299, *R. etli* strains CE3 and CNPAF512, *Azorhizobium caulinodans* ORS571, *R. trifolii* ANU843, *R. meliloti* 1021, *Rhizobium* sp. NGR234, and *R. leguminosarum* A34, to test the occurrence of *nodO*-like sequences in other rhizobia. Only the latter two contained sequences that showed weak hybridization to the probe compared to *Rhizobium* sp. BR816 (data not shown).

pBRF2 and pBRF2Δ110 were then introduced into *Rhizobium* sp. NGR234Ω25, a *nodS* mutant, and tested for nodulation. In both cases, wild-type nodulation was restored (Table 3). However, the *nodS* gene of BR816, which is located downstream of the *nodABC* operon (van Rhijn 1994), was not isolated by the introduction of pSym plasmid library into *R. etli*. The reasons for this are not clear.

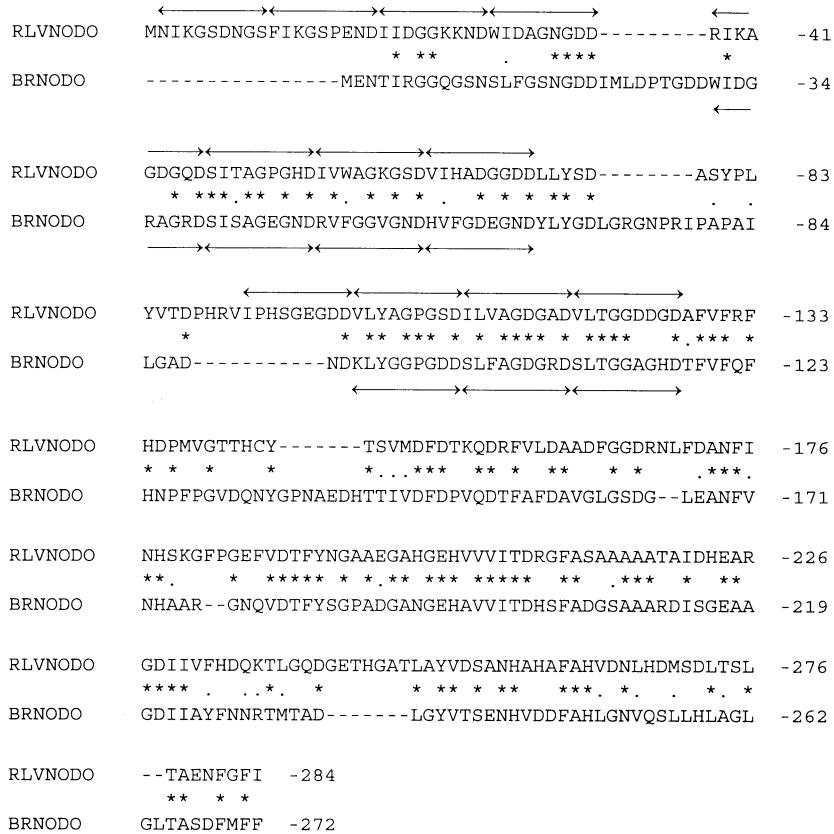

Fig. 2. Alignment of the amino acid sequences of the NodO protein of *Rhizobium leguminosarum* (RLVNODO) and the NodO protein of BR816 (BRNODO). Perfectly conserved positions are indicated by an asterisk, conserved substitutions by a dot. Homology between the two NodO proteins consists of 43.4% identical and additionally 8.5% similar amino acids. The horizontal arrows indicate the nonapeptide repeats that are thought to be involved in Ca^{2+} binding (de Maagd et al. 1990).

Table 3. Nodulation phenotype[a] of NGR234 *nodS* mutant, harboring pBRF2 or a derivative plasmid on *Leucaena leucocephala*

Inoculum	Nodules per plant	Fixation phenotype
NGR234	23.4 A	+
NGR234 Ω25	0.0 B	−
NGR234 Ω25 (pBRF2)	25.4 A	+
NGR234 Ω25 (pBRF2Δ110)	22.2 A	+

[a] Mean values for five plants. The plants were grown in vermiculite (van Rhijn et al. 1994) and further analyzed for acetylene reduction activity (fixation phenotype). Results indicated by the same letter are not significantly different (Duncan $P \leq 0.05$).

The cross complementation between *nodS* of NGR234 and *nodO* of BR816 suggests that both determine specificity for nodulation of *Leucaena* and that they are interchangeable for their function. A similar situation is seen in *R. leguminosarum* bv. *viciae*, where *nodO* partially complements a deletion of *nodEFL* of *R. leguminosarum* bv. *viciae* for the nodulation of *Pisum* and *Vicia* (Downie and Surin 1990), and also extends the host range of nodulation of a *nodE* mutant of *R. trifolii* to *Vicia* (Economou et al. 1994). Whereas *nodE* is involved in the production of the lipo-oligosaccharides (Spaink et al. 1991), *nodO* appears to have another function. Research by Sutton and co-workers (1994) indicates that *nodO* codes for a secreted protein with no detectable cellulase, pectinase, or protease activity, but which could form Ca^{2+}-regulated ion channels in an artificial membrane. They conclude, that because pure lipo-oligosaccharides induce ion fluxes across plant membranes (Ehrhardt et al. 1992), NodO must have a complementary role to the nodulation factors.

To our knowledge, this is only the second *Rhizobium* strain in which *nodO* has been found. Although the derived amino acid sequences of various *nodO*s share a high degree of similarity, there is sequence divergence in certain domains. These differences can be of great importance in determining the structure-function relationships of the two NodO proteins.

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