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Identification and Characterization of a Functional *nodD* Gene in *Azorhizobium caulinodans* ORS571

KOEN GOETHALS, GUY VAN DEN EEDE, MARC VAN MONTAGU, AND MARCELLE HOLSTERS*

Laboratorium voor Genetica, Rijksuniversiteit Gent, B-9000 Ghent, Belgium

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***Azorhizobium caulinodans* ORS571, a bacterium capable of nodulating roots and stems of the tropical legume *Sesbania rostrata*, has been shown to have no *nodD*-like gene located immediately upstream from its common *nodABC* locus. A clone carrying a functional *nodD* gene of strain ORS571 has now been isolated from a pLAFR1 gene library by screening for naringenin-induced expression of the common *nod* genes in an *Agrobacterium* background. Tn*5* mutagenesis of the cloned insert DNA delimited the inducing activity to a ±0.8-kilobase-pair fragment. One of the Tn*5* insertions in the activator locus was homogenotized in the ORS571 genome. This resulted in a mutant strain (ORS571-3) that was unable to induce common *nod* gene expression in the presence of host plant exudate or the flavanone naringenin and that had lost the capacity to nodulate the roots and stems of *S. rostrata*. Complementation of both mutant phenotypes was achieved upon introduction of the cloned *nodD* gene. Sequencing of the *nodD* locus indicated the presence of a single, 942-base-pair-long open reading frame (ORFD) with significant homology to the *nodD* genes of (brady)rhizobia. The level of homology, however, is the lowest thus far reported for this kind of gene. ORFD most likely initiates translation with a TTG start codon. Upstream from ORFD, a divergently oriented *nod* box-like sequence is present, the function of which remains to be determined.**

The interaction between leguminous plant species and soil bacteria belonging to the genera *Bradyrhizobium* and *Rhizobium* can lead to the development of nodules on the roots of the host plant. In these new plant organs, differentiated bacteria (bacteroids) encounter favorable conditions for symbiotic nitrogen fixation. Several bacterial and plant genes involved in the establishment of this symbiosis have been studied extensively during the last few years (for reviews, see references 23 and 26).

More recently, *Azorhizobium caulinodans* ORS571 has been described as the archetypical species of a new genus of soil bacteria which, apart from being diazotrophic in free-living conditions, is also able to nodulate effectively the tropical legume *Sesbania rostrata* (11). A unique feature of the host plant is the occurrence of dormant root primordia in vertical rows all along the stem. Upon infection by *A. caulinodans*, these primordia develop into N_2-fixing aerial or stem nodules (9).

To understand the factors that contribute to the formation of these N_2-fixing aerial nodules, we started identifying and characterizing *Azorhizobium* genes involved in the nodulation process. In previous reports, we described the identification and characterization of common *nodABC*-related genes (14, 34) that are essential for the interaction with the host plant and are highly conserved among rhizobia, bradyrhizobia, and, to a lesser extent, the genus *Azorhizobium*.

In (brady)rhizobia, the common *nod* genes are organized in an operon that is coregulated with other *nod* operons by a positive, regulatory function encoded by the *nodD* gene. The NodD proteins typically have a molecular mass of approximately 34 kilodaltons and are classified in the recently described LysR family of transcriptional activators (18). On the basis of sequence alignments, each member of this protein family is predicted to have an amino-terminally located helix-turn-helix motif involved in DNA binding. It has indeed been demonstrated that NodD proteins bind specifically at a particular, conserved DNA sequence (the *nod* box consensus) located upstream from inducible *nod* operons (12, 22, 27). Activation of *nod* gene expression by NodD occurs only in the presence of specific (iso)flavonoid-type inducer molecules that are exuded by the host plant. Mutational and recombinational experiments suggest a direct interaction between the inducer molecules and the NodD protein (5, 19, 32). In most cases documented, a *nodD* gene is linked to, but divergently transcribed from, the common *nod* genes. In *A. caulinodans*, common *nodABC*-related genes were shown to be very likely organized in an operon, but no evidence for an upstream located *nodD* gene was found (14). However, the regulation of expression of the *A. caulinodans* common *nod* genes is very similar to that of the (brady)rhizobial *nod* operons. Using *lacZ* fusions, it was demonstrated that the expression of ORS571 *nod* locus *1* genes is activated in the presence of host plant exudate or the flavanone naringenin (14). This led us to suspect that a related regulatory mechanism is present in ORS571. In this report, we present confirmation of this hypothesis by showing the existence in *A. caulinodans* of a *nodD*-homologous gene that is essential for plant-inducible *nod* gene expression as well as for nodulation of *S. rostrata* stems and roots.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. Strains, plasmids, and phages used are listed in Table 1.

Growth media and culture conditions. Cultures of ORS571 or derivatives were grown at 37°C on LSR medium (34) or MMO medium (14). When necessary, antibiotics were applied in the following concentrations (micrograms per milliliter): carbenicillin, 100; tetracycline, 10; kanamycin, 20; and spectinomycin, 20.

Cultures of *Agrobacterium* strain GV3101 were grown at 28°C on YEB medium or PA medium (36). When necessary, rifampin was added to a final concentration of 100 μg/ml, tetracycline was added to 5 μg/ml, spectinomycin was added

* Corresponding author.

TABLE 1. Bacterial strains, phages, and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
ORS571	*A. caulinodans* type strain capable of nodulating roots and stems of *S. rostrata*	10, 11
ORS571-S	Spontaneous mutant of ORS571 unable to regulate the expression of the common *nod* genes	This work
ORS571-3	Tn*5* insertion mutant in the *nodD* regulatory gene	This work
GV3101	Rif^r derivative of *Agrobacterium tumefaciens* cured of its pTiC58 plasmid	35
MC1061	*araD139* Δ(*ara leu*) Δ*lacX74 galU galK hsr hsm*$^+$ *strA*	6
CSH2110	*polA* Nal^r	17
Phages		
M13mp8	M13 cloning vector	37
M13mp8-*nodD1*	M13mp8 clone carrying a 249-bp *Bam*HI-*Bgl*II insert containing the 5′ section of the *R. meliloti nodD1* gene	16
Plasmids		
pBR325	repColE1 Cb^r Tc^r Cm^r	4
pRK2013	repColE1 Km^r Tra$^+$ *mob*$^+$ used as a helper plasmid in conjugations	8
pUC8	Cb^r ColE1 cloning vector	37
pUC8-4	pUC8 with a 4-kb *Eco*RI-*Bam*HI subfragment carrying ORFD	This work
pJS144	pACYC184 carrying a 1.7-kb *Eco*RI fragment containing the *nodD1* of *R. meliloti*	J. Schmidt and M. John (personal communication)
pBH264	pRK290 derivative carrying the *nodD* gene from MPIK3030	19
pRK290	Wide-host-range cloning vector; Tc^r Tra$^-$ *mob*$^+$ IncP	8
pLAFR1	Wide-host-range cosmid, derived from pRK290; Tc^r	13
pGV910	rep pVS1 Cb^r Cm^r Sm^r/Sp^r *mob* ColE1 *mob* RP1	R. Deblaere (personal communication)
pRG910-12	pGV910 with a 12.7-kb *Eco*RI fragment carrying the ORS571 *nod* locus *1*	This work
pRG290-12	pRK290 with a 12.7-kb *Eco*RI fragment carrying the ORS571 *nod* locus *1*	14
pRG290-12::M3	pRG290-12 containing a *nodC-lacZ* fusion	14
pRG290-12::M21	pRG290-12 containing a *nodB-lacZ* fusion	14
pRG290-12::T20	pRG290-12 containing a *nodA-lacZ* fusion	14
pRG290-12::M63	pRG290-12 containing an ORF4-*lacZ* fusion	14
pRG910-12::M3	pGV910-12 containing a *nodC-lacZ* fusion	14
pRG701::M3	pBR325 containing a 12.7-kb *Eco*RI fragment carrying the ORS571 *nod* locus *1* genes with *lacZ* fused to the *nodC* gene	14
pRG701::M63	pBR325 containing a 12.7-kb *Eco*RI fragment carrying the ORS571 *nod* locus *1* genes with *lacZ* fused to ORF4	14
pRG100	pLAFRI clone isolated from the ORS571 gene bank carrying a functional *nodD* gene	This work
pRG910-16	pGV910 containing a 16-kb *Eco*RI fragment of the pRG100 insert	This work
pRG910-4	Subclone of pRG910-16 containing a 4-kb *Eco*RI-*Bam*HI fragment carrying the ORS571 *nodD* gene	This work
pRG910-16::Tn*5*-3	pRG910-16 containing a Tn*5* insertion in ORFD	This work

to 100 μg/ml, kanamycin was added to 25 μg/ml, and carbenicillin was added to 100 μg/ml.

Escherichia coli cultures were grown on LB medium (24) with the addition of antibiotics, when needed, at the following concentrations (micrograms per milliliter): tetracycline, 10; kanamycin, 25; carbenicillin, 100; streptomycin, 100; spectinomycin, 100; and nalidixin, 60.

Molecular cloning techniques. Standard molecular biology techniques for restriction enzyme digests, cloning, electrophoresis, fragment isolation from gels, and labeling of DNA fragments were as described previously (24). In pGV910, no useful *Bam*HI site is available for subcloning the 4-kilobase-pair (kb) *Eco*RI-*Bam*HI subfragment of pGV910-16, carrying open reading frame D (ORFD). Therefore, a *Hin*dIII site was generated immediately next to the *Bam*HI site of this 4-kb fragment by subcloning in pUC8 and digestion of the resulting clone (pUC8-4) with *Eco*RI-*Hin*dIII. Cloning of this fragment in *Eco*RI-*Hin*dIII-cut pGV910 resulted in the recombinant plasmid pGV910-4.

DNA sequence determination and analysis. A 1.1-kb DNA fragment of the pUC8-4 insert was sequenced, using M&G paper to bind DNA according to a modified Maxam-Gilbert sequencing procedure of Amersham (U.K.). Compilation and analysis of the sequence data was done by using the IntelliGenetics suite version 5.3 software for SUN.

DNA hybridizations. ORS571 total DNA preparation, labeling of double-stranded DNA probes, and DNA hybridizations were done as described previously (34). M13mp8-derived, single-stranded DNA was labeled by using the New England BioLabs sequencing primer 1211 and DNA polymerase I Klenow fragment as described by New England BioLabs.

When single-stranded DNA of phage M13mp8-*nodD1* (harboring a 249-nucleotide fragment of the amino-terminal part of *Rhizobium meliloti nodD1*; Table 1) was used as a hybridization probe against *Eco*RI-digested ORS571 total DNA, a large number of ORS571 fragments were found to hybridize. However, a similar pattern of homology was observed when labeled M13 DNA (without a *nodD* insert) was used as probe. This is reminiscent of observations described in literature where sequences in the M13 gene III

were shown to hybridize under low-stringency conditions to eucaryotic as well as to bacterial DNA fragments (20, 28).

Triparental matings. Matings were done for ORS571 as well as for *Agrobacterium* sp. by using the helper plasmid pRK2013 (8).

In vivo construction of pRG910-12::M3. Plasmid pRG701::M3 was transformed into the *polA E. coli* derivative CSH2110(pRG910-12). Since pRG701::M3 is a ColE1-derived replicon, it cannot be maintained in a *polA* strain but it can be rescued by cointegration via homologous recombination with the pRG910-12 insert. Cointegrates were screened for segregation of the pBR325 marker Tc^r and maintenance of the M3 (Mu dIIPR13) insertion marker Cm^r. The plasmid DNA of several putative double recombinants (pRG910-12::Mu3) was analyzed by restriction enzyme digest to verify the structure.

Tn*5* insertion mutagenesis of pRG910-16. The Tn*5* insertions in the clone pRG910-16 were isolated in *E. coli* MC1061 by the λ::Tn*5* mutagenesis method (7).

Isolation of an ORFD::Tn*5* homogenote. The 22-kb *Eco*RI insert from pRG910-16::Tn*5*-3 was cloned in pBR325. The resulting plasmid, pBR325-16::Tn*5*-3, was introduced into wild-type ORS571 by triparental mating.

From Tc^r Km^r transconjugants harboring a cointegrate, Km^r Tc^s derivatives were isolated after replica plating on medium with and without tetracycline. Total DNA of putative homogenotes was isolated, cut with *Eco*RI, and hybridized to a pBR325-16::Tn*5*-3 probe to verify their true structure.

β-Galactosidase assays. Quantitative β-galactosidase assays (using *o*-nitrophenyl-β-D-galactoside [ONPG] as a substrate) and screening of induced β-galactosidase expression on MacConkey agar (Difco Laboratories) plates were carried out as described previously (25). In vivo plate assays in the presence of *S. rostrata* plantlets or explants were performed as described before (14).

Nodulation tests. Nodulation tests were done as described previously (34).

Chemicals. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was purchased from Research Organics Inc., and ONPG was purchased from J. T. Baker Chemicals N.V. Naringenin was purchased from CarlRoth GmbH+Co.

RESULTS

Failure to find a *nodD*-related ORS571 gene by DNA hybridization studies. Since the expression pattern of the ORS571 common *nod* genes is very similar to that of the (brady)rhizobial *nod* operons, we thought it very likely that strain ORS571 could harbor one or more *nodD*-related genes. In a first attempt to identify such genes, we carried out hybridization experiments of *Eco*RI-digested total ORS571 DNA with well-characterized *nodD* clones (or subfragments) as probes. The same low-stringency hybridization conditions that had allowed us previously to detect a *nodC*-related ORS571 gene (34) were used. When the purified insert fragment of pJS144, carrying the *R. meliloti nodD1* gene, or the pBH264 insert fragment, carrying *nodD1* from strain MPIK3030 (Table 1), was used as a hybridization probe, no homology to ORS571 DNA could be detected (see Materials and Methods). Therefore, if *nodD*-related sequences are present in ORS571, we calculated them (according to reference 1) to be more than 44% divergent in nucleotide sequence as compared with the probes used.

Identification of a cloned ORS571 DNA fragment involved in naringenin-induced expression of ORS571 *nod* genes in an *Agrobacterium* background. Because of the negative results in the hybridization experiments, we switched to a functional approach based on a search for clones capable of activating in *trans* the expression of a reporter *lacZ* fusion in the presence of the inducing flavanone naringenin. Because we did not have at our disposal the equivalent of a $NodD^-$ ORS571 strain (such as, for instance, would be provided for *Rhizobium* strains by *sym* plasmid-cured derivatives), these experiments had to be carried out in a different bacterial background. For that purpose, *Agrobacterium tumefaciens* GV3101 (a Ti plasmid-cured C58C1 derivative) was chosen, since it has been shown previously (38) that *R. meliloti nodD1* can activate transcription of the common *nod* genes in an *Agrobacterium* background in the presence of alfalfa root exudate.

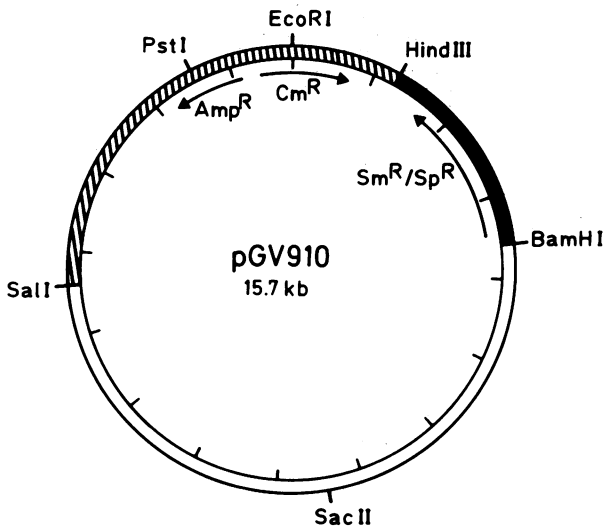

FIG. 1. Restriction map of the plasmid vector pGV910 (Van den Eede et al., unpublished data). Symbols: ■, R702 derived; □, pVS1 derived; ▥, pBR325 derived. The indicated restriction sites are unique. The scale is in kilobases.

A further prerequisite for this approach was the ability to maintain two different plasmids in the same bacterium: one containing a reporter *lacZ* fusion in a common *nod* gene and the other carrying a putative activator locus from ORS571. This could be achieved by combining two different wide-host-range plasmids: pRK290 (or the derived cosmid pLAFR1), selecting for tetracycline resistance, and pGV910, selecting for spectinomycin-streptomycin resistance. These plasmids are compatible and have approximately equal copy numbers (R. Deblaere, personal communication). Plasmid pGV910 is a derivative of pVS1 (21); its construction, features, and further applications are described elsewhere (G. Van den Eede et al., unpublished data), but for the sake of clarity a restriction map of pGV910 is given in Fig. 1.

A GV3101 derivative was constructed that harbors the pGV910-type plasmid pRG910-12::M3 carrying a *lacZ* gene fused to the common *nodC* gene of ORS571 (Fig. 2; see Materials and Methods). In this strain, no effect of naringenin on the expression of the *nodC-lacZ* fusion M3 could be measured (Table 2, line 1). Into this strain, a partial *Eco*RI gene bank of ORS571 in the cosmid vector pLAFR1 was introduced via triparental mating. To identify pLAFR1 clones allowing naringenin-inducible expression of the reporter gene, Tc^r Sp^r transconjugants were individually screened by spotting on MacConkey agar plates with and

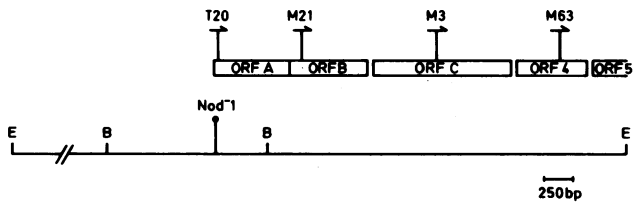

FIG. 2. Simplified restriction map of the 12.7-kb *Eco*RI fragment carrying the *nod* locus *1* of ORS571. Symbols: ⚲, position of the *nod*-Tn*5* insertion originally defining the *nod* locus *1*; →, positions and orientations of *lacZ* fusions T20, M21, M3, and M63. For details, see reference 14. Abbreviations for restriction enzymes: B, *Bam*HI; E, *Eco*RI.

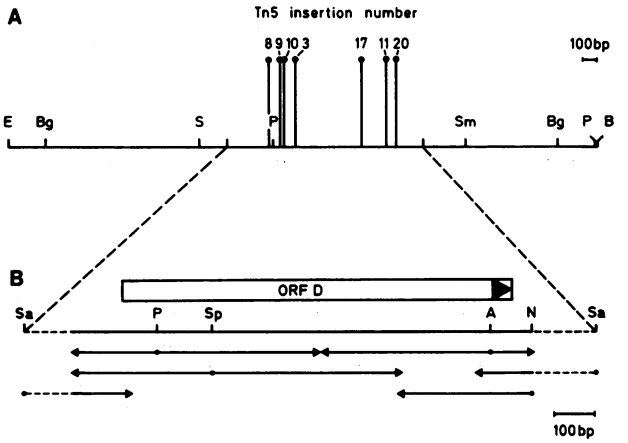

FIG. 3. Restriction map and sequencing strategy of *nod* locus *3*. (A) Restriction map of the 4-kb *Eco*RI-*Bam*HI fragment carrying the *nodD* gene of ORS571. ⚲, Positions of different Tn*5* insertions abolishing ORFD activity. (B) Strategy for sequencing the 4-kb *Eco*RI-*Bam*HI fragment carrying ORFD. Arrows indicate directions and lengths of the sequences determined; the arrowhead indicates the orientation of ORFD. Abbreviations for restriction enzymes: A, *Ava*I; B, *Bgl*II; E, *Eco*RI; N, *Nru*I; P, *Pst*I; S, *Sal*I; Sa, *Sau*I; Sm, *Sma*I; Sp, *Spl*I. Not necessarily all sites are indicated for each restriction enzyme.

without 20 μM naringenin. On this medium, colonies of the acceptor strain GV3101(pRG910-12::M3) were white-pinkish. Several hundred transconjugants were screened and found to be indistinguishable from the recipient strain except for one transconjugant that became bright red on the naringenin-containing medium.

From this transconjugant, the cosmid DNA (called pRG100) was isolated and transformed to *E. coli* MC1061 for further physical analysis. The pRG100 insert contained four *Eco*RI fragments of 16, 11, 1.5, and 1 kb, respectively. By subcloning, the 16-kb *Eco*RI fragment was found to be responsible for the transactivating capacity. For this subcloning and further characterizations, the putative activator loci were always cloned in the pGV910 vector so that they could be combined with the different *nod* locus *1* reporter fusions (Fig. 2) that were available in the pRK290-type vector (a situation opposite from the one used to identify the activating clone from the gene library).

A restriction map of the 16-kb *Eco*RI fragment was constructed, and by further subcloning the activating function was allocated to a 4-kb *Eco*RI-*Bam*HI fragment (Fig. 3).

Data presented in Table 2 (lines 1 to 7) illustrate the above-mentioned steps by quantitative measurements of the expression of the *nodC-lacZ* fusion M3 and the *nodA-lacZ* fusion T20. In the presence of the activator locus, a three- to fourfold increase in β-galactosidase units was observed 4 h after the addition of naringenin. Similar results (data not

TABLE 2. Quantitative β-galactosidase measurements of *nod-lacZ* fusions in different backgrounds[a]

Strain	Reporter plasmid	Activator plasmid	β-Galactosidase activity (U)	
			−Nar	+Nar
Agrobacterium tumefaciens				
1. GV3101	pRG910-12::M3 (*nodC-lacZ*)		49	50
2. GV3101	pRG910-12::M3	pRG100	63	145
3. GV3101	pRG290-12::M3		52	53
4. GV3101	pRG290-12::M3	pRG910-16	47	113
5. GV3101	pRG290-12::T20 (*nodA-lacZ*)		45	47
6. GV3101	pRG290-12::T20	pRG910-16	40	180
7. GV3101	pRG290-12::T20	pRG910-4	58	220
8. GV3101	pRG290-12::T20	pRG910-16::Tn*5*-3	39	43
Escherichia coli				
9. MC1061	pRG290-12::T20		51	50
10. MC1061	pRG290-12::T20	pRG910-4	70	64
Azorhizobium caulinodans				
11. ORS571 (wild-type)	pRG290-12::T20		27	1,081
12. ORS571-S (spontaneous mutant)	pRG290-12::T20		17	17
13. ORS571-S	pRG290-12::T20	pRG910-16	21	1,209
14. ORS571-S	pRG290-12::T20	pRG910-4	90	1,548
15. ORS571-S	pRG290-12::T20	pRG910-16::Tn*5*-3	10	10
16. ORS571-3 (homogenote Tn*5*-3)	pRG290-12::T20		16	16
17. ORS571-3	pRG290-12::T20	pRG910-16	47	1,320
18. ORS571-3	pRG290-12::T20	pRG910-4	92	1,375

[a] The β-galactosidase assays were carried out as described previously (14). For *Agrobacterium tumefaciens* and *E. coli*, *lacZ* activity was measured 4 h after induction with 20 μM naringenin (Nar); *A. caulinodans* fusions were measured after 12 h of induction with 10 μM naringenin. Media were as described in Materials and Methods. Background levels of *lacZ* activity were less than 5 U in GV3101, MC1061, and ORS571.

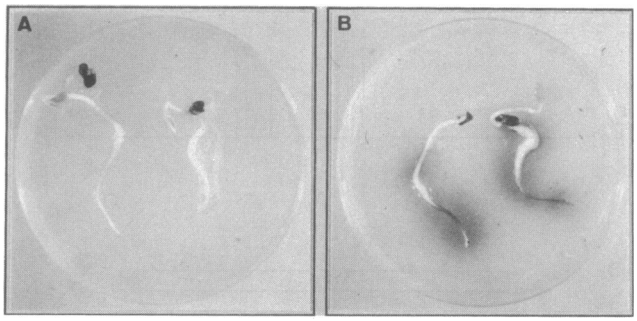

FIG. 4. In vivo assay for *nod* gene induction in *Agrobacterium* strain GV3101. On PA plates containing X-Gal (80 μg/ml) and a lawn of a GV3101 derivative, sterile seedlings of *S. rostrata* were incubated in the dark at 28°C for 12 h. (A) GV3101(pRG290-12::T20); (B) GV3101(pRG290-12::T20, pRG910-4).

shown) in *Agrobacterium* sp. were obtained with *lacZ* fusions in *nodB* (M21) and ORF4 (M63) (Fig. 2). In an *E. coli* MC1061 background, no such induction could be obtained. Inducing factors exuded by *S. rostrata* likewise enhanced the expression in *Agrobacterium* sp. of *nod* locus *1* gene fusions if an activator locus was present (Fig. 4).

Isolation of a spontaneous noninducible ORS571 mutation complementable by pRG910-16. While characterization of the ORS571 activator locus in *Agrobacterium* sp. was in progress, we isolated a spontaneous ORS571 mutant (ORS571-S) that had lost the ability to activate *nod* locus *1* genes in the presence of naringenin or host plant exudate. This mutant was isolated starting from an ORS571 derivative containing a pBR325 replicon-based reporter plasmid pRG701::M63 (Table 1) cointegrated in the genome. When this strain was plated on a medium containing naringenin, X-Gal, and tetracycline, colonies turned dark blue because of the induced expression of the ORF4-*lacZ* fusion M63. A pale blue colony was detected that, upon further investigation, was found to harbor a chromosomally located mutation affecting the expression of the *nod* locus *1* genes.

Indeed, after excision and subsequent loss of the pRG701::M63 plasmid, a Tc^s derivative strain resulted (ORS571-S) into which reporter plasmids with *lacZ* fusions in ORFA, ORFB, ORFC, or ORF4 were introduced. In all of the resulting strains, expression of the *lacZ* fusion could be induced neither by naringenin nor by host plant exudate. The mutant ORS571-S could be phenotypically complemented by introducing plasmid pRG910-16 or pRG910-4 (Table 1). Expression of the ORFA-*lacZ* fusion T20 in wild-type ORS571, in ORS571-S, in ORS571-S(pRG910-16), and in ORS571-S(pRG910-4) is presented in Table 2 (lines 11 to 14).

The spontaneous mutant ORS571-S was used as a recipient strain to delimit the activator locus by Tn*5* insertion mutagenesis.

Tn*5* mutagenesis of pRG910-16 to delimit the activator locus. Plasmid pRG910-16 encodes one or more functions involved in transactivation of the expression of *nod* locus *1* gene fusions in *Agrobacterium* sp. and is capable of complementing the spontaneous noninducible mutant ORS571-S.

A population of pRG910-16 derivatives harboring a Tn*5* insertion was generated in *E. coli* MC1061 (see Materials and Methods; 7). The mutated plasmids were introduced into strain ORS571-S(pRG290-12::T20) by triparental mating, and transconjugants were selected and simultaneously screened by plating on minimal medium (MMO) with X-Gal (40 μg/ml), spectinomycin (20 μg/ml), tetracycline (10 μg/ml), and naringenin (10 μM). Colonies that remained pale blue on this medium (indicating possible absence of complementation) were isolated; plasmid DNA was prepared and transformed to *E. coli* MC1061, with selection for the pRG910-16::Tn*5* markers (spectinomycin and kanamycin). The position of the Tn*5* insertion in each plasmid was determined via restriction mapping (Fig. 3).

The different Tn*5* insertions delimit a DNA stretch of approximately 0.8 kb that is essential for the transactivation of *nod* gene expression in the presence of the inducer. This conclusion was confirmed by introducing the individual mutant plasmids in strain GV3101(pRG290-12::T20), leading to transconjugants that were unable to induce β-galactosidase expression in the presence of naringenin or host plant exudate (Table 2, line 8).

Sequence of the activator locus. Using a modified Maxam-Gilbert procedure (see Materials and Methods), 1,110 nucleotides of the regulatory locus carried by the pRG910-4 insert and delimited by the Tn*5* insertion mutagenesis were sequenced (Fig. 3). Analysis of the sequence revealed a 942-base-pair (bp)-long ORF (ORFD) (Fig. 5), the location of which coincides with the mapped positions of the different Tn*5* insertions that inactivate the *trans*-acting regulatory function of pRG910-4 (Fig. 3).

Computer-assisted analysis of the sequence revealed that the nucleotide sequence as well as the deduced amino acid sequence of ORFD show significant homology with the corresponding sequences of (brady)rhizobial *nodD* genes. At the nucleotide level, the overall homology, comparing ORFD with *R. meliloti nodD1* and MPIK3030 *nodD*, adds up to 52 and 48%, respectively. In the protein sequence, about 51% of the amino acids are either identical residues (32%) or conservative substitutions compared with the sequence of eight other NodD proteins (Fig. 5). Long stretches of conserved residues are found in the amino-terminal half, whereas the rest of the protein shows a more dispersed type of conservation. The most proximally located conserved stretch encompasses 19 amino acids containing 13 identical residues and six conservative substitutions, one of which is a TTG-encoded leucine residue at position 1 (position 127 in the nucleotide sequence), which corresponds to the presumed methionine start residue of all other NodD proteins. Moreover, immediately upstream from this TTG codon are an in-frame stop codon and a strongly conserved Shine-Dalgarno sequence (33) (Fig. 5). These observations suggest that the TTG codon is the actual methionine start codon of the ORFD gene product.

ORFD ends at nucleotide 1079 with a TAG stop codon and as such encodes a 314-amino-acid polypeptide with a molecular mass of 35,565 daltons, a size comparable to that of other NodD proteins. By sequencing some 1,000 nucleotides downstream from ORFD (data not shown), no evidence was found for the presence of a large, cotranscribed ORF.

Analysis of the ORFD 126-bp upstream sequence revealed the presence of a divergently oriented *nod* box-related sequence (Fig. 5). This *nod* box is positioned relative to the TTG start codon very similarly to the *nod* box-*nodD* organization found in (brady)rhizobia (19, 30).

Genomic hybridization using ORFD as a probe. To find out whether ORFD is a unique gene or whether cryptic *nodD* sequences are present in the ORS571 genome, we carried out genomic hybridizations. Southern blots of *Eco*RI-, *Hin*dIII-, or *Bam*HI-digested total DNA of ORS571 were prepared and used in low-stringency hybridization experiments (see Materials and Methods). As a radioactive probe, the 896-bp *Pst*I-*Sac*I fragment (the DNA spanning nucleotides 204 to

```
1     TCGTGCAGAGATACCATGCGCTGTGCGCCTACAGCGAAGCAAGATGTGACGCTGGACAATCTTTCGTAGCT

72    ACGGCAAAATTCACCGTGGGACGACACTGCGGATGCTGGGTAGATGGAGAGTTAG TTG CGA TTT AAG
                                                    Met Arg Phe Lys

139   GGA CTT GAT CTG AAT CTG CTT GTC GCA CTG AAT GCT CTG CTT AGC GAG CAC AGC
      Gly Leu Asp Leu Asn Leu Leu Val Ala Leu Asn Ala Leu Leu Ser Glu His Ser

193   GTG ACA TCT GCA GCG AAG AGC ATC AAT CTC AGT CAG CCA GCC ATG AGC GCC GCA
      Val Thr Ser Ala Ala Lys Ser Ile Asn Leu Ser Gln Pro Ala Met Ser Ala Ala

247   GTC CAG AGA CTG CGG ATA TAT TTC AAC GAC GAT CTA TTC ACG ATT AAT GGG CGG
      Val Gln Arg Leu Arg Ile Tyr Phe Asn Asp Asp Leu Phe Thr Ile Asn Gly Arg

301   GAG CGC GTA TTT ACG GCT CGC GCC GAG TCC CTC GCA CCC GCC GTA CGA GAC ATC
      Glu Arg Val Phe Thr Ala Arg Ala Glu Ser Leu Ala Pro Ala Val Arg Asp Ile

355   CTT TCT CGT ATA CAG TCC AGC ATT ATT AAA GGG GAT CTG TTC GAG GCC GAC AGA
      Leu Ser Arg Ile Gln Ser Thr Ile Ile Lys Gly Asp Leu Phe Glu Ala Asp Arg

409   AGT GAG CGG GTA TTT CGG ATA ATT TCA TCA GAT TAT TCG ACA TCC ATA TTC ATT
      Ser Glu Arg Val Phe Arg Ile Ile Ser Ser Asp Tyr Ser Thr Ser Ile Phe Ile

463   AGA GGG GTT ATT TCC GCC GCC AGC ACC TCA TTA CCC CTA CTG AGA TTT GAA TTG
      Arg Gly Val Ile Ser Ala Ala Ser Thr Ser Leu Pro Leu Leu Arg Phe Glu Leu

517   ATT TCA CCC GAT GAC AAT TGC CAT GAT TTA CTC AAC AAG TCA GAG GTG GAC GCT
      Ile Ser Pro Asp Asp Asn Cys His Asp Leu Leu Asn Lys Ser Glu Val Asp Ala

571   TTG ATT ATG CCG GAA ATA TTT ATG TCG TCA GCC CAT CGA TTT GTA CCA CTG TTT
      Leu Ile MET Pro Glu Ile Phe MET Ser Ser Ala His Pro Phe Val Pro Leu Phe

625   GAG GAG AAA ATG GTT TGC GTT GGA TGC GCC AGA AAT CAT GAA GAT CGG AAC ATT
      Glu Glu Lys MET Val Cys Val Gly Cys Ala Arg Asn His Glu Asp Arg Asn Ile

679   TCT AGC ATT CAG GAG TAT TTG TCA ATG CGC CAC GTT GTT GCG AAG TTT GGT CGT
      Ser Ser Ile Gln Glu Tyr Leu Ser MET Arg His Val Val Ala Lys Phe Gly Arg

733   GGG ATG CGT CCC TCT CTT GAG GAA TGG TTT ATG GCG GAA AAC GGA ATG AGG AGG
      Gly MET Arg Pro Ser Leu Glu Glu Trp Phe MET Ala Glu Asn Gly MET Arg Arg

787   CGC ATC GAT ATA GTA GTA CAG TCG TTT TCG ATG ATT CCG CCT GTT ATT CAG GGA
      Arg Ile Asp Ile Val Val Gln Ser Phe Ser MET Ile Pro Pro Val Ile Gln Gly

841   ACG GAG CGT ATT GCG ATA ATG CCA TAT CGT CTT GTT GAA CAT TTT TCA AAA TTT
      Thr Glu Arg Ile Ala Ile MET Pro Tyr Arg Leu Val Glu His Phe Ser Lys Phe

895   ATG CCA TTA AAA GTC TTT GCG CTA CCA TTT CCT CTT CCG AGA TTC ACA GAA TGC
      MET Pro Leu Lys Val Phe Ala Leu Pro Phe Pro Leu Pro Arg Phe Thr Glu Cys

949   CTG CAA TGG CCT TCC ATT GCA ACC CCC GAT CTG GGT AAT CGC TGG TTG CGA GCA
      Leu Gln Trp Pro Ser Ile Ala Thr Pro Asp Leu Gly Asn Arg Trp Leu Arg Ala

1003  TAC CTA GCG GAC CAT ACA TCC CAA ATG ATG ATT TTG GAC AGC GCA GAA TAT TCG
      Tyr Leu Ala Asp His Thr Ser Gln MET MET Ile Leu Asp Ser Ala Glu Tyr Ser

1057  GGA GCT TCC ATA TAG TCGTTTTCCGATATGCAGAGATCAAGAGCTCTCAATCGC
      Gly Ala Ser Ile  *
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FIG. 5. Sequence of ORFD (*nod* locus *3*) of strain ORS571. The sequence of a 1,110-bp fragment is shown. Underlined bases indicate the Shine-Dalgarno-like sequences. The stop codon is indicated by an asterisk. The predicted amino acid sequence of the ORFD gene product is given in three-letter code under the nucleotide sequence. Shaded amino acids are those that are identical (boxed) or similar in the published sequences of *R. meliloti* 41 *nodD1* and *nodD2* (16), *Rhizobium leguminosarum* biovar *viciae* and biovar *trifolii nodD* (29, 31), *Bradyrhizobium parasponiae nodD1* (30), *Rhizobium* strain MPIK3030 *nodD1* (19), and *Rhizobium japonicum nodD1* and *nodD2* (3). Similar residues are those belonging to the groups DEQN, AGST, VLIM, KR, and WYF (one-letter code). The bases upstream from the TTG translational start codon that constitute a divergently oriented *nod* box-like sequence are boxed.

1101 in Fig. 5) was used. Upon exposure, a single hybridizing band became apparent in all cases tested, consistent with ORFD being present as a unique gene in ORS571 (Fig. 6). Exactly the same pattern of hybridization was visible with total DNA prepared from the mutant ORS571-S (data not shown), indicating the absence of large deletions or rearrangements in the ORFD region of this strain.

Symbiotic phenotype of an ORFD::Tn*5* homogenote and of the spontaneous mutant ORS571-S. A Tn*5* insertion (Tn*5*-3; Fig. 3) in ORFD was chosen for homogenotization in the ORS571 genome in order to study the effect of the inactivation of this gene on the symbiotic properties of the strain. A homogenote, ORS571-3, was isolated and characterized as described in Materials and Methods. When ORS571-3 was used in a triparental mating as an acceptor strain for *nod* locus *1-lacZ* reporter plasmids, the resulting transconjugants

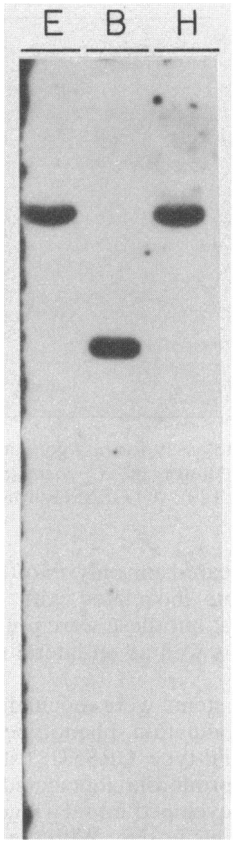

FIG. 6. Radiogram obtained after hybridization of Southern-blotted ORS571 genomic DNA to an ORFD specific probe (see text). The DNA was digested with *Eco*RI (lane E), *Hin*dIII (lane H), and *Bam*HI (lane B).

were unable to induce *lacZ* expression either by naringenin or in the presence of *S. rostrata* exudate (Table 2, line 16; Fig. 7). As expected, the noninducible phenotype was complemented by the introduction of either pRG910-16 or pRG910-4 carrying the wild-type *nodD* locus (Table 2, lines 17 and 18; Fig. 7).

Strain ORS571-3 was tested for its symbiotic phenotype by inoculation on *S. rostrata* roots and stems (Fig. 8). In neither case could nodules be obtained. Root inoculations occasionally yielded a few, very delayed-appearing nodules. Bacteria isolated from these nodules were ORS571 according to growth behavior, colony morphology, and the high level of carbenicillin resistance, but they had lost the Tn*5*-encoded kanamycin resistance marker. Upon reinoculation, these bacteria showed a normal nodulation behavior; by these criteria, we presume that the exceptional root nodules were due to the occurrence of rare revertants that had lost the Tn*5* insertion by excision. It can be concluded that inactivation of ORFD by Tn*5* insertion leads to a complete Nod^- phenotype on *S. rostrata* roots and stems. Introduction of pRG910-4 in the $NodD^-$ mutant ORS571-3 restored the nodulation capacity on roots as well as stems.

The spontaneous mutant ORS571-S, undistinguishable from the homogenote ORS571-3 with respect to absence of plant-inducible common *nod* gene expression, nevertheless differed in its nodulation phenotype: instead of being Nod^-, it showed delayed stem and root nodulation. Five days after inoculations, wild-type ORS571 yielded small nodules on all inoculated roots, whereas with the mutant ORS571-S, a few

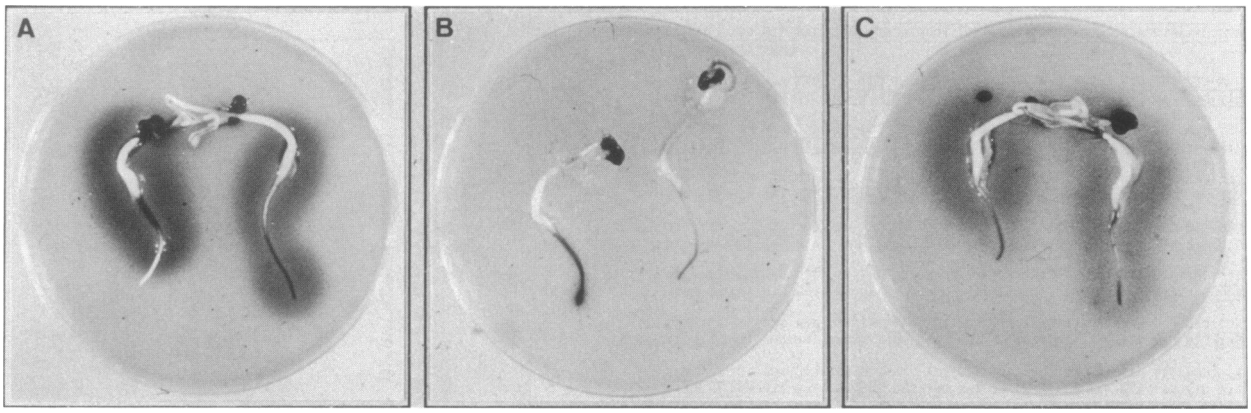

FIG. 7. In vivo plate assay for *nod* gene induction in ORS571. On MMO plates containing X-Gal (80 μg/ml) and a lawn of an ORS571 derivative, sterile seedlings of *S. rostrata* were incubated in the dark at 37°C for 12 h. (A) ORS571(pRG290-12::T20); (B) ORS571-3(pRG290-12::T20); (C) ORS571-3(pRG290-12::T20, pRG910-4).

small swellings appeared on only two-thirds of the inoculated roots. All roots inoculated with ORS571-S showed nodules after 10 days, but these were present on lower parts of the root system as well as on lateral roots and they had very irregular sizes.

When *S. rostrata* stems were inoculated with ORS571-S, again a delayed nodulation phenotype was apparent in comparison with wild-type ORS571. After 10 days, most infection sites (root primordia) inoculated with the wild-type strain had already developed into dark-green, beadlike nodules very homogeneous in size. With the ORS571-S mutant, far fewer infection sites developed into nodules, and the nodules that appeared were very heterogeneous in size, either remaining very small or growing abnormally large (Fig. 8).

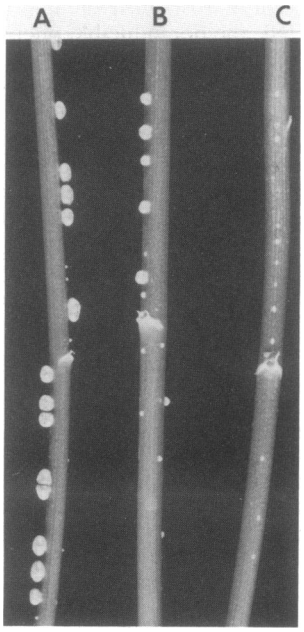

FIG. 8. Stem nodulation with ORS571 derivatives. Tests were done as described previously (34). Shown are sections of the stem 10 days after infection with the indicated strain. (A) Wild-type ORS571; (B) ORS571-S; (C) ORS571-3.

DISCUSSION

The (brady)rhizobial *nodD* genes play a central role in the flavonoid-induced expression of the *nod* regulon. In a previous report, we documented the presence of a flavonoid-regulated common *nod* operon in *A. caulinodans* ORS571 (*nod* locus *1*; 14). Upstream from this operon, no *nodD*-like gene was present. Furthermore, hybridization experiments with ORS571 total DNA using the *R. meliloti nodD1* gene or the NGR234 *nodD1* gene as a probe did not allow us to detect related sequences in the ORS571 genome. Here, we described the isolation by a functional approach of an ORS571 activator gene which, by several criteria, can be considered a *nodD* gene.

The activator locus was isolated from an ORS571 gene bank by screening for transactivation of an *Azorhizobium nodC-lacZ* fusion in an *Agrobacterium* background and in the presence of the inducer naringenin. Subcloning of the locus delimited its location to a ±4-kb *Eco*RI-*Bam*HI fragment, and by Tn*5* mutagenesis the regulatory function was further delimited to a 0.8-kb fragment. Homogenotization in the ORS571 genome of a Tn*5* insertion in the activator region resulted in a mutant strain, ORS571-3, that was unable to induce the expression of common *nod-lacZ* fusions in the presence of *S. rostrata* exudate or the inducing flavanone naringenin and unable to elicit nodules on the roots or the stems of *S. rostrata*. Both mutant phenotypes were complemented upon introduction of a clone carrying the wild-type activator locus. These data together with the results of hybridization experiments indicate the presence in ORS571 of a unique *nodD*-like regulatory gene (*nod* locus *3*). There is no evidence for a close linkage between the activator locus and *nod* locus *1* or *2* (34).

Further confirmation of the *nodD*-like character of the activator gene was found by DNA sequence analysis. A single, 942-bp-long ORF is present (ORFD) which encodes a 35,565-dalton protein. At the nucleotide level, ORFD shows some 50% homology with several published *nodD* genes. Although this level of similarity is significant, it is the lowest observed thus far for this gene family. Therefore, it seems that in *Azorhizobium* spp. the *nodD* gene is evolutionary more divergent, a situation also reflected in the low degree of conservation exhibited by the common *nod* genes (14). At the protein level, the overall amino acid identity between the putative ORFD gene product and eight other NodD proteins

adds up to 32%. The highest level of homology was found in the amino-terminal part of the protein, possibly containing a helix-turn-helix domain involved in the binding of DNA.

Translation of ORFD most probably starts at a TTG initiation codon. There are several arguments that support this unusual situation. The strong amino-terminal homology between the ORFD-derived protein and all other tested NodD proteins places the methionine start codon of the latter at precisely the position of this TTG codon in the former. Furthermore, immediately upstream from the TTG codon is an in-frame stop codon. Finally, at the appropriate position upstream from the presumed TTG start codon, a well-conserved Shine-Dalgarno sequence is located. The use of this alternative start codon has been reported in only a few exceptional cases (15). Interestingly, one example is the regulatory *virG* gene of *Agrobacterium rhizogenes* and possibly *Agrobacterium tumefaciens* (2). Perhaps this situation reflects a kind of translational control involved in the synthesis of optimal concentrations of these regulatory proteins. Immediately upstream from the ORS571 *nodD* gene is a divergently oriented *nod* box-related sequence, the function of which is unknown. A *nod* box with the same relative orientation and position was also found upstream from several other *nodD* genes (19).

In this report we have also described the isolation of a spontaneous ORS571 mutant (ORS571-S) with the same noninducible phenotype as the NodD$^-$ Tn*5* homogenote ORS571-3 but with a different nodulation phenotype. The spontaneous mutant was not completely Nod$^-$ but showed delayed stem and root nodulation. The aberrant nodulation as well as the absence of plant-inducible common *nod* gene expression were complemented upon introduction in ORS571-S of a clone carrying the wild-type *nodD* locus. When such a clone carried a Tn*5* insertion in ORFD, no complementation occurred. These genetic data indicate that the spontaneous mutation may be located in the *nodD* gene. Possibly the defective phenotype of ORS571-S is a result of a point mutation in ORFD or its promoter, since no major rearrangements or deletions in the ORFD region were evident upon hybridization analysis, although the presence of a small deletion or rearrangement cannot be excluded for the moment. From sequencing data, we know that ORFD is not followed by another cotranscribed gene; therefore, the difference in nodulation behavior between ORS571-3 and ORS571-S cannot be explained by a polar effect of the Tn*5* insertion on expression of a downstream gene involved in nodulation. A possible explanation that is worth investigating is that whereas in ORS571-3 the *nodD* function is completely abolished by Tn*5* insertion, in ORS571-S the mutation affects only part of the function (inducible activation) of the regulatory protein but leaves intact another function that contributes in an as yet unknown way to nodulation.

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