Identification of a New Inducible Nodulation Gene in Azorhizobium caulinodans

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The narrow host range bacterial strain *Azorhizobium caulinodans* ORS571 induces the formation of nitrogen-fixing nodules on the root and stem of the tropical legume *Sesbania rostrata*. Here, a new flavonoid-inducible locus of ORS571 is described, locus 4. The locus was identified and isolated via the occurrence of particular sequences, the γ and δ elements. These elements are reiterated in the ORS571 genome, linked to symbiotic loci. Sequencing of locus 4 showed the presence of an open reading frame (ORF6) that is flanked downstream by a γ element and upstream by a δ element. The γ element is approximately 180 bp in size, and shows homology to the insertion element ISRm3, an insertion sequence belonging to a distinct class of IS elements. The δ element is about 300 bp in size and has homology with repeated sequences found in other Rhizobiaceae. The ORF6 gene product shows a low, but significant homology to the mouse mastocytoma antigen P35B (Szikora et al., EMBO J. 9: 1041-1050, 1990) and to a class of NAD/NADP-binding sugar epimerase/dehydrogenases (Pisowotzki et al., Mol. Gen. Genet. 231: 113-213, 1991). Immediately upstream from ORF6, a nod box-related sequence is present, the arrangement of which is fully consistent with a recently presented model for the nod box structure (Goethals et al., Proc. Natl. Acad. Sci. USA 89: 1646-1650, 1992). Insertional inactivation of ORF6 did not affect the nodulation and fixation performance on *S. rostrata*. However, on *S. formosa* roots the nodulation kinetics of such a mutant was clearly affected (about 5 days delay). We propose to call this new symbiotic gene *nolK*.

The genera *Rhizobium* and *Bradyrhizobium*, together with the more recently described genus *Azorhizobium* (type species *A. caulinodans* strain ORS571; Dreyfus et al. 1988) constitute a group of soil bacteria that are capable of inducing the formation of nitrogen-fixing nodules on the roots of leguminous plants. The host plant for *A. caulinodans* is the tropical legume *Sesbania rostrata* Brem which grows during the rain season in the Sahel region of West Africa and, as an adaptation to waterlogged conditions, carries vertical rows of dormant root primordia all along its stem. These primordia will develop into roots when submerged in water; they develop into nitrogen-fixing nodules upon infection by *A. caulinodans* (Dreyfus and Dommergues 1981). A characteristic of strain ORS571 is its narrow host range of infection: Only on *S. rostrata* fully nitrogen-fixing nodules are formed. On other *Sesbania* species nodules may be induced that are, however, ineffective (Fix⁻) (Ndoye 1990).

In *Bradyrhizobium* and *Rhizobium* species sets of nodule (nod) genes have been identified that are involved in the nodulation process and are organized in nod operons under the positive transcriptional control of one or more regulatory nodD genes (for a review, see Long 1992). These nodD genes encode LysR-type regulatory proteins (NodD) that respond to signal molecules present in host plant exudates. Upon expression of the nod operons, the bacteria produce a return signal (the nod factor), a lipo-oligosacchride of specific structure that is released in the environment and induces changes related to the nodulation process in the host plant cells (Lerouge et al. 1990; Spank et al. 1991; Truchet et al. 1991; Dénarié and Roche 1992). In *A. caulinodans*, an essential nod locus (locus 1) carries genes homologous to the (brady)rhizobial common nodABC genes and is transcribed in the presence of *S. rostrata* seedling exudate (Van den Eede et al. 1987; Goethals et al. 1989). The most prominent inducer molecule in this exudate was shown to be 7,4'-dihydroxyflavanone (liquiritigenin; Messens et al. 1991). Also, the related flavanone naringenin turned out to be a good inducer of the azorhizobial nodABC operon (Goethals et al. 1989). The induction process is mediated by the azorhizobial locus 3, encoding the NodD activator protein (Goethals et al. 1990) that binds to conserved motifs (NodD boxes) found in nod promoter sequences (nod boxes) (Goethals et al. 1992).

As an approach to studying nod factor biosynthesis, we want to identify and sequence the bacterial genes that are expressed early in the interaction, under the control of the nodD gene. The identification of these genes in *A. caulinodans* is hampered by the absence of a Sym plasmid and by the fact that the symbiotic loci found until now are dispersed over the chromosomes. Moreover, sequencing of several ORS571 nod genes revealed them to be too divergent from (brady)rhizobial homologues to be picked up by DNA hybridizations.

Another way to proceed is to make use of repeated sequences that often seem to be linked to symbiotic loci (reviewed by Martinez et al. 1990). Here, we give an example of such an approach: The isolation of a new plant-inducible nod gene is described, which was identified by the presence of flanking repeated sequences that are also located upstream from locus 1 and downstream from locus 3.
MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used are listed in Table 1. Growth conditions and antibiotic concentrations for ORS571 and Escherichia coli were as described before (Goethals et al. 1989).

Molecular biology techniques. Standard molecular biology techniques for DNA isolation, restriction analysis, DNA labeling, cloning, Southern blotting, and hybridization were as described by Sambrook et al. (1989). Low-stringency hybridizations were carried out at 60°C using 6× SSC (1× SSC is 0.18 M NaCl plus 0.015 M sodium citrate). Electroporation of E. coli cells was done with a gene pulser (Bio-Rad, Richmond, CA) according to the manufacturer’s instructions.

Insertion mutagenesis of the locus 4 was achieved by cloning of the Ω cassette of pPH45Ω (see Table 1) in the unique BssHII site of pRG9011 (carrying itself the 2.3-kb SalI subfragment of pRG901 cloned in pBR325; Table 1) yielding pRG9011-ΩB. Simultaneously, the 8.4-kb EcoRI insert fragment of pRG901 was subcloned in the EcoRI site of the broad host range vector pLAFR1 (Table 1) yielding pRG9010, and this construct was electroporated into the polA− E. coli strain CSH2110. Next, pRG9011-ΩB was electroporated to CSH2110(pRG9010) and, as pBR325 cannot replicate in a polA− background, growth on a medium containing tetracycline (pLAFR1), spectinomycin, and streptomycin (Ω) resulted in the selection of single recombinants in which pRG9010 and pRG9011-ΩB are cointegrated. Double recombinants, whereby the Ω cassette is introduced in the 8.4-kb EcoRI fragment, were isolated after screening for the loss of the pBR325 carbenicillin marker and were inspected by restriction enzyme analysis (data not shown). From a resulting recombinant, pRG9010-ΩB, the 10.4-kb EcoRI insert fragment was isolated and recloned in the EcoRI site of pBR325, generating construct pRG9011ΩB. Finally, this plasmid was mobilized by triparental mating to ORS571 and, because pBR325 cannot replicate in this strain, screening for tetracycline-sensitive and streptomycin/spectinomycin-resistant colonies resulted in the isolation of homogenates where the wild-type DNA is exchanged for the mutant DNA as controlled by hybridization analysis (data not shown). The mutant strain ORS571-ΩS, carrying an Ω element in the Smal site upstream from nolK (Fig. 1) was generated in a similar way.

DNA sequencing procedures were basically as described by Sanger et al. (1977). Sequence processing and interpretation was done with the IG Suite and PC/GENE packages from IntelliGenetics, Inc., Mountain View, CA. Oligonucleotide synthesis was carried out on an Applied Biosystems (Foster City, CA) model 394 DNA/RNA synthesizer according to the manufacturer’s recommendations.

Triparental matings and β-galactosidase tests. Triparental mating using pRK2013 as a helper plasmid (Table 1) and quantitative β-galactosidase assays using o-nitrophenyl β-D-galactoside as a substrate were carried out as described before (Goethals et al. 1989).

Nodulation assays. Sterilization of S. rostrata seeds, growth of S. rostrata seedlings, nodulation, and acetylene reduction assays were done according to Van den Eede et al. (1987). For the other plants tested, essentially the same approach was followed. Nodulation was scored every 2 days. For every strain tested, 10 individual plants were inoculated; each experiment was done at least twice.

RESULTS

Identification of nod locus 4. The expression of A. caulinodans nod locus 1 is naringenin inducible, and the

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<td>Strains</td>
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<td>Azorhizobium caulinodans type strain able to nodulate stems and roots of Sesbania rostrata</td>
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<td>ORS571-3 A nodD− derivative of ORS571</td>
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Fig. 1. Physical-genetic map of locus 4. A, Schematic representation of the locus 1 upstream region (left) and the locus 3 downstream region (right). Stripped box, γ1 element; dotted box, δ1 element. Black arrowheads indicate the direction of translation of the respective genes or the arbitrary orientation of a repeated element. An open arrowhead indicates the position and the polarity of a nod box sequence. Scale is the same as for B. B, Physical-genetic map of the locus 4. Black arrowheads point the direction of ORF6 (white box) expression or the relative orientation of the repeated elements γ and δ. An open arrowhead indicates the position and orientation of a nod box sequence. ΩB and ΩS indicate the positions of the Ω cassette insertions in the respective mutants (see Results). Stripped box, γ1 element; dotted box, δ1 element. Abbreviations: B, BssHII; Bg, BglII; Sa, SalI; Sm, SmaI; Sp, SphI; X, XhoI. Scale is in kilobases. C, Representation of the different fragments (relative to B) cloned in pMP220. The arrows indicate the orientation of the lacZ gene. The table shows the β-galactosidase units (Miller units, see Materials and Methods) for the respective cloned fragment in a wild type ORS571 (W. T.) and in the nodD− strain ORS571-3 in the presence (+) and absence (−) of 10 μM naringenin. ND, not determined. |
locus contains a nod box-type promoter (NB1) (Goethals et al. 1992). To identify other similarly regulated ORS571 genes an oligonucleotide spanning the NB1 was synthesized, end-labeled, and used as a probe in hybridizations under different stringencies against several digestes of total ORS571 DNA. Apart from the expected bands, no other related sequences could be identified using this approach. However, when a 12.8-kb EcoRI fragment containing part of locus I and approximately 8 kb upstream (Goethals et al. 1989) was used as a probe, many fragments from an EcoRI-digested ORS571 total DNA cross-hybridized, even at high stringencies. This observation was taken as an indication of the presence of repeated sequences in the ORS571 genome. A preliminary characterization of cross-hybridizing sequences showed the occurrence of several classes of repeats, some of which are present in only two copies, others in five or more (M. Holsters, unpublished results). One particular element, called γ, was present in two copies only, one copy located immediately upstream of the nodABC operon (γ₁) (Fig. 1), another copy located on an 8.4-kb EcoRI fragment (γ₂). This 8.4-kb EcoRI could be isolated as part of a cosmid clone upon colony hybridization against an ORS571 pLAFRI genomic library. It turned out that the same 8.4-kb EcoRI fragment cross-hybridized to another sequence δ₁, present downstream from the ORS571 nodD gene in locus 3. Like the γ elements, the δ elements are present in only two copies in the ORS571 genome. On the 8.4-kb fragment, γ₂ and δ₂ are separated by approximately 1.5 kb. The linkage of the δ and γ₁ elements to ORS571-symbiotic loci prompted us to investigate the possible occurrence of symbiotic genes, perhaps similarly regulated as locus 1, linked to γ₂. A screening for naringenin-inducible promoter activity was done by cloning of subfragments of the 8.4-kb EcoRI fragment in the promoter probe plasmid pMP220 (Table 1), upstream from a promoterless β-galactosidase gene and determining the levels of lacZ expression in Azorhizobium in the presence and the absence of naringenin. As shown in Figure 1, an inducible promoter activity could indeed be ascribed to an approximately 2.3-kb Sall-BgIII fragment and was further delimited to be contained within an approximate 400-bp Sphi-Smal fragment. This inducible

Fig. 2. Sequence of the locus 4 2,333-bp Sall fragment. The repeated sequences γ₁ and δ₁ are underlined. The orientation of the fragment is opposite to that of Figure 1. The boxed sequence upstream of ORF6 is the nod box-related sequence. Immediately upstream of the ATG and GTG possible start codons, the potential Shine-Dalgarno sequences are boxed. The potential GTG start codon is boxed. Three asterisks indicate the ORF6 stop codon.
expression was dependent on the presence of an intact nodD gene (Fig. 1). As it turned out from mapping of homologies and sequencing data (see further), the inducible promoter activity should not be ascribed to either of the two repeated elements but to a nod box-type sequence.

**Sequence of nod locus 4.** The sequence of the entire 2.3-kb SalI fragment was determined and is presented in Figure 2. An open reading frame (ORF6 in Fig. 1) starts either at an ATG start codon or at a GTG codon located 48 bp further downstream. In the former case (ORF6 ATG), a 329-amino acid protein would be encoded with a molecular mass of 36,225 Da and a pI of 5.88. In the latter case (ORF6 GTG), a 313-amino acid protein of molecular mass 34,378 Da and a pI of 6.08 would be produced. We favor the latter start codon (GTG) as the actual in vivo start codon because it is preceded by a more elaborate Shine-Dalgarno (SD) sequence (Stormo et al. 1982). Furthermore, using the PC/GENE computer program for detection of protein-coding regions, a higher coding score was obtained for ORF6 GTG (score 4) than for ORF6 ATG (score 7). Databank (SWISSPROT release 20 and PIR release 30) searches with the ORF6 gene product as a query revealed a low, but significant homology with the mouse mastocytoma transplantation antigen P35B (Szikora et al. 1990) (Fig. 3). Further examination of the sequence revealed the presence of a putative NAD/NAP-binding domain in the amino-terminal part of the protein. Low, but significant, homology was found with a class of nucleotide sugar metabolizing enzymes that bind NAD/NAP and of which UDP-glucose-4-epimerase (Lemaire and Müller-Hill 1986) showed the best fit (Fig. 3; for more details, see Discussion).

Upstream from ORF6, in a 422-bp SalI-SphI fragment associated with the inducible promoter activity, a correctly oriented nod box-related sequence is present. Upon comparison of this nod box (NB2) with nod boxes from (brady)rhizobia and with the ORS571 nod box from nod locus 1 (NB1), a high sequence divergence was found in all cases (about 50% homology; Fig. 4).

**Fig. 3.** Homology of the ORF6 gene product with the mouse mastocytoma transplantation antigen P35B and UDP-glucose-4-epimerase. a, P35B antigen; b, ORF6 gene product; c, UDP-glucose-4-epimerase (Eschericia coli). Double points indicate identical amino acids or conserved substitutions belonging to either of the groups EQDN, WYF, HKR, or AGST. Amino acid positions identical in the three sequences are shaded. The putative β-turn-α-helix-8-turn motif found in NAD/NAP-interacting proteins is boxed. The invariant glycine residues are indicated separately in bold.

**Fig. 4.** The nod box-related sequence of locus 4. A, Alignment of the nod boxes of: Rm(abc), the R. meliloti common nod operon; Ac(ORF6), the A. cauliformans locus 4 (NB2); Ac(abc), the A. cauliformans common nod operon (NB1). The boxed bases indicate the nodD boxes: the repeat ATC-N4-GAT or a related motif. B, Indication of the inverted repeat structure of the NodD boxes found in the nod box of: Rm(abc), the R. meliloti common nod operon; Ac(ORF6), the A. cauliformans locus 4 (NB2); Ac(abc), the A. cauliformans common nod operon (NB1). A point indicates the center of the palindrom.
two types of bacteria were present in the nodules. We also used a dilution series of wild type and mutant to nodulate roots and observed no differences.

To test the possible involvement of ORF6 in host specificity of nodulation, we compared the nodulation behavior of wild type and mutant on several tropical legumes, some of which allow the formation of non-nitrogen-fixing (Fix') nodules with ORS571. The plants tested were *S. grandiflora*, *S. cannabina*, *S. emerus*, *S. formosa*, *Acacia albida*, *A. radiana*, *Aeschynomene indica*, and *Neptunia oleracea*. The only difference in nodulation behavior occurred on root nodulation of *S. formosa*. On this *Sesbania* species, *A. cauliformis* can induce large, white, nonfixing nodules. An approximate 5-day delay in nodule appearance was observed with ORS571-1B over a 25-day period (Fig. 6). Because of this phenotype on the one hand and the expression pattern on the other hand, we propose to call the described locus a nod locus (nod locus 4) and propose the name nolK for ORF6.

Using a nolK internal BstHII-Stul fragment as a probe for hybridization against ORS571 total DNA, we observed some faintly hybridizing bands at lower stringencies (data not shown). These data might indicate the presence of different nolK-related genes in OR571, a finding that could explain the weak effect of the nolK mutation on nodulation.

An ORS571 derivative with an Ω insertion in the δ2 element of locus 4 (ORS571-1; Fig. 1) was also constructed and tested on *S. rostrata* and the other plant species listed above. No differences in nodulation or nitrogen fixation characteristics were observed.

**DISCUSSION**

In this paper, a new symbiotic locus of *A. cauliformis* strain OR571 is described. The expression of this locus (locus 4) is induced by the flavanone naringenin. This induction is mediated by the *A. cauliformis* nodD gene product (NodD) and could be correlated with the presence of a nod box-related sequence (NB2). The sequence of NB2 displays only about 50% homology to (brady)rhizobial nod boxes as well as to the *A. cauliformis* nod box from the common nod locus (NB1 in locus 1). This might indicate that locus 1 and locus 4 were acquired independently from different strains, or it might illustrate the ancient nature of the ORS571 nod genes.

The nucleotide conservation between NB1 and NB2 is in concordance with a recently proposed model concerning the fundamental structure of a nod box (Goethals et al. 1992). In this model, the nod box as originally defined (Rostas et al. 1986) represents a modular structure with different juxtaposed NodD boxes acting as the binding sites for the NodD proteins and having the typical inverted repeat structure ATC-N2-GAT. Also, in NB2 two such consecutive NodD boxes occur (Fig. 4). The most 5'located NodD box of NB2 constitutes a perfect inverted repeat over its entire length (Fig. 4).

Downstream from NB2, an open reading frame (ORF6) is present, which is derived from the protein of which showed a low, but significant homology with the mouse mastocytoma transplantation antigen P35B (Szikora et al. 1990), the function of which is unknown. In the amino-terminal part of the ORF6 protein, a motif could be discerned that corresponds to the so-called β-turn-α-helix-β-turn domain.

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**Fig. 5.** Sequence alignment of the γ1 and δ1 elements. A. Sequence alignment of the γ1 and γ2 repeated elements. Some flanking bases are included to show the divergence at the borders. B. Sequence alignment of the δ1 and δ2 repeated elements. Some flanking bases are included to show the divergence at the borders.

**Fig. 6.** Nodulation kinetics on *Sesbania formosa*. +, ORS571 nodulation; ■, ORS571-1B nodulation. For each strain, the data are the mean of 10 plants tested.
involved in NAD/NADP binding by several proteins (Rossman et al. 1975). Typical for this domain is the presence of a stretch of invariant glycine residues. As these motifs are characteristically located at the very amino-terminus of a protein, this again argues for the GTG start codon being the actual translation start. A low, but significant, homology was also found between the ORF6 gene product and a class of sugar epimerases that use NAD/ NADP as cofactor and of which UDP-glucose-4-epimerase (GalE) of E. coli (Lemaire and Müller-Hill 1986) was taken as an example (Fig. 3). The amino acid conservation between the ORF6 gene product and both the P35B and the UDP-glucose-4-epimerase protein, corresponds well with the amino acid residues noted by Pissowitzki et al. (1991) to be more or less conserved between nucleotide sugar metabolizing enzymes. Therefore, we postulate that the ORF6 gene product encodes a nucleotide sugar epimerase/dehydrogenase that uses NAD/NADP as a cofactor.

Insertional inactivation of ORF6 resulted in a derivative strain (ORS571-ΔB) that was able to nodulate the stems and the roots of S. rostrata equally well as the wild type ORS571. Also, no differences in fixation capacities of the nodules were evident. Different other Sesbania species (which form Fix’ nodules with ORS571) and other genera of tropical Leguminosae were used in root nodulation tests to investigate the possible involvement of the locus in host range determination. The only difference scored was a reduced nodulation kinetics on S. formosa; nodules appeared on this plant with an approximate 5-day delay. This phenotype suggests the involvement of ORF6 in nodule induction and, combined with the expression pattern, led us to assign the locus as a nod locus and to propose the name nolK for ORF6. Possibly, the locus is involved in the optimization of the nodulation process under conditions occurring in the natural environment, the rain season in the Sahel region, and as such functions in the ecology of the nodulation process.

Hybridization studies suggested the occurrence of other nolK-related sequences in ORS571. Perhaps this could explain the weak effect of nolK inactivation on the nodulation behavior as shown before for the R. meliloti nodFQ and nodM genes (Schwedock and Long 1990; Baev et al. 1991).

The nolK gene is flanked at both sides by sequences that are repeated once in the ORS571 genome, linked to other symbiotic loci. The γ sequence is approximately 180 bp long with one copy present downstream of nolK and the other copy (84% homologous) located upstream of the common nod locus of ORS571. Databank searches with this region as a query revealed an intriguing homology with ISRm3, an insertion sequence that was described in R. meliloti and that is a member of a distinct class of IS elements (Wheatcroft and Laberge 1991). The ISRm3 homology extends 280 bp to the left of γ1 (Fig. 1) until the end of the available sequence information. The degree of homology adds up to an overall 50%, but in the last approximate 200 bp of the sequence this increases up to 68%. At the protein level, a clearcut homology (about 60%) with the ISRm3 transposase is evident when one ignores three frameshift mutations in the 5′ end of the γ1-linked sequence. These data imply the occurrence in A. caulino-
dans of two related IS elements (or their relics), belonging to the ISRm3 family. It is tempting to speculate on the possible involvement of these elements in the evolution and dispersion of the ORS571 nodulation genes.

Upstream of nolK, the 300-bp δ1 element is present, a 95% homologous copy of which (δ1) is located downstream of the ORS571 nodD gene. When the δ element was used as a query against a databank, we found some 53% homology with a R. fredii repetitive element that is associated with the nodABC genes of this strain (Krishnan and Pueppke 1991). The homology was confined to a part of this repetitive element carrying two overlapping ORFs, which are also found in the Agrobacterium rhizogenes Ri T-DNA, although at the protein level no obvious homology could be shown. Whereas the R. fredii repetitive element is homologous to the B. japonicum RSRβ insertion sequences, we could only detect a weak homology between the δ element and RSRβ sequences. Again the true nature of the δ elements remains obscure, although one can argue that they are derived from insertion elements and may have played (or still play) a role in the evolution of the nodulation genetics of A. caulindans.

Currently, we are investigating the structure and biosynthesis of the azorhizobial nod factor. In the future, we would like to correlate structural properties of the factor with the function of specific nod genes. As a first approach we can study the effect of the nolK mutation on structural modifications of the ORS571 nod factor.

Isolation of other ORS571 nod genes involved in factor biosynthesis is impeded by the absence of a Sym plasmid, the divergence of the nod sequences compared to the ones of (brady)rhiizobia and their dispersed localization on the ORS571 chromosome. Furthermore, the divergence of the nod box sequence in A. caulindans itself precludes the possibility of using a nod box probe as a tag for flavonoid-inducible nod genes. In fact, hybridization experiments with such a probe gave no useful results. As an alternative, we can make use of the peculiarity of some repeated elements to be linked to symbiotic loci. As an illustration, we showed the isolation of nod locus 4 (nolK) using the γ element. Still other classes linked to symbiotic loci are present in the ORS571 genome and may be exploited further to clone other genes involved in nodulation and eventually factor biosynthesis.

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**LITERATURE CITED**


