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Research Note

Sequence and Mutational Analysis of the 6.7-kb Region Containing *nod*AFEG Genes of *Rhizobium* sp. Strain N33: Evidence of DNA Rearrangements

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A 6.7-kb region upstream of *nod*BC genes in *Rhizobium* sp. strain N33 was shown to contain the *nod*AFEG genes and an open reading frame designated orfZ. The open reading frames for these genes contain 591, 282, 1209, 738, and 1,338 nucleotides respectively. Homologues of these genes were found in other rhizobia with the exception of orfZ, for which there was no counterpart found in the GenBank/EMBL database. Tn*5* mutagenesis in *nod*EG and in the intergenic *nod*G-B region has shown a Nod$^+$ phenotype on their temperate hosts *Onobrychis viciifolia* and *Astragalus cicer*. The nodules formed on *O. viciifolia* plants by these mutants were altered in shape and size. However, on *A. cicer* there was only a reduction in the number of nodules formed, compared with the wild-type strain. Sequence analysis of the orfZ-*nod*A and *nod*G-B intergenic regions indicates the presence of truncated *nod*D genes.

Additional keywords: nodulation, symbiosis.

The symbiosis between bacteria of the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* with their leguminous host plant results in the formation of root and stem nodules in a species-specific way, in that they have a narrow or a broad host range. Many of the genes involved in the symbiotic association that results in nodulation are well characterized (for review see Dénarié et al. 1992, 1996; Carlson et al. 1994). The so-called common *nod*ABC genes are required for nodule formation (Long 1989) and encode enzymes that synthesize the Nod factor core molecule, which is then modified by the host-specific nodulation gene products. Nod factors are β-(1-4) linked oligomers of N-acetylglucosamine to which a fatty acyl chain is linked at the nonreducing terminal end (Spaink et al. 1991). Species-specific structural modifications or variations include, for example, the length of the N-acetylglucosamine oligomer, which may vary from three to five residues. Moreover, the length and the degree of unsaturation of the fatty acyl chain and modifications of the reducing terminal end are determinant for host-specific recognition (Lerouge et al. 1990; Spaink et al. 1991; Sanjuan et al. 1992; Price et al. 1992; Poupot et al. 1993; Carlson et al. 1993; Mergaert et al. 1993; Bec-Ferte et al. 1993). Mutations in these host-specific nodulation genes cause a variety of phenotypes, including delayed nodulation, reduced number of nodules, and altered host specificity.

Homologues of the *nod*FE genes are found in *R. meliloti, R. leguminosarum* bv. *viciae,* and *R. leguminosarum* bv. *trifolii*. The NodF protein is similar to the acyl carrier protein of *Escherichia coli* (Shearman et al. 1986) and, like the acyl carrier protein, NodF carries a phosphopantetheine group (Geiger et al. 1991). The NodE protein has been localized in the cytoplasmic membrane (Spaink et al. 1989) and is similar to the fatty acid synthases of *E. coli* (FabB) and *Saccharomyces cerevisiae*; it is also similar to the putative β-ketoacyl synthase of a *Streptomyces* sp. (Bibb et al. 1989). Therefore, it is proposed that these proteins (NodFE) are involved in the synthesis and transfer, via NodA, of the polyunsaturated fatty acid to the nonreducing terminal end of the N-glucosamine residue (Spaink et al. 1989; Geiger et al. 1991; Spaink et al. 1993; Demont et al. 1993). In *R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii* the *nod* operon containing the *nod*FE genes is important for host specificity. It was shown by Djordjevic et al. (1985) that *R. leguminosarum* bv. *trifolii* strain ANU843, which contains a Tn*5* insertion in the *nod*E gene, poorly nodulates white and red clover but has acquired the ability to nodulate peas. In *R. leguminosarum* bv. *viciae*, mutation in the same gene greatly reduces nodulation of peas or *Vicia hirsuta* (Downie et al. 1985). It has been shown, at the molecular level, that a mutation within the *nod*E gene of *R. leguminosarum* bv. *viciae* results in the replacement of the highly unsaturated acyl chain (C18:4) by a mono-unsaturated (C18:1) acyl chain (Spaink et al. 1991). It has been reported up to now that the *R. meliloti nod*G gene has no counterpart among rhizobia (Dénarié et al. 1992). Inconsistent results have been obtained for the *nod*G gene containing a Tn*5* insertion in

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R. meliloti. One report showed that this mutation had no effect (Swanson et al. 1987), whereas another indicated a reduction in the number of nodules and a delay in nodule appearance (Horvath et al. 1986).

Rhizobium sp. strain N33 is among many rhizobia isolates from the Canadian high arctic that have been characterized for their adaptation to low temperature. These strains were shown to nodulate many arctic and temperate legumes of various genera, such as *Astragalus*, *Onobrychis*, and *Oxytropis* (Prévost et al. 1987b). Moreover, in another study it was shown that *Onobrychis viciifolia* (sainfoin) plants nodulated by arctic rhizobia and grown at 15°C had greater nitrogenase activity when tested at 5 and 10°C than sainfoin plants nodulated by temperate rhizobia (Prévost et al. 1987a). Arctic rhizobia also promoted better growth of sainfoin at low temperature, compared with temperate strains (Prévost et al. 1994). Here, we report the DNA sequence and mutational analysis of a 6.7-kb region containing the *nod*AFEG genes and an open reading frame orfZ, and we show evidence of DNA rearrangements in strain N33 involving *nod*D genes.

Identification of *nod*AFEG genes and sequence analysis.

We have recently reported the identification of the common nodulation genes *nod*BCIJ (Cloutier et al. 1996b) and the host-specific nodulation genes *nod*HPQ in *Rhizobium* sp. strain N33 (Cloutier et al. 1996a). By Southern hybridization, with the *nod*ABC genes from *Bradyrhizobium japonicum* as a probe on total genomic DNA of strain N33, we showed previously that this probe hybridized with a 4.1-kb *Eco*RI fragment (pJC1) coding potentially for the *nod*A gene (Cloutier et al. 1996b; Table 1). We have sequenced this 4.1-kb *Eco*RI fragment and showed that it encodes for *nod*F, *nod*E, and *nod*G genes and part of the *nod*A gene. The rest of the *nod*A gene was found on a contiguous 0.4-kb *Eco*RI fragment (pJC7) and next to this fragment we identified a 2.4-kb *Eco*RI fragment (pJC4) that contains an open reading frame of 1,338 nucleotides designated orfZ (Fig 1; Table 1). The nucleotide sequence data are to be found at GenBank/EMBL as accession number U53327.

The deduced amino acid sequences encoded by *nod*FEG of *Rhizobium* sp. strain N33 were compared with those of other *Rhizobium* spp. (Table 2). The deduced amino acid sequence encoded by *nod*A gene of strain N33 was also compared: there was 55 to 69% amino acid identity found with the corresponding proteins from *Azorhizobium caulinodans* (Goethals et al. 1989), *R. meliloti* (Török et al. 1984; Egelhoff et al. 1985), *R. fredii* (Krishnan and Pueppke 1991), *R. leguminosarum* bv. *viciae* (Rossen et al. 1984), *R. leguminosarum* bv. *trifolii* (Schofield and Watson 1986), *Rhizobium* sp. NGR234 (Relić et al. 1994), *R. leguminosarum* bv. *phaseoli* (Vázquez et al. 1991), and *Bradyrhizobium* sp. *parasponia* (Scott 1986). No homology to the orfZ was found in the GenBank/EMBL database.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Rhizobium		
N33	Wild type	Prévost et al. 1987b
N33	Wild type, Sm^r	This study
JC20	*nod*G::Tn*5*, Sm^r Nm^r	This study
JC47	orfZ::Tn*5*, Sm^r Nm^r	This study
JC62	orfZ::Tn*5*, Sm^r Nm^r	This study
JC72	*nod*E::Tn*5*, Sm^r Nm^r	This study
JC226	Intergenic *nod*G-B::Tn*5*, Sm^r Nm^r	This study
JC306	Intergenic *nod*G-B::Tn*5*, Sm^r Nm^r	This study
Plasmids		
pJC1	4.1-kb *Eco*RI fragment containing *nod*FEG genes and part of *nod*A gene cloned into pUC18	This study
pJC4	2.4-kb *Eco*RI fragment containing orfZ cloned into pUC18	This study
pJC7	0.4-kb *Eco*RI fragment containing part of *nod*A gene cloned into pUC18	This study

[a] Nm = neomycin; Sm = streptomycin.

Shape and size of nodules induced on *O. viciifolia* and *Astragalus cicer* by *Rhizobium* sp. strain N33 mutants and their nodulation kinetics.

Various mutants in the *nod*EG genes and in orfZ were obtained following Tn*5* mutagenesis (Fig. 1) and tested for nodulation kinetics and phenotypes as described previously (Cloutier et al. 1996b). Derivatives of *Rhizobium* sp. strain

Table 2. Percent protein identity of NodFEG proteins between *Rhizobium* sp. strain N33 and other *Rhizobium* spp.

		R. leguminosarum	
***Rhizobium* sp. strain N33**	***R. meliloti***	**bv. *viciae***	**bv. *trifolii***
NodF	70.2	63.4	61.3
NodE	80.4	79.9	77.4
NodG	87.0		

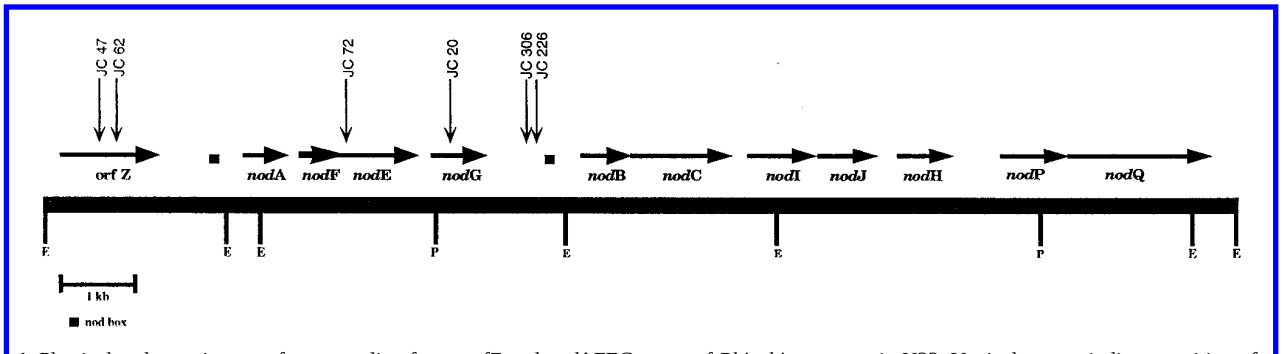

Fig. 1. Physical and genetic map of open reading frame orfZ and *nod*AFEG genes of *Rhizobium* sp. strain N33. Vertical arrows indicate position of various Tn*5* insertions in orfZ and in the *nod*AFEG genes. The *nod* box and positions and direction of transcription of orfZ and *nod*AFEGBCIJHPQ are indicated. Restriction sites for mapping are indicated: E, *Eco*RI; P, *Pst*I.

N33 harboring mutations in the *nod*E gene (JC72), *nod*G gene (JC20), and in the intergenic *nod*G-B region (JC306) have shown a Nod^+ phenotype on their temperate hosts *A. cicer* and *O. viciifolia* (Fig. 2; Table 1). These mutants showed a reduced number of nodules on *A. cicer* plants, compared with the wild-type strain N33. This reduction in the number of nodules was not observed on *O. viciifolia* plants except for the intergenic *nod*G-B mutant (JC306), which showed a slightly reduced number of nodules (Fig. 2). The same nodulation phenotype was also obtained for another intergenic *nod*G-B mutant (JC226, data not shown). Mutations in the orfZ region (JC47 and JC62) caused no detectable change in nodulation phenotype, compared with the wild-type strain N33, indicating that this gene is not required for the nodulation of *A. cicer* and *O. viciifolia* plants (data not shown).

Nodules induced by the wild-type strain N33 on *O. viciifolia* are generally torpedo shaped and occasionally branched (Fig. 3A, B, and C). They occur singly or in cluster. The nodules induced by *nod*G mutant (JC20) were generally smaller but similar in shape to those induced by the wild-type strain N33 (Fig. 3D, E, and F). The nodules induced by *nod*E mutant (JC72) were also smaller but whiter, oval, and often clustered (Fig. 3G, H, and I). The intergenic *nod*G-B mutant (JC306) induced the formation of smaller, round nodules (Fig. 3J, K, and L). The same result was obtained with JC226 (data not shown). This phenotype could be due to a polar effect on downstream genes where, near the site of Tn*5* insertion, a consensus *nod* box is present before the *nod*BC genes. This polar effect, if present, did not totally block transcription of downstream genes since we have previously reported that mutation in *nod*BC genes caused a Nod^- phenotype on their temperate hosts (Cloutier et al. 1996b).

We have been able to recover bacteria from crushed nodules induced by all the mutants with a 50 to 75% recovery at day 40 on *O. viciifolia* plants, compared with strain N33, in which the recovery was 100%. However, fewer bacteria were recovered from nodules induced by all mutants compared with the wild type after 6 days of growth on yeast mannitol agar plates. This lower level of bacteria correlates with the smaller size of the nodules formed by the mutants. On *A. cicer* plants, the nodules induced by all the mutants were identical in size and shape to those formed by strain N33 (data not shown). The recovery of bacteria was the same (40%) for the wild-type strain and for all the mutants tested.

Analysis of the orfZ-*nod*A and *nod*G-B intergenic regions.

Sequence analysis indicated there was a region of homology shared between the intergenic regions orf*Z-nod*A and *nod*G-B in *Rhizobium* sp. strain N33. This region comprises three contiguous but distinct subregions of 92, 84, and 62% homology (Fig. 4). The *nod* boxes are part of the region that is 84% homologous. Further analysis of the intergenic regions orfZ-*nod*A and *nod*G-B indicated extensive rearrangements. In the orf*Z-nod*A region there are two contiguous regions (D-1 and D-2) corresponding, respectively, to nucleotides 1 to 254 and nucleotides 522 to 678 of the *nod*D3 gene (coding region)

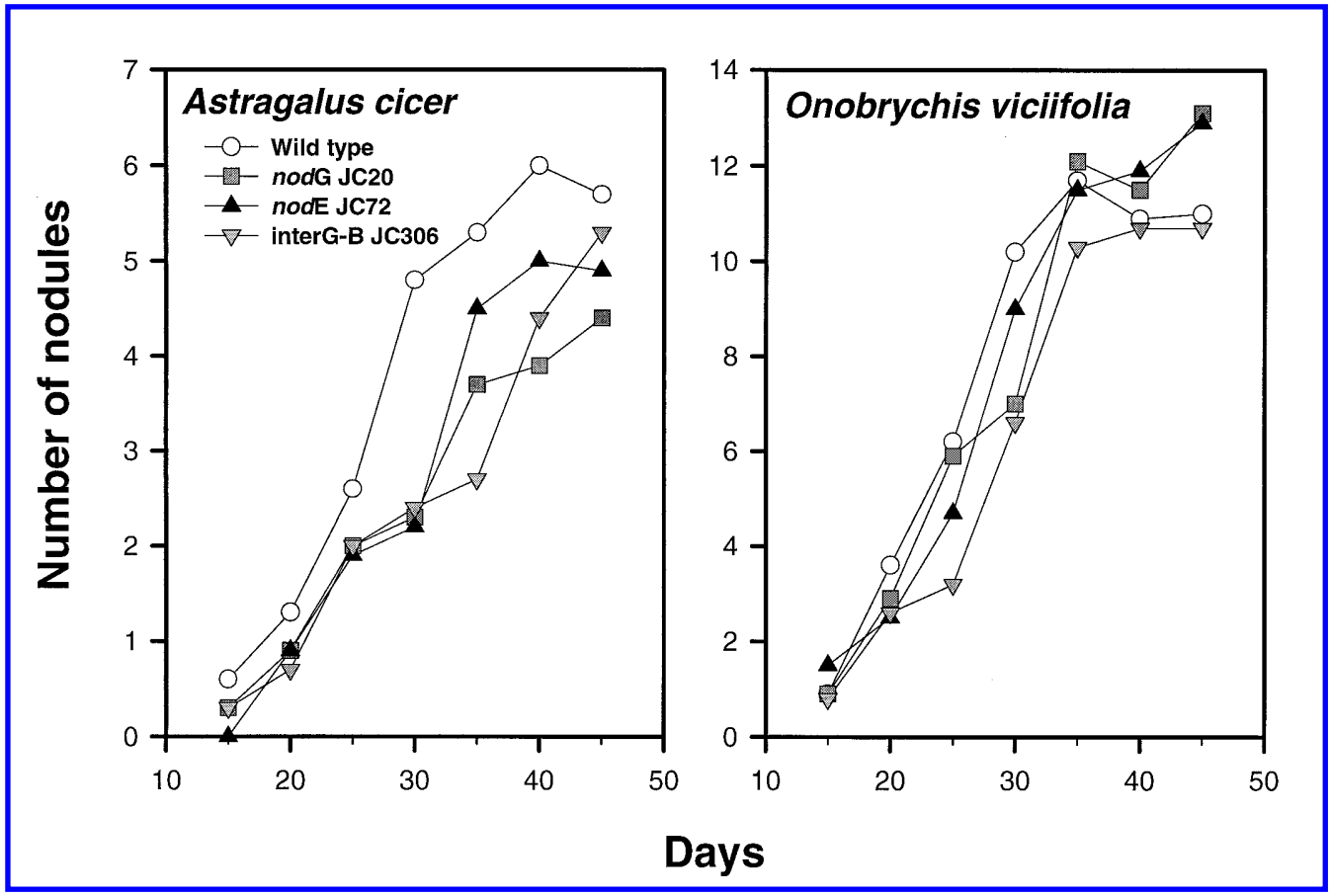

Fig. 2. Nodulation kinetics of *Rhizobium* sp. strain N33 and mutants carrying Tn*5* insertions in the *nod*EG genes and in the intergenic *nod*G-B region. Host plants tested are indicated. Each value represents the mean of 10 plants.

from *R. loti* (accession number U22899). These D-1 and D-2 regions are also homologous to the *nod*D gene from various rhizobia (data not shown). This result indicates that at least the C-terminal part of the *nod*D gene was deleted in this bacteria, as well as a region corresponding to nucleotides 254 to 522. In strain N33, these *nod*D pseudogene segments contain many mutations that created stop codons and frameshifts.

The intergenic *nod*G-B region contains two DNA segments (D-3 and D-4) corresponding, respectively, to nucleotides 1 to 93 and nucleotides 59 to 223 of the *nod*D3 gene from *R. loti* (Fig. 4). These D-3 and D-4 segments are separated by 312 bp in the *nod*G-B region. These data indicate also that extensive rearrangements have occurred. The *nod*G-B region contains also a *nod*A pseudogene encoding for the last 24 amino acids of the C-terminal part, indicating that at one point there was a *nod*A gene present beside *nod*B in this bacteria. The *nod*A

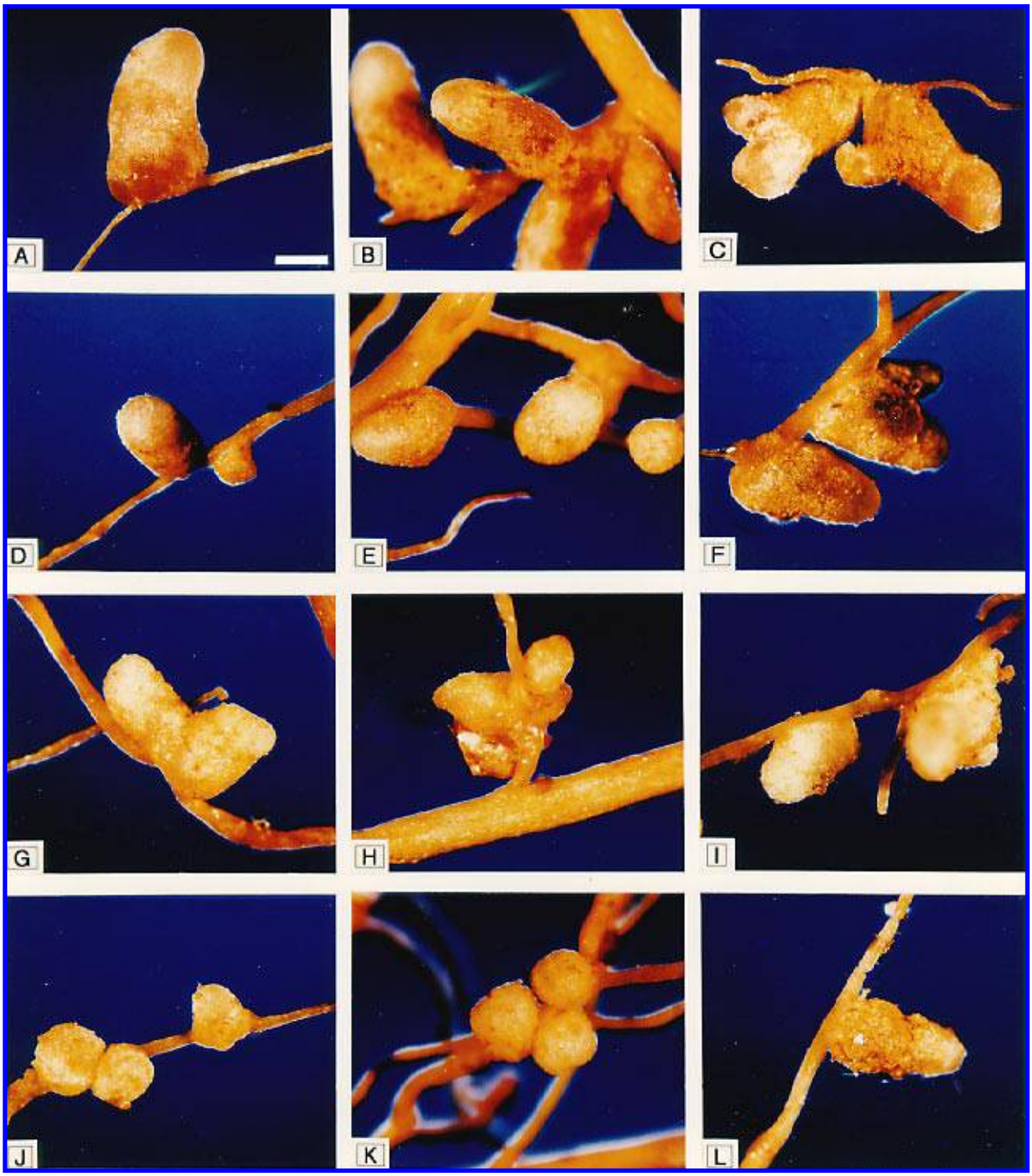

Fig. 3. Representative nodules induced on *Onobrychis viciifolia* by *Rhizobium* sp. strain N33 **(A, B,** and **C)** and mutants of strain N33 carrying Tn5 insertions in the *nod*G gene **(D, E,** and **F)**, the *nod*E gene **(G, H,** and **I),** and the intergenic *nod*G-B region **(J, K,** and **L)**. Scale bar corresponds to 0.28 cm.

gene is now present beside the *nod*F gene in *Rhizobium* sp. strain N33. It is noteworthy that the *nod*A pseudogene and the complete *nod*A gene of strain N33 are not more homologous with each other than with any other *nod*A genes, indicating that there might have been two totally different *nod*A genes in strain N33. There is also a region in these two intergenic regions that is highly homologous (74%) to an intergenic region of *R. loti* (accession number X65629) corresponding in this bacteria to a region located between the *nod*B and a *nod*A pseudogene. This arrangement thus appears to be conserved in *R. loti* and *Rhizobium* sp. strain N33, indicating either a common ancestry or that a transfer of DNA has taken place between the forebears of these bacteria.

The identification of this new 6.7-kb nodulation region containing *nod*AFEG genes indicates that strain N33 is very similar in *nod* gene content to *R. meliloti* since both species possess the *nod*AFEGBCIJHPQ genes. Thus, we expect that the Nod factor(s) produced by these species could be similar. We are currently working to determine the structure of the Nod factor(s) of *Rhizobium* sp. strain N33. Elucidation of this structure should tell us if other nodulation gene(s) could be present in this strain.

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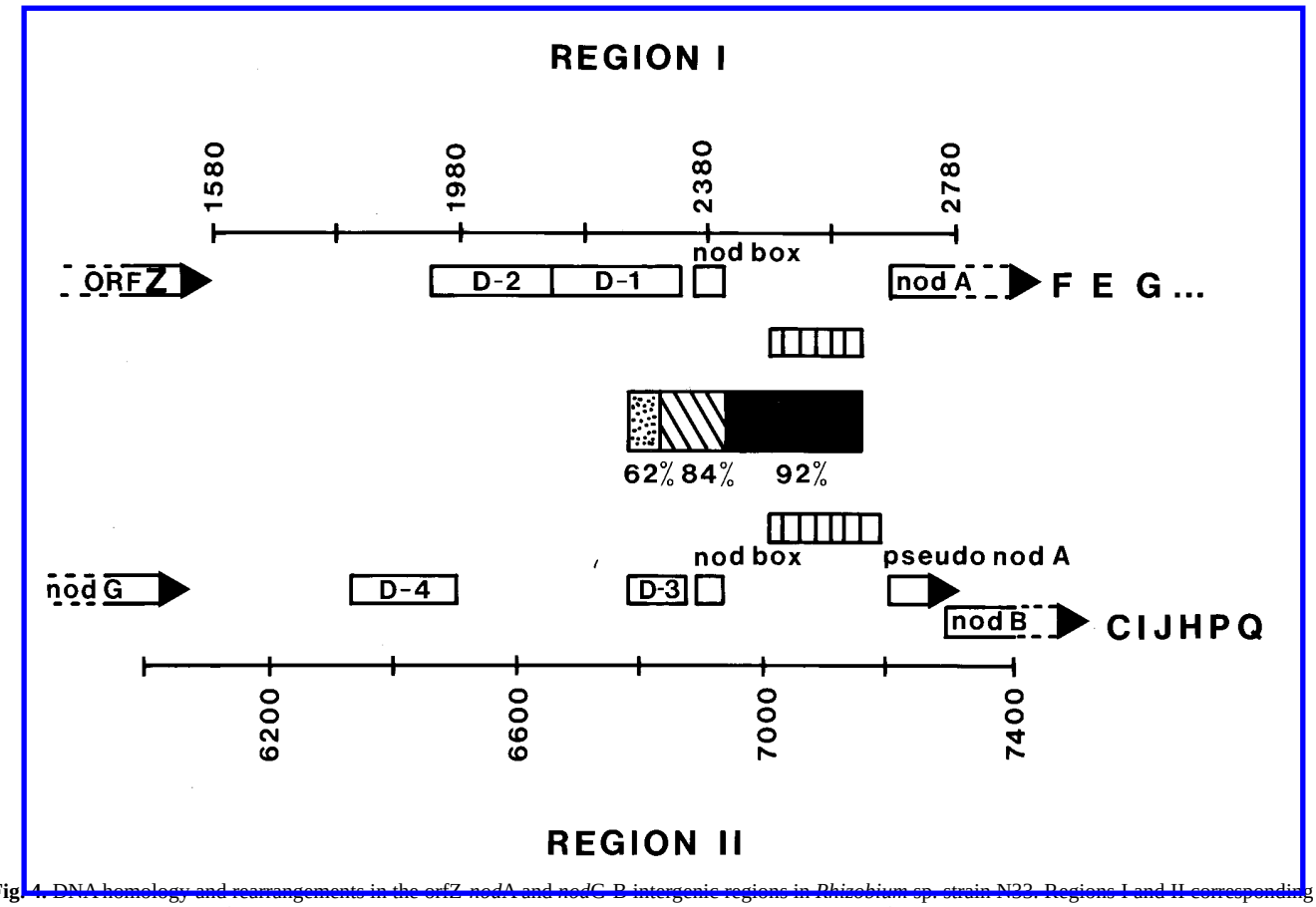

Fig. 4. DNA homology and rearrangements in the orfZ-*nodA* and *nodG*-B intergenic regions in *Rhizobium* sp. strain N33. Regions I and II corresponding to the intergenic regions orfZ-*nod*A and *nod*G-B are enlarged: numbers correspond to the nucleotides of the *nod* genes under GenBank/EMBL accession number U53327. Region I: between orfZ and *nod*A are indicated a *nod* box and two *nod*D pseudo genes (D-1 and D-2). Region II: between *nod*G and *nod*B are indicated a *nod* box and two *nod*D pseudo genes (D-3 and D-4). Three contiguous boxes and their percent identity between regions I and II are indicated: the two boxes above and below these three boxes correspond to a region that is homologous between *Rhizobium* sp. strain N33 and *R. loti*.

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