

Characterization and Mutational Analysis of *nodHPQ* Genes of *Rhizobium* sp. Strain N33

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We have shown, by sequencing the nodulation gene region of *Rhizobium* sp. strain N33 previously isolated from the Canadian high arctic, that the *nodHPQ* genes are located in a 4.8-kb region downstream of *nodBCIJ*. The open reading frames of *nodHPQ* are 747, 906, and 1941 nucleotides long, respectively. The strain N33 genome contains one copy of *nodH* and two copies of *nodPQ* that are homologous to those genes in *Rhizobium meliloti*. Tn5 insertions in the *nodHPQ* genes of strain N33 did not affect the formation of nodules on the two homologous hosts, *Astragalus cicer* and *Onobrychis viciifolia*. Since strain N33 contains the *nodBCIJHPQ* genes and the recently sequenced *nodAFEG* genes, we looked for similar host range with *R. meliloti*. Strain N33 and *R. meliloti* strains A2 and RCR2011 were shown to induce the formation of root nodules on plants of *O. viciifolia*. However, strain N33, compared with *R. meliloti* strains, was able to elicit a few, white, empty, root nodules on *Medicago sativa*. *R. meliloti* strains, compared with strain N33, were shown to induce only few nodules containing bacteria on *A. cicer*. Induction of *nod* genes transcription in strain N33 was shown to be induced by a variety of flavonoid compounds that are different from those inducing *nod* genes from *R. meliloti*.

Rhizobia are bacteria that induce the formation of nodules on the roots of leguminous plants in which they fix atmospheric nitrogen. They are characterized as having a broad or narrow host range according to the number of plant species with which they can form nodules (Young and Johnston 1989).

Many rhizobial species have in common the nodulation genes *nodABCIJ*, which are usually grouped in one transcriptional unit (Göttfert 1993; Carlson et al. 1994; Schultze et al. 1994). Transcription is activated by specific plant exudates, which interact in an indirect way with NodD protein (Mulligan and Long 1985; Peters et al. 1986). Nodulation genes from fast-growing rhizobia are generally induced by plant fla-

vonones or flavanones (Firmin et al. 1986; Peters et al. 1986; Redmond et al. 1986; Spaink et al. 1987), whereas slow-growing rhizobia nodulation genes are induced by isoflavones (Kosslak et al. 1987; Göttfert et al. 1988). The interaction may be complex; for example, it has also been reported that isoflavones are inhibitors of *nodD*-dependent induction in fast-growing rhizobia (Firmin et al. 1986; Redmond et al. 1986; Djordjevic et al. 1987). Mutation in any of the *nodABC* genes results in the absence of nodule formation (Long 1989); this mutation can be complemented by the homologous genes from other *Rhizobium* spp. without altering the host range (Kondorosi et al. 1984; Jacobs et al. 1985; Debelle et al. 1986). Recent evidence indicates that *nodABC* genes are involved in the synthesis of the lipo-oligosaccharide backbone of the plant-activating signal molecule (Nod factor): *nodA* encodes for an acyltransferase (Atkinson et al. 1994; Röhrig et al. 1994), *nodB* for a chitin oligosaccharide deacetylase (John et al. 1993), and *nodC* for a chitin oligosaccharide synthase (Geremia et al. 1994; Spaink et al. 1994).

Rhizobium spp. possess other nodulation genes (*hsn*) that confer host specificity properties. These are involved in the decoration of the Nod factor signal molecule. Specificity of the Nod factor molecules resides in the structure of the acyl group added at the nonreducing end and by various substitutions on the lipo-oligosaccharide backbone (for review see Carlson et al. 1994; Dénarié et al. 1992). Mutations in *hsn* genes cannot be fully complemented by genes from other *Rhizobium* spp. or biovars (Kondorosi et al. 1984; Debelle et al. 1986; Horvath et al. 1986). Their phenotype is often an alteration or extension of the host range (Debelle et al. 1986; Faucher et al. 1988; Cervantes et al. 1989). For example, *nodH* in *R. meliloti* is required for nodulation of particular host plants. Indeed, not only are *R. meliloti nodH* mutants not able to nodulate *Medicago sativa* L. but they are found to be able to nodulate *Vicia sativa* subsp. *nigra* (L.) Ehrh., which is not a usual host (Faucher et al. 1988). Further examples of host range determinant genes are *nodPQ*, of which there are two copies in *R. meliloti* (Schwedock and Long 1992). Tn5 insertions in only one of the *nodPQ* copies result in the simultaneous production of both sulfated and nonsulfated Nod factors, allowing infection of both *M. sativa* and *V. sativa* subsp. *nigra* (Roche et al. 1991). Furthermore, double *nodP₁Q₁-nodP₂Q₂* mutants, like *nodH* mutants, excrete only a nonsulfated factor (Roche et al. 1991; Schwedock and Long

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1992). It has been demonstrated by Schwedock and Long (1990) that *nodPQ* encode, for an ATP sulfurylase (*nodP*) and an adenosine 5'-phosphosulfate kinase (*nodQ*), the enzymes responsible for the synthesis of the activated sulfate donor 3'-phosphoadenosine 5'-phosphosulfate. These data indicate that the capacity of *R. meliloti* to nodulate *M. sativa* is determined by a sulfated Nod factor. It has been recently shown that NodH has an in vitro sulfotransferase activity and catalyzes the transfer of sulfate from 3'-phosphoadenosine 5'-phosphosulfate to the terminal 6-O position of *R. meliloti* Nod factors (Ehrhardt et al. 1995).

Rhizobium sp. strain N33, isolated from the Canadian high arctic, is a slow-growing, nitrogen-fixing organism. It has been shown to be able to induce root nodules on both arctic and temperate legumes of various genera, such as *Astragalus*, *Onobrychis*, and *Oxytropis* (Prévost et al. 1987a, 1987b). We have shown previously, by Tn5 insertions, that *nodBC* genes of strain N33 are essential for nodulation and that *nodIJ* genes determine the size and number of nodules that are formed (Cloutier et al. 1996). In this paper we have characterized the *nodHPQ* genes in strain N33 by DNA sequencing and report the phenotypes of Tn5 insertion mutants in these genes.

RESULTS AND DISCUSSION

Identification of *nodHPQ* genes and sequence analysis.

We have shown previously that the nodulation genes *nodBCIJ* of *Rhizobium* sp. strain N33 are located on a 8.2-kb *Pst*I fragment (Cloutier et al. 1996). By DNA sequencing the region downstream of *nodJ*, we found a sequence with homology to *nodH* of *R. meliloti*. The DNA sequence of a 5.7-kb

*Eco*RI fragment (contained partly in the 8.2-kb *Pst*I fragment) revealed the presence of the *nodH* and *nodP* genes and part of the *nodQ* gene. The rest of the *nodQ* gene was found on a 0.6-kb *Eco*RI fragment contiguous to the 5.7-kb *Eco*RI fragment. The nucleotide sequences of the *nodH*, *nodP*, and *nodQ* genes are to be found at GenBank/EMBL accession number U53327. The nucleotide sequences of the *nodH*, *nodP*, and *nodQ* genes are 77.2, 74.8, and 68.2% homologous, respectively, to the corresponding genes of *R. meliloti*. The putative protein encoded by the strain N33 genes *nodH*, *nodP*, and *nodQ* shares 71, 75, and 65% amino acid identity, respectively, with its *R. meliloti* counterpart and 67, 69.3, and 63.3% amino acid identity, respectively, with its *R. tropici* counterpart (Debellé et al. 1986; Folch-Mallol et al. 1996). We have also shown by Southern hybridization of total genomic DNA from *Rhizobium* sp. strain N33 digested with *Eco*RI that *nodH* is present in one copy per genome while the *nodPQ* genes are present in two highly homologous copies (data not shown).

Nodulation characteristics of *Rhizobium* sp. strain N33 and *R. meliloti* on *Onobrychis viciifolia*.

We have shown previously that strain N33 contains the *nodBCIJ* genes (Cloutier et al. 1996). In this paper, we have shown that strain N33 contains the *nodHPQ* genes, and recently we have shown that it also contains the *nodAFEG* genes (J. Cloutier, S. Laberge, and H. Antoun, unpublished). Since strain N33 possesses overall the *nodAFEGBCIJHPQ* genes, which are homologous to those of *R. meliloti*, we were prompted to compare the host range of strain N33 with that of *R. meliloti*. Strain N33 and *R. meliloti* strains A2 and RCR2011 induce the formation of nodules on *Onobrychis vi-*

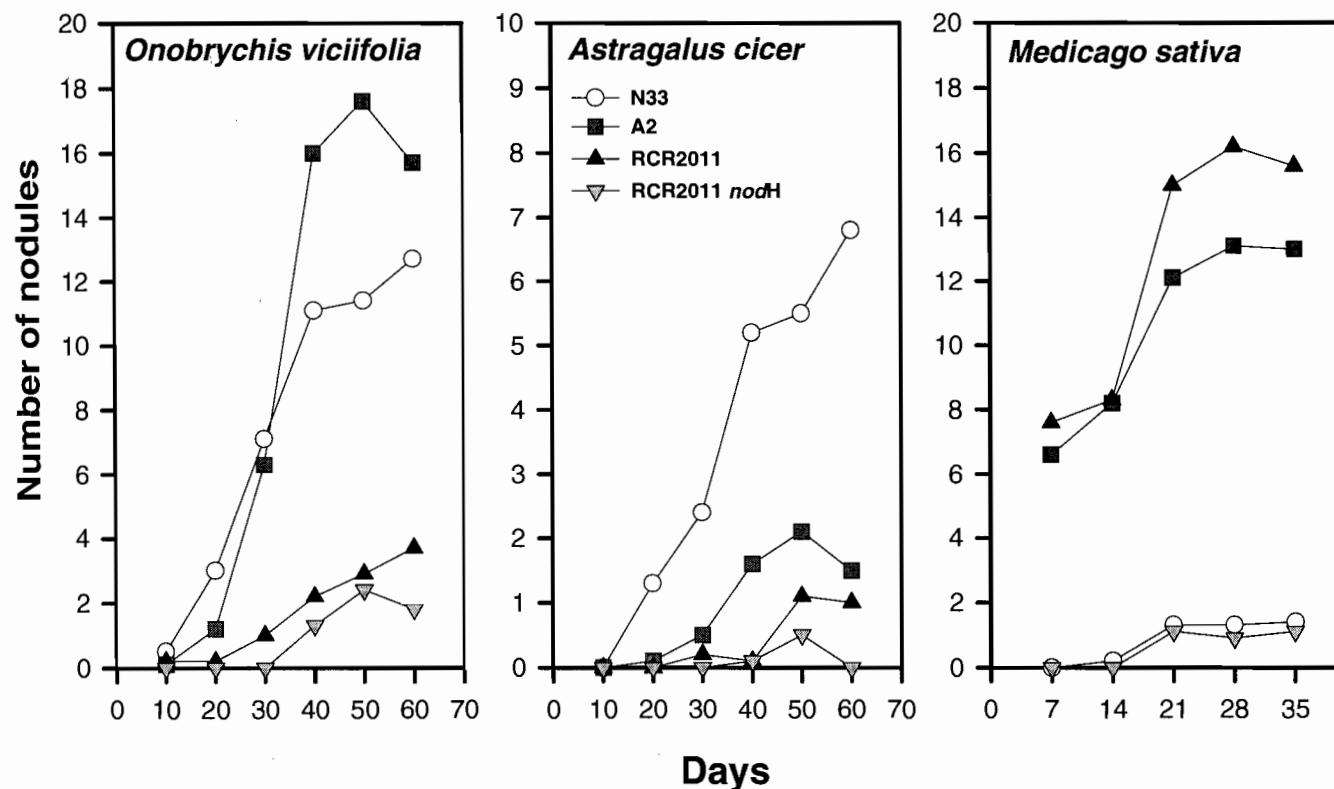


Fig. 1. Nodulation kinetics of *Rhizobium* sp. strain N33 and *Rhizobium meliloti* strains A2 and RCR2011 on different host plants. Each value represents the mean of 10 plants scored.

ciifolia Scop. We observed differences between two *R. meliloti* strains tested for nodulation on *O. viciifolia*. *R. meliloti* strain A2 was able to infect and nodulate *O. viciifolia* plants after 30 days; this was just as efficient as *Rhizobium* sp. strain N33 (Fig. 1). Nodule formation was not delayed but fewer plants were induced to form nodules with strain A2 than with strain N33 after 20 days; however, at the end of the nodulation period (40 to 60 days), the number of nodules was higher with strain A2 than with *Rhizobium* sp. strain N33 (Fig. 1). The nodules induced by *R. meliloti* strain A2 (Fig. 2C and H) were smaller and different in shape than the nodules induced by strain N33 (Fig. 2A and F). *R. meliloti* strain RCR2011 was also able to induce the formation of nodules on *O. viciifolia*, but the appearance of nodules was delayed and the number of nodules was lower during the entire nodulation period (Fig. 1). The size and shape of the nodules induced by strain RCR2011 (Fig. 2D and I) were comparable to the size and shape of those formed by strain A2 (Fig. 2C and H). *R. meliloti* strain GMI5431 (RCR2011 *nodH* mutant) also induced fusiform root nodules (Fig. 2E) beginning at day 20 of the nodulation period, and white nodules (Fig. 2J) were formed 40 to 60 days after inoculation (Fig. 1). We have been able to recover bacteria from crushed nodules induced by both

wild-type strains of *R. meliloti* (A2 and RCR2011) and *Rhizobium* sp. strain N33 but not from crushed nodules induced by *R. meliloti* strain GMI5431 (RCR2011 *nodH* mutant).

Overall, these results indicate that out of two *R. meliloti* wild-type strains, one (A2) is able to nodulate *O. viciifolia* as efficiently as strain N33, while the other (RCR2011) nodulates to a lesser extent even though each nodule contains recoverable bacteria.

Nodulation characteristics of *Rhizobium* sp. strain N33 and *R. meliloti* on *Astragalus cicer* and *M. sativa*.

The appearance of nodules induced by *R. meliloti* strains A2 and RCR2011 on *Astragalus cicer* L. plants was delayed, compared with *Rhizobium* sp. strain N33, and evident at 30 days after inoculation (Fig. 1). The size and shape of the nodules induced by wild-type *R. meliloti* strains (Fig. 2M and N) were similar to the size and shape of the nodules induced by strain N33 (Fig. 2K). *R. meliloti* strain GMI5431 (RCR2011 *nodH* mutant) also induced fusiform root nodules (Fig. 2O) beginning at day 20, and white nodules were also formed beginning at day 50 after inoculation (Fig. 1). The recovery of bacteria from crushed nodules induced by both wild-type strains of *R. meliloti* (A2 and RCR2011) and strain N33 was

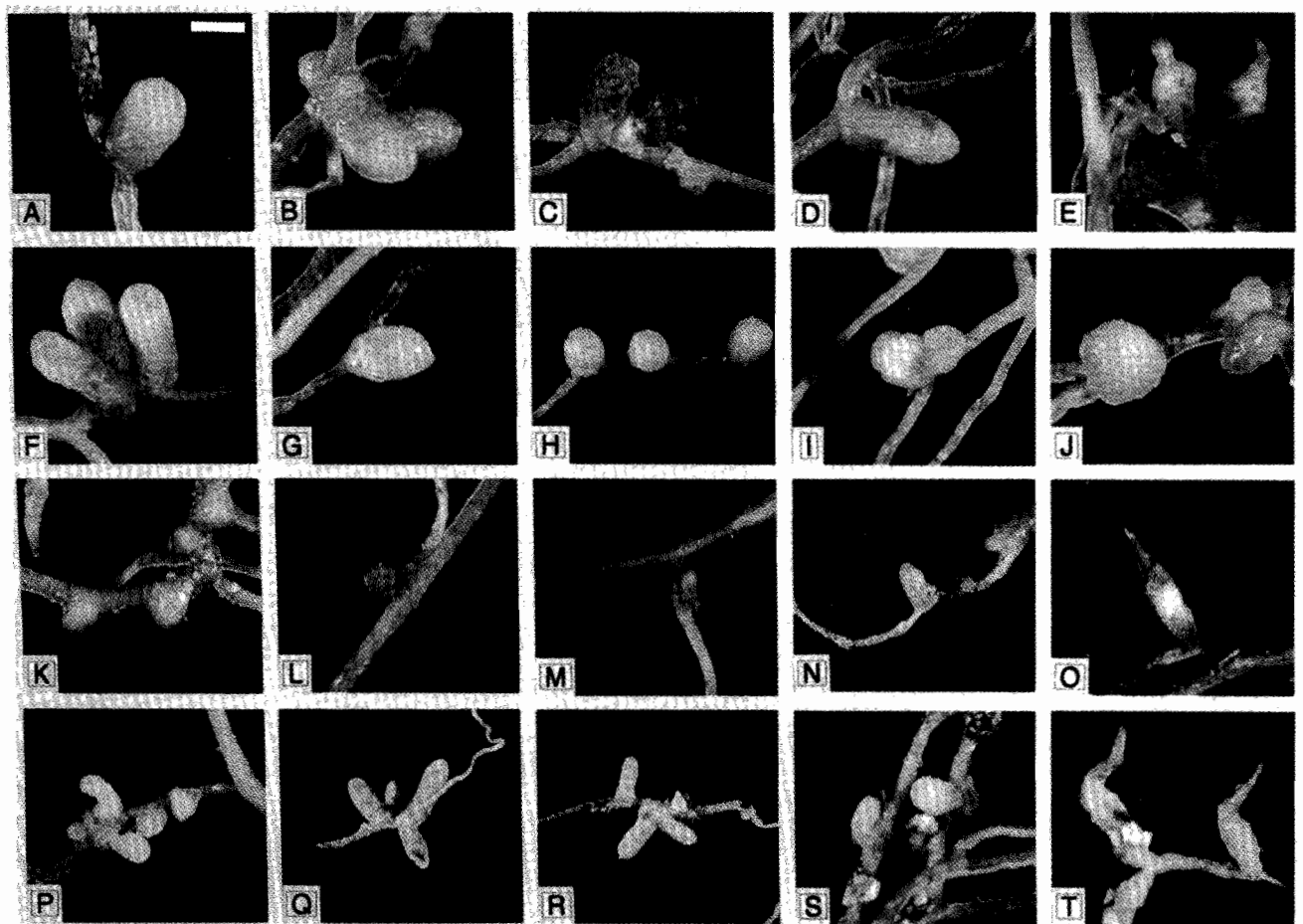


Fig. 2. Representative nodules induced on *Onobrychis viciifolia* by *Rhizobium* sp. strain N33 (A and F), *Rhizobium* sp. strain N33 *nodH* mutant (B and G), *R. meliloti* strain A2 (C and H), *R. meliloti* strain RCR2011 (D and I), *R. meliloti* RCR2011 *nodH* mutant (E and J); on *Astragalus cicer* by *Rhizobium* sp. strain N33 (K), *Rhizobium* sp. strain N33 *nodH* mutant (L), *R. meliloti* strain A2 (M), *R. meliloti* strain RCR2011 (N), *R. meliloti* RCR2011 *nodH* mutant (O); and on *Medicago sativa* by *Rhizobium* sp. strain N33 (P); *R. meliloti* strain A2 (Q), *R. meliloti* strain RCR2011 (R), and *R. meliloti* RCR2011 *nodH* mutant (S and T). The scale bar corresponds to 0.33 cm.

possible from 50% of the nodules, except for *R. meliloti* strain GMI5431 (RCR2011 *nodH* mutant), with which no recovery of bacteria was possible. Overall, these results indicate that both *R. meliloti* wild-type strains nodulate *A. cicer* poorly, compared with strain N33, even though the nodule shape and bacterial content were similar.

The appearance of nodules induced by *Rhizobium* sp. strain N33 on *M. sativa* was delayed and evident at 21 days after inoculation, compared with both *R. meliloti* strains, for which 50% of the nodules were already formed at day 7 (Fig. 1). Only 40% of the plants were nodulated 21 days after inocula-

tion by strain N33 (Fig. 1). The size and shape of the nodules induced by strain N33 (Fig. 5P) were similar to the size and shape of the nodules induced by both *R. meliloti* strains (Fig. 2Q and R) except that strain N33-induced nodules were white. *R. meliloti* strain GMI5431 (RCR2011 *nodH* mutant) induced fusiform root nodules (Fig. 2T) beginning at day 7 and white nodules (Fig. 2S) were also formed beginning at day 21 after inoculation (Fig. 1). We were unable to isolate bacteria from crushed nodules induced by strain N33 and *R. meliloti* strain GMI5431 (RCR2011 *nodH* mutant) on *M. sativa*. Overall, these results indicate that strain N33 nodulates

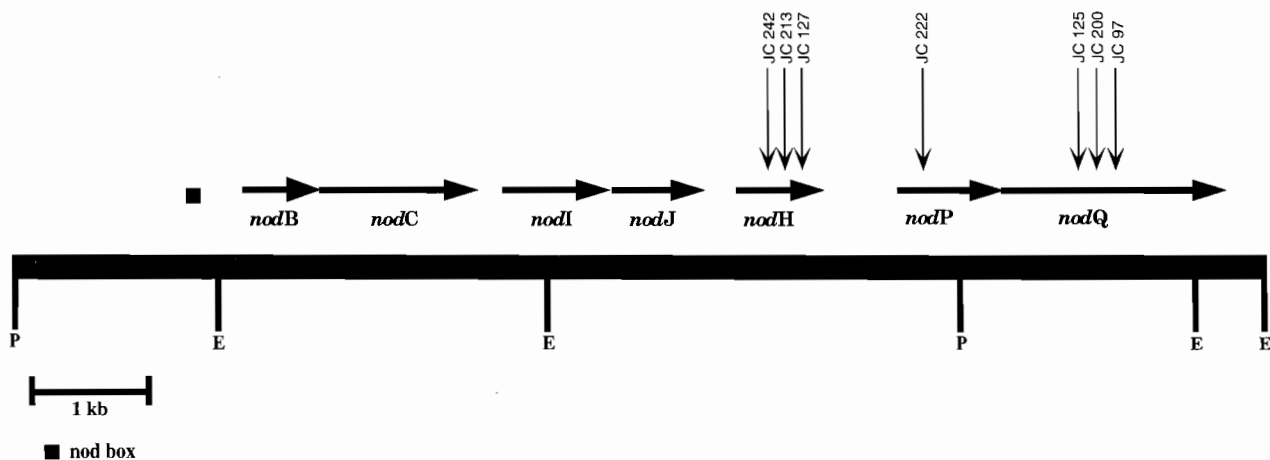


Fig. 3. Physical and genetic map of the nodulation genes *nodBCIJHPQ* of *Rhizobium* sp. strain N33. Vertical arrows indicate position of various Tn5 insertions. The *nod* box and direction of transcription are indicated. Restriction sites: E, *EcoRI*; P, *PstI*.

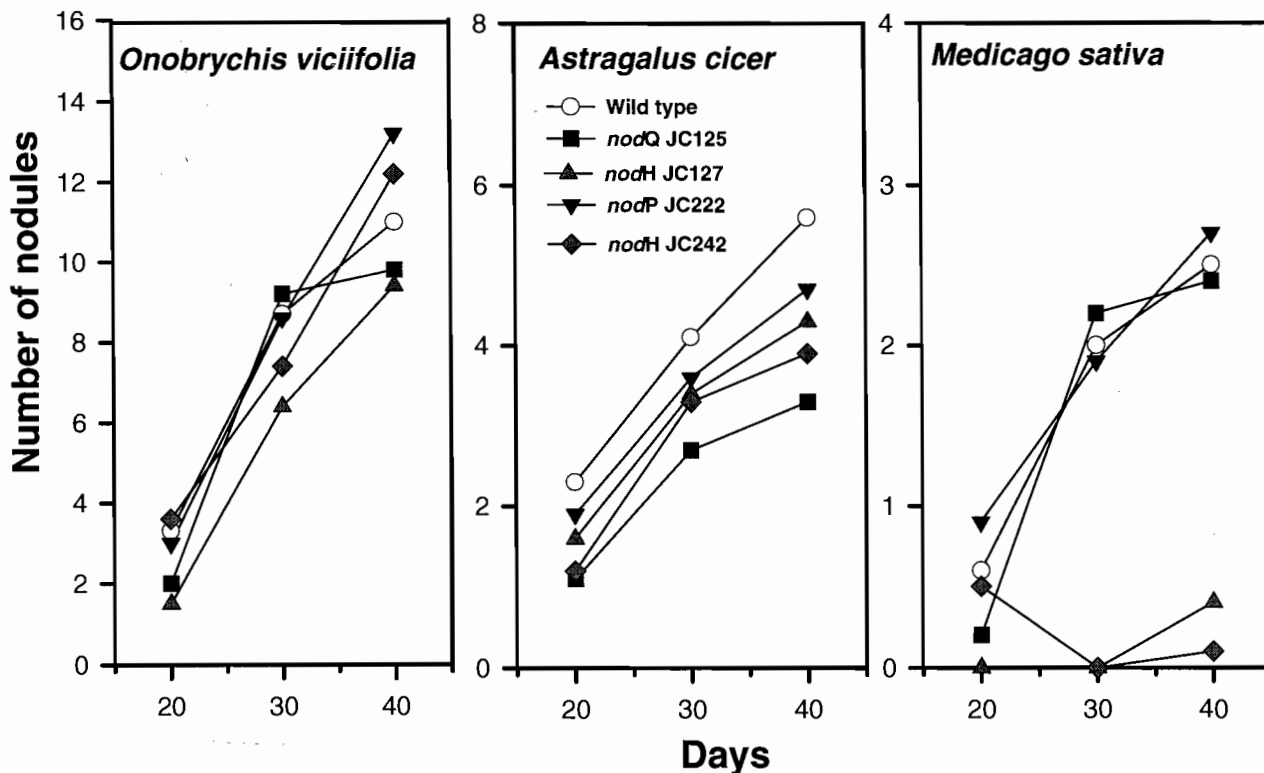


Fig. 4. Nodulation kinetics of *Rhizobium* sp. strain N33 and mutants carrying Tn5 insertion in the *nodHPQ* genes on different host plants. Each value represents the mean of 10 plants scored.

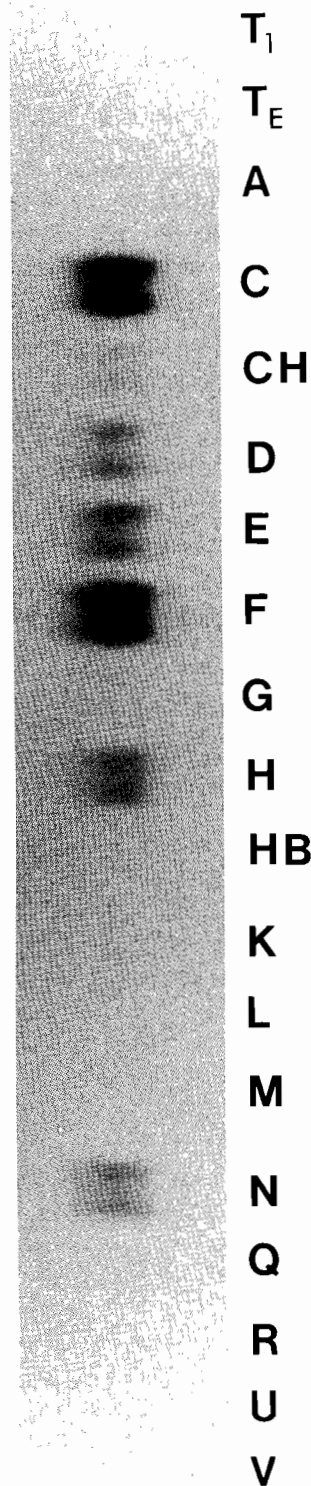


Fig. 5. Northern (RNA) hybridization of total RNA isolated from *Rhizobium* sp. strain N33 following induction with several flavonoid compounds. Homologous *nodBC* genes were used as probe. Transcript size is approximately 9 kb. T₁, (control); T_E, (control with 1% ethanol); A, (apigenin); C, (p-coumaric acid); CH, (chrysin); D, (daidzein); E, (eriodictyol); F, (formononetin); G, (genistein); H, (hesperetin); HB, (p-hydroxybenzoic acid); K, (kaempferol); L, (luteolin); M, (morin); N, (naringenin); Q, (quercetin); R, (rutin); U, (umbelliferone); V, (vanillic acid).

alfalfa poorly, but is capable of forming a few, oval-shaped, empty nodules similar to those formed by *R. meliloti*.

Nodulation kinetics of *Rhizobium* sp. strain N33 *nodHPQ* mutants.

Various mutants in the *nodHPQ* region were obtained following Tn5 mutagenesis (Fig. 3). Mutations in the *nodP* (JC222) and *nodQ* (JC125) genes have shown a Nod⁺ phenotype on their homologous hosts, *A. cicer* and *O. viciifolia*, with a slight reduction in the number of nodules formed on *A. cicer* (Fig. 4). On *M. sativa* these mutants were able to form a few, white, empty nodules, as was the wild-type strain N33 (Fig. 4). Other *nodQ* mutants (JC97 and JC200) showed the same nodulation phenotype (data not shown). These results were expected since we have identified a reiterated copy of the *nodPQ* genes in *Rhizobium* sp. strain N33, as was previously found in *R. meliloti*. It has been shown in *R. meliloti* that the inactivation of one of these two copies allows *R. meliloti* to infect its homologous host *M. sativa* and a nonhomologous host, *V. sativa* subsp. *nigra* (Cervantes et al. 1989; Faucher et al. 1989; Roche et al. 1991). This phenotypic effect was due to the secretion from *R. meliloti* of both sulfated and nonsulfated Nod factors. However, wild-type strain N33 and mutants in either the *nodP* or *nodQ* gene have shown a Nod⁺ phenotype on their homologous hosts *O. viciifolia* and *A. cicer* (Fig. 4) and were unable to elicit root nodules on *V. sativa* subsp. *nigra* (data not shown). Surprisingly, mutants in the *nodH* gene of *Rhizobium* sp. strain N33 (JC127 and JC242) were able to induce effective root nodules on their homologous hosts *O. viciifolia* (Fig. 4; Fig. 2B and G) and *A. cicer* (Fig. 4; Fig. 2L). On the latter host, the number of nodules formed by *nodH* mutants was slightly reduced. The same result was obtained with another *nodH* mutant, JC213 (data not shown). In contrast to these results, it has been shown that *nodH* mutants of *R. meliloti* have lost the ability to elicit root hair curling (Hac⁻), infection thread formation (Inf⁻), and nodule formation (Nod⁻) on their homologous host *M. sativa*, but have acquired the ability to be Hac⁺, Inf⁺, and Nod⁺ on their nonhomologous host *V. sativa* subsp. *nigra* (Faucher et al. 1988). Moreover, *nodH* mutants of strain N33 have not been able to elicit root nodules on *V. sativa* subsp. *nigra* (data not shown). These results clearly indicate that the *nodH* gene of strain N33 is not required for effective symbiotic association with *A. cicer* and *O. viciifolia* host plants. The *Rhizobium* sp. strain N33 *nodH* mutants (JC127 and JC242) have lost the ability, compared with the wild-type strain, to induce a few, white, empty, root nodules on *M. sativa* (Fig. 4). These results show the importance of the *nodH* gene for strain N33 in the formation of nodules on *M. sativa*.

Assay for *nod* gene induction by various flavonoids.

Rhizobium sp. strain N33 has a content in nodulation genes similar to that of *R. meliloti*. Accordingly, both bacteria can form nodules on *O. viciifolia*. *R. meliloti* can form a few nodules on *A. cicer* (a host of strain N33) and strain N33 can form a few, white, empty nodules on *M. sativa*. These results indicate overall that similar Nod factors are excreted by these bacteria and/or that their *nod* genes are activated by different compounds. To test the latter hypothesis, 14 flavonoid compounds from diverse classes (flavones, flavanones, flavanols,

and isoflavanones), a phenylpropane derivative (*p*-coumaric acid), and two aromatic acid derivatives (*p*-hydroxybenzoic acid and vanillic acid) were assayed for *nod* gene induction in *Rhizobium* sp. strain N33. The isoflavanone formononetin and the phenylpropane derivative *p*-coumaric acid were found to be the strongest inducers (Fig. 5). The induction by eriodictyol, hesperetin, naringenin, and the isoflavanone daidzein was moderate, whereas the induction by chrysin was slight (Fig. 5). All the other compounds tested have not been able to induce the *nodBC* genes of *Rhizobium* sp. strain N33. It has been reported that the flavanones eriodictyol and hesperetin were found to be the best inducers of nodulation genes for *Rhizobium leguminosarum* bv. *viciae* (Firmin et al. 1986) and that the flavone luteolin isolated from seeds of *M. sativa* was found to be the major *nod* gene inducer interacting with NodD1 of *R. meliloti* (Peters et al. 1986). Others flavones such as apigenin and 4',7 dihydroxyflavone have also been able to induce *nod* genes of *R. meliloti* (Peters and Long 1988; Maxwell et al. 1989; Györgypal et al. 1991). Our results showed that luteolin and apigenin, which are able to induce *nod* genes from *R. meliloti*, are not able to induce the transcription of *nodBC* genes of strain N33 (Fig. 5). These results could partly explain why *Rhizobium* sp. strain N33 does not form an effective symbiotic association with *M. sativa*, since no induction of *nod* genes has been observed with known inducers of alfalfa. Furthermore, it has been shown that the isoflavone formononetin is not a potential inducer of *R. meliloti nodD* genes (Maxwell and Phillips 1990; Györgypal et al. 1991). This may indicate that *O. viciifolia* plants possess both

inducers able to activate the transcription of *nod* genes from *Rhizobium* sp. strain N33 and *R. meliloti* strains, but inducers for strain N33 could be absent in *M. sativa*. It is noteworthy to observe that, for slow-growing, nitrogen-fixing symbionts such as strain N33 and *Bradyrhizobium japonicum*, the major class of inducing compounds were found to be isoflavones. It has been shown by Krishnan and Pueppke (1991) that *R. fredii*, another symbiont of soybean, also responds to the isoflavone inducers daidzein and genistein.

In this paper, we have shown that *Rhizobium* sp. strain N33 contains the *nodHPQ* genes. Recently, we have shown that it also contains the *nodBCIJ* (Cloutier et al. 1996) and *nodAFEG* (J. Cloutier, S. Laberge, and H. Antoun, unpublished) genes. These results indicate that the 11 nodulation genes characterized so far have homologues in *R. meliloti*. Consistent with these results, strain N33 and *R. meliloti* strain A2 are able to nodulate *O. viciifolia* to the same extent. At a much lower level compared with strain N33, *R. meliloti* is able to form nodules on *A. cicer*. Also, strain N33 is able to form a few, white, empty nodules on *M. sativa*. These results, overall, indicate that strain N33 and *R. meliloti* probably excrete similar Nod factors. We are presently investigating whether other specific nodulation genes could be present in the vicinity of the *nodAFEGBCIJHPQ* genes in strain N33. Since *Rhizobium* sp. strain N33 nodulation genes are induced by compounds widely different from those that induce *R. meliloti* nodulation genes, it is also probable that the lack of effective nodulation on alfalfa is hampered by the absence in this plant of an effective inducer toward strain N33 nodulation genes.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference
<i>Escherichia coli</i> strains		
DH5 α	<i>endA1 hsdR17 supe44 thi-1 recA1 gyrA96 relA1 Δ(argF-lacZYA)U169 Φ80dlacZAM15</i>	Gibco BRL, Bethesda, MD
J53	<i>pro met nal</i>	E. Johansen
MT607	<i>pro-82 thi-1 hsdR17 supE44 endA1 recA56</i>	Finan et al. 1986
MT609	<i>thyA36 polA1, Sp^r</i>	T. M. Finan (McMaster University, Canada)
MT614	MT607 Ω Tn5	T. M. Finan (McMaster University, Canada)
MT616	MT607 containing (pRK600), mobilizer	Finan et al. 1986
Arctic rhizobia strains		
N33	Wild type	Prévost et al. 1987b
N33	Wild type, Sm ^r	This study
JC97	<i>nodQ::Tn5, Sm^r, Nm^r</i>	This study
JC125	<i>nodQ::Tn5, Sm^r, Nm^r</i>	This study
JC127	<i>nodH::Tn5, Sm^r, Nm^r</i>	This study
JC200	<i>nodQ::Tn5, Sm^r, Nm^r</i>	This study
JC213	<i>nodH::Tn5, Sm^r, Nm^r</i>	This study
JC222	<i>nodP::Tn5, Sm^r, Nm^r</i>	This study
JC242	<i>nodH::Tn5, Sm^r, Nm^r</i>	This study
<i>Rhizobium meliloti</i> strains		
A2	Wild type	Bordeleau et al. 1977
RCR2011	Wild type	Rosenberg et al. 1981
GMI5431	<i>nodH::Tn5-2313</i>	Debellé et al. 1986
Plasmids		
pPH1J1	pRK2 derivative (IncP), Cm ^r , Gm ^r , Sp ^r	Beringer et al. 1978
pRK600	pRK2013 Nm ^r ::Tn9, Cm ^r , Nm ^r	Finan et al. 1986
pRK7813	pRK2 derivative (IncP) carrying <i>cos</i> site, pUC9 polylinker, Tc ^r	Jones and Gutterson 1987
pUC18/19	Cloning vector, ColE1 oriV bla, Ap ^r	Yanisch-Perron et al. 1985
pJC3	5.7-kb <i>EcoRI</i> fragment containing <i>nodIJHPQ</i> genes cloned into pUC18	This study
pJC5	8.2-kb <i>PstI</i> fragment containing <i>nodBCIJHPQ</i> genes cloned into pUC18	This study
pJC6	0.6-kb <i>EcoRI</i> fragment containing part of <i>nodQ</i> gene cloned into pUC18	This study

^a Ap, ampicillin; Cm, chloramphenicol; Gm, gentamycin; Nm, neomycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Bacterial strains and plasmids used in this study are listed in Table 1. *Rhizobium* sp. strain N33 was previously isolated from the arctic legume *Oxytropis arctobia* (Prévost et al. 1987b).

Media, antibiotics, and growth conditions.

Rhizobium sp. strain N33 and *R. meliloti* strains A2 and RCR2011 were grown on yeast mannitol broth (YMB) at 25°C (Vincent 1970). *R. meliloti* strain GMI5431 (RCR2011 *nodH* mutant) was grown on tryptone yeast broth at 25°C (Beringer 1974); *Escherichia coli* was grown at 37°C on Luria broth (Miller 1972). Antibiotics were used at the following concentrations (micrograms per milliliter) for *Rhizobium* sp. strain N33: chloramphenicol, 20; neomycin, 20; streptomycin, 200; tetracycline, 5. For *R. meliloti*: neomycin, 100; streptomycin, 100. For *E. coli*: ampicillin, 80; chloramphenicol, 20; neomycin, 20; spectinomycin, 50; tetracycline, 10.

DNA manipulations.

DNA cloning, transformation, restriction endonuclease digestion analysis, agarose gel electrophoresis, and Southern transfer and hybridization were carried out as described by Sambrook et al. (1989). Hybridizations were done at 68°C with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.5% sodium dodecyl sulfate (SDS) and 0.25% wt/vol low fat milk powder. DNA probes were labeled with α -³²P-dCTP (3,000 mCi/ml, Amersham), using the oligolabeling procedure (Feinberg and Vogelstein 1984).

Total genomic DNA from *Rhizobium* sp. strain N33 was prepared as described by Laberge et al. (1989). Plasmid DNA was prepared by the method of Brun et al. (1991) and lambda phage DNA was prepared by the method of Davis et al. (1986).

Construction of a genomic bank from *Rhizobium* sp. strain N33.

Total genomic DNA from *Rhizobium* sp. strain N33 was partially digested with the restriction endonuclease *Sau*3A. DNA within the 15- to 20-kb range was isolated by centrifugation (26,000 rpm, Beckman rotor SW28 [Beckman, Palo Alto, CA], 16 h, 10°C) on a sucrose gradient (10 to 40% wt/vol), dialyzed to remove sucrose and concentrated by ethanol precipitation. DNA was ligated into the compatible *Bam*HI sites of EMBL3 phage arms and packaged in vitro into lambda heads by the method suggested by the supplier (Promega, Madison, WI). Lysate plaque (4,000 in total) DNA was transferred to Hybond-N membranes (Amersham) and screened by hybridization with a DNA probe containing the *nodII* genes from *B. japonicum* (Göttfert et al. 1990). Positives clones were selected and multiplied, and their DNA isolated and digested with *Eco*RI. Following agarose gel electrophoresis, Southern hybridization was performed with the *nodII* probes. A 5.7-kb *Eco*RI positive fragment was cloned as pJC3 and is shown in this paper to contain the *nodHP* genes and most of *nodQ* gene.

DNA sequencing and computer analysis.

DNA sequencing was done by the dideoxy chain termination method (Sanger et al. 1977; Sambrook et al. 1989). The

complete nucleotide sequence of both DNA strands of fragments was determined with the Nested Deletion Kit procedure from Pharmacia (Montreal).

DNA and protein sequence analysis were done with the UWGCG (Genetics Computer Group of the University of Wisconsin, Madison) software package version 7.1 (Devereux et al. 1984). Nucleotide and amino acids comparisons were determined with the Gap and Fasta programs (UWGCG). Data base searches were done with GenBank (release 73.0) and EMBL (release 32.0).

Transposon mutagenesis and homogenitization of *nodHPQ* genes containing Tn5 insertions.

The 5.7-kb *Eco*RI fragment from pJC3 was subsequently cloned into pRK7813 and transformed in *E. coli* strain MT614 carrying Tn5. Transposition was identified by mobilizing the resulting plasmids into *E. coli* MT609 (*polA*) by triparental mating with *E. coli* MT616. The position of Tn5 insertions was determined following single and double digestions with various restriction enzymes: *Bam*HI, *Eco*RI, *Hind*III, *Sal*I, and *Sma*I. Plasmids containing Tn5 insertion were transformed into *E. coli* DH5 α and mobilized into *Rhizobium* sp. strain N33 by triparental mating with *E. coli* MT616. Transconjugants were selected on YMB media containing neomycin, tetracycline, and streptomycin (Yarosh et al. 1989). Marker exchange was performed by biparental mating with *E. coli* strain J53 containing the IncP plasmid pPH1J1. Homogenotes were selected that had retained the transposon (Nm^r) and the incoming plasmid pPH1J1 (Cm^r) and that had lost tetracycline resistance. Homogenotes were cured of plasmid pPH1J1 by repeated subculturing in the absence of antibiotic selection (Ruvkun and Ausubel 1981). Marker exchange DNA was confirmed by a Southern blot probe.

Nodulation assays and isolation of bacteria from nodules.

Seeds of *O. viciifolia* cv. Nova, *A. cicer* cv. Oxley, *M. sativa* cv. DK120, and *V. sativa* subsp. *nigra* were obtained from Agriculture Canada Research Branch at Saskatoon (Saskatchewan) and Sainte-Foy (Québec). Seeds were surface sterilized by soaking once in ethanol 95% for 30 s and twice in sodium hypochlorite (3.0% vol/vol) for 2 min, and then washed three times with sterile, distilled water and dried. Seeds were germinated for 3 days at room temperature on 1.5% wt/vol agar and incubated in darkness. Single seedlings were transferred to vermiculite tubes (25 × 200 mm) supplemented with 20 ml of nitrogen-free Hoagland's solution containing 0.1% wt/vol calcium carbonate (Vincent 1970). An aliquot (1 ml) of bacterial suspension ($\geq 10^8$ cells) was added to each of 10 replicate tubes. Plants were grown at 20°C under fluorescent light on a 16-h day/8-h night cycle for 35 or 60 days and scored at weekly intervals for number of plants showing nodules and number of nodules on each plant. Ten nodules formed by *R. meliloti* strains, *Rhizobium* sp. strain N33, and various mutant derivatives were surface sterilized (Vincent 1970), crushed, and streaked on yeast extract mannitol agar with Congo red. Each nodule containing recoverable bacteria was scored as 10% recovery.

Assays for *nod* gene induction and total RNA isolation.

The induction of *nodBC* transcripts of *Rhizobium* sp. strain N33 by various flavonoids was assayed by the following pro-

cedure. Strain N33 was first grown in a 25-ml YMB flask until the desired optical density (OD₆₀₀ = 0.2) was reached. Then, 250 µl of a 10 mM solution (final concentration = 100 µM) of each flavonoid (dissolved in 95% ethanol) was added and cells were cultured for an additional 24 h until an OD₆₀₀ of 1.0 was attained. The flavonoids used were apigenin, chrysin, daidzein, eriodictyol, formononetin, genistein, hesperetin, kaempferol, luteolin, morin, naringenin, quercetin, rutin, and umbelliferone, (all except daidzein, eriodictyol, and formononetin [from Spectrum Chemical Co., Gardena, CA] were from Sigma Chemical Co., St. Louis, MO). Three other compounds were also used: the phenylpropane derivative p-coumaric acid and the aromatic acids p-hydroxybenzoic acid and vanillic acid (all purchased from Sigma).

Total RNA was extracted from each cell culture in order to determine the levels of *nodBC* transcripts. RNA isolation was performed as described by Gray et al. (1990) with minor modifications. Total RNA (10 µg) was size fractionated on a 0.8% formaldehyde agarose gel (Fourney et al. 1988), transferred to a nylon membrane (Hybond N⁺, Amersham) and hybridized overnight at 68°C in 2× SSC, 0.5% SDS, 0.25% (wt/vol) low fat powder milk (Sambrook et al. 1989) with an α-³²P-dCTP-labeled DNA probe containing *nodBC* of *Rhizobium* sp. strain N33 (Cloutier et al. 1996).

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