

# Sequence and Mutational Analysis of the Common *nodBCIJ* Region of *Rhizobium* sp. (*Oxytropis arctobia*) Strain N33, a Nitrogen-Fixing Microsymbiont of Both Arctic and Temperate Legumes

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By heterologous hybridization, we have identified the common nodulation genes *nodBCIJ* of *Rhizobium* sp. strain N33 within a 8.2-kb *Pst*I fragment. The *nodBCIJ* genes are located within a 4,620-bp region which also included a consensus *nod* box promoter. The four open reading frames coding for the *nodBCIJ* genes contain 657, 1,353, 915, and 789 nucleotides, respectively. We found that the *nodA* gene was not adjacent to the *nodB* gene, unlike the situation in many rhizobia. The DNA of the *nodBCIJ* genes of *Rhizobium* sp. strain N33 were found to be homologous to the corresponding genes of other rhizobia except for the 3'-coding region of the *nodC* gene. The deduced NodC protein was the longest of the rhizobia except *Bradyrhizobium japonicum*. Tn5 mutagenesis of the common *nod* region of strain N33 revealed that the *nodBC* genes were essential for nodulation on their temperate hosts *Onobrychis viciifolia* and *Astragalus cicer*. By contrast, mutations in the *nodI* and *nodJ* genes produced a Nod<sup>+</sup> phenotype with a reduced number of nodules on the temperate hosts. Nodules formed on *Onobrychis viciifolia* by either *nodI* or *nodJ* mutants were approximately 10 times smaller than nodules formed by the wild type strain; this reduction in nodule size was not observed on *Astragalus cicer*.

*Additional keywords:* symbiosis.

Soil bacteria from the rhizobiaceae family are capable of forming root nodules on leguminous host plants. In a symbiotic form within the nodule they provide nitrogen to the plant by the process of nitrogen fixation. The bacterial nodulation (*nod*) genes involved in this symbiotic association are classified into two distinct categories. The first category, referred to as the common nodulation genes, includes the *nodABC* genes. These genes are interchangeable among *Rhizobium* species without altering the nodulation host range and are essential for

nodule formation (Kondorosi et al. 1984; Fisher et al. 1985; Marvel et al. 1985). Mutation in any of these genes results in a Nod<sup>-</sup> (absence of nodule) phenotype (Kondorosi et al. 1984; Rossen et al. 1984; Djordjevic et al. 1985; Debelle et al. 1986). Recent evidence indicates that *nodA*, *nodB*, and *nodC* genes encode, respectively, for an acyltransferase (Röhrig et al. 1994; Atkinson et al. 1994), a chitin oligosaccharide deacetylase (John et al. 1993; Spaink et al. 1994) and a chitin oligosaccharide synthase (Geremia et al. 1994; Spaink et al. 1994). These common *nod* genes are involved in the synthesis of the Nod factor core molecule (Spaink et al. 1991). Two other nodulation genes located downstream of *nodC*, *nodI*, and *nodJ*, exist in several fast- and slow-growing rhizobia (Evans and Downie 1986; Djordjevic et al. 1986; Nieuwkoop et al. 1987; Surin et al. 1990; Geelen et al. 1993; Vázquez et al. 1993). These genes are involved at least partially in the extracellular transport of the Nod factor (Spaink et al. 1992; McKay and Djordjevic 1993; Spaink et al. 1995). The second set of nodulation genes are referred to as host specific nodulation (*hsn*) genes and cannot be complemented by *hsn* genes from other *Rhizobium* species. In fact, these *hsn* genes are involved in the decoration of the Nod factor, the lipooligosaccharide released by *Rhizobium* in response to its host plants (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).

Arctic rhizobia were first isolated from nodules of various plants in the Canadian high arctic, i.e., *Oxytropis arctobia*, *Oxytropis maydelliana*, and *Astragalus alpinus* (Prévost et al. 1987a). These bacteria are also able to form root nodules on the temperate legumes *Onobrychis viciifolia* (sainfoin), *Oxytropis monticola*, and *Astragalus cicer*. Prévost et al. (1987b) have shown in another study that sainfoin plants nodulated by arctic rhizobia and grown at 15°C had greater nitrogenase activity when tested at 5°C and 10°C, than sainfoin plants nodulated by temperate rhizobia: arctic rhizobia also promoted better growth of sainfoin at low temperature compared to temperate strains (Prévost et al. 1994). It has also been shown, that at 9°C, arctic rhizobia are more competitive than temperate rhizobia to form nodules on sainfoin (Prévost and Bromfield 1991).

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As a first step toward understanding the nodulation specificity of arctic rhizobia, we initiated a physical and genetic study of the nodulation genes in strain N33. In this paper we report the DNA sequence and the characterization of a symbiotic region, and the nodulation phenotypes of mutants altered in the *nodBCIJ* genes.

## RESULTS

### Identification of symbiotic regions.

To characterize and locate the common nodulation genes from the *Rhizobium* sp. strain N33, heterologous DNA probes containing the *nodABC* and *nodIJ* genes from *Bradyrhizobium japonicum* were hybridized with total genomic DNA. The *nodABC* genes hybridized with 2.8- and 4.1-kb *EcoRI* frag-

ments, whereas the probe containing the *nodIJ* genes hybridized with 2.8- and 5.7-kb *EcoRI* fragments (data not shown). Both DNA probes hybridized with an 8.2-kb *PstI* fragment (data not shown).

### Cloning and sequence analysis of *nodBCIJ* genes.

A lambda EMBL3 phage bank of *Rhizobium* sp. strain N33 was screened with the *B. japonicum nodABC* DNA probes and 20 hybridizing phages were purified and their DNA isolated. Southern hybridizations (using the *B. japonicum nodABC* or *nodIJ* probes) of DNA from several phages digested with either *EcoRI* or *PstI* showed the same banding pattern as with total genomic DNA, thus indicating that these phages contained full-length copy of the 2.8- and 5.7-kb *EcoRI* fragments and also the 8.2-kb *PstI* fragment. These

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1   TTCGAAAGGGCTCGTGTATCCGATGCTTCGGCTTGGCCGGCAGTGAGGCCGGCCGGCGAGCAGCCGCTCGTGCACCAGATGAGATT 90
91   TACATCGAGCCGTTTGAAGCCGATGTCATCAGCTATCCAAAGCGTGGATGCACTGTGAAACAAACGATTTTACCGATTTCGAGAA 180
181  CGTCGCATAGGGAGAATCAAGGAAGTCTAAAGGAACGTTGTTCGATGTTTGGCTTAGCTCCGAGGAGCACTTTCGGCTCCGAGGAGCAT 270
271  TTTCTGGTGTACGCCCTTCGGCATAAGCCCAACATCAATCAAGTCGCATGTCGATTGGCGCTTGGTTCCTCGGTGACCCGTCAGAGTCC 360
361  ATCGCGTCGACACGAGCCGACAACTCTCCATTCCATCCGGCAGCGTTCCGCAAGCGATGCCGAACGATGTTGGCCAAATTCGATGCTCG 450
      G T V R Q A M P N D V A P I R C S
451  ATGGGGAGTGGCCGCCCGCCACCCTAATCGATCGGAGCGTTCGCAACTATGAAAAATGTGGACTACATGTGGCAAGTGGCCAGTGGACT 540
      M K N V D Y M C E V P S D
      M G E W P P G T L I D R S V P N L *
541  GCGCTGACGGCACTCAAGATCGCAGCGTCTATTTGACGTTTGACGACGGGCCAATCCAGTTCACACCCGAGATCCCGATTGTTGG 630
      C A D G T Q D R S V Y L T F D D G P N P V C T P Q I L D L L
631  CGCAACATCEGGTGCCGCGACGTTCTTCGTGATCGGTGCCACGACGAGACCAGCCGGAGCTCATCCGACGAATGATTGCAAGAGGCC 720
      A Q H R V P A T P F V I G A H A A D Q P E L I R R M I A E G
721  ACGAGGTAGCCAAATCACACGATGACTATCCGGACCTGTCTCAGATGCGAACCCGGCGAAGTCGAACGTAAATAGTCGAGGCAAGCAAG 810
      H E V A N H T M T H P D L S R C E P G E V E R E I V E A S N
811  CCATCAGGATGGCGTGTCCCAAGGCCAGGTCGGCGCATGCGCGCGGTATGGGGTCTGGACCCGAGGACGCTGCTACGTCGGCCGC 900
      A I R M A C P Q A T V R R M R A P Y G V W T E D V L T T S A
901  GCGCTGGATTGGCATGCTCCATTGGTCAGTTGACCGCGGAGACTGGGCTCGCCGCGCTCGACCGGATGTCGATGAGGTGCTACCG 990
      R A G L A C V H W S V D P R D W A R P G V D A I V D E V L T
991  GTGTTGAGCCGGCGCAATTGTGCTCTTGCACGACGGGTGGCCGAGGAGTTGAAATCGGGCCACTTACGCCAGTCTGCGTACCCAGACT 1080
      G V E P G A I V L L H D G W P E E L K S A T Y A S L R D Q T
1081  TCACGGCGCTATCCCGCCTGATTCCAGCGCTGCATCACCGGGTTTGTAAATCCGGCCGCTTCCTCAACACTCACTGAACATACGAGATCC 1170
      V T A L S R L I P A L H H R G F V I R P L P Q H H *
      nodC
1171  CATGGACCTGCTTACCACGACAGTACTGTCGCCGTCGCGTCTATGCACTGCTCTGCACTGTTTATAAGGGCATGACAGGCGGTTTATTC 1260
      M D L L T T T S T V A V A C Y A L L S T V Y K G M Q A V Y S
1261  CCTGCCCAACCGTTGCACCGCGCTCGGAAGACTGGTCCGCTCCGACCTCTGGCCGAGCGTGGATGTCATTCCTCCCTGTACAACGA 1350
      L P P T V A P A S E D L V G S D L W P S V D V I A I P C Y N E
1351  AGGCCGCTTACGCTCTCGCGCTGCCTAGATTCCATTCCAAACAGGAATACCGCGAAGACTACGTGCTCACTGGTGTATGACGGTTC 1440
      G P L T L S A C L D S I A N Q E Y A G K L R V Y V V D D G S
1441  TGGAAATCGCGACCGCTCATTCGCATTACGACAAATACCGCGGACCGCGGATTTCGACTTCATCTGCTCCAGAGAAATGTCGGCAA 1530
      G N R D A V I P I H D N Y A G D P R F D I L L P E N V G K
1531  GCGCAAAGCGAGATCGCCCGGATACGTCGCTATCTGGAGATTGGTGTCAACGTCGACTCGGACACGACACTTGCCTCCGACGCTCAT 1620
      R K A Q I A A I R R S S G D L V L N V D S D T T L A S D V I
1621  CAGGAAGCTTGCACGGAAGATGACGAGTCCAGCAATCGCGCTGCCATGGGCCAGTTGACGGCAAGCAACCGGAGCGACACTTGGCTGAC 1710
      R K L A R K M Q D P A I G A A M G Q L T A S N R S D T W L T
1711  CCGATTGATCGATATGCACTACTGGCTGGCCTGCAACGAGGAGAGGGCGCGCAAGCCGCTTCGGTGGCCGCTCATGTGCTGTTGGCGCC 1800
      R L I D M E Y W L A C N E E R A A Q A R F G A V M C C C G P
1801  CTGTGCTATGTACCGCCGATCTTCGCTGCTTCGCTGCTGACACAGTACGAGACGAGATGTTTCGGGGCAAGCCAGCGACTTCGGTGA 1890
      C A M Y R R S S L S L S L L D Q Y E T Q M F R G K P S D F G E
1891  GGATCGCCATCTTACGATCCTCATGCTGGAGGCAAGCTTTCGAAACCGAGTACGTTCCGGACGCTATTGCTGTAACCGTGGTTCGCGATAG 1980
      D R H L T I L M L E A G F R T E Y V P D A I A V T V V P D R
1981  GTTAGGACCTTATCGCCCAACAACCTGGCCTGGGACCGGAGCACTTTCGGGACACGCTGCTGGCGTGGCCCTGTTGGCCGGCTCCGA 2070
      L G P L R Q Q L R W A R S T F R D T L L A L R L L P G L D
2071  TCGTATCTTACATGGACCTCGTCGGACAGAATCTGGCCCGTACTTCTTCGCTGCTGTAATAGCCGGGATTCGCGAGTTTGGACT 2160
      R Y L T L D V V G Q N L G P L L L A L S V I A G I A Q F A L
2161  GACAGCCAGCTGCGCTGGCCGCAATCCCTCGTTATTGAGCAATGACCATTCCTGGTGCACCGTGCACATGTCGAGCTCGGCAAGC 2250
      T A I L P W P T I A A M T I I R C T V T A C C A R A Q A

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Fig. 1. Nucleotide sequence of the *nodBCIJ* region of *Rhizobium* sp. strain N33. The sequence begins upstream of the *nod* box region (bold) and covers 4,620 nucleotides including four open reading frames corresponding to the *nodB* gene (nucleotides 501 to 1158), *nodC* gene (nucleotides 1172 to 2525), *nodI* gene (nucleotides 2756 to 3671) and *nodJ* gene (nucleotides 3674 to 4463). The derived amino acid sequence encoded are shown using the one-letter code. Conserved amino acids of partial *nodA* gene are indicated in bold character upstream of *nodB* gene. Dots above the *nod* box indicate the two ATC<sub>N</sub>GAT motifs. The nucleotide sequence correspond to nucleotides 6781 to 11400 in the Genbank/EMBL accession number U53327. (Continued on next page).

fragments were isolated and subcloned into pUC18. Sequences of the 2.8-kb *EcoRI* fragment were homologous to *nodB* and *nodC* genes and part of the *nodI* gene from many rhizobia species (Fig. 1). By synthesizing an oligonucleotide (20 mers) corresponding to the end of *nodI* gene present on the 2.8-kb *EcoRI* fragment and annealing it on the 8.2-kb *PstI* fragment, we found by DNA sequencing the contiguous part of the *nodI* gene. We also showed that the 5.7-kb *EcoRI* fragment previously cloned is adjacent and downstream of the 2.8-kb *EcoRI* fragment. By further sequencing the 5.7-kb fragment we found the rest of the *nodI* gene and the *nodJ* gene (Fig. 1). Analysis of sequence upstream of the *nodB* gene indicates that a peptide is encoded which is homologous to the C-terminal NodA proteins from many rhizobia (Fig. 1). No homology was found upstream of that sequence. The DNA

junction between this partial *nodA* gene and the *nodB* gene is similarly organized to the DNA junction found in many rhizobia, i.e., the sequence ATGA encodes for the start codon of the *nodB* gene and the stop codon of the *nodA* gene. Furthermore, at a position 376 nucleotides upstream of the *nodB* gene, a consensus *nod* box was identified (Wang and Stacey 1991), thus indicating that the *nodA* gene is not adjacent to the *nodBCIJ* genes in *Rhizobium* sp. strain N33 (Fig. 1). Strain N33 contains one megaplasmid that hybridizes with the 2.8-kb *EcoRI* fragment containing the homologous *nodBC* and part of *nodI* genes (data not shown).

The deduced protein sequences encoded by *nodB* and *nodC* in strain N33 were compared by computer analysis with sequences from other *Rhizobium* species. There was 61 to 76% amino acid identity found with the corresponding proteins

Fig. 1. (Continued from preceding page).

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2251  TCGATTATCGGCTTTTCACTGCGACACTTTTATCAACATCTTTCTGTACTCCCTTGAAAGCCTATGCTTTGTGCACCTTGAGCAATAG 2340
      R F I G F S L H T F I N I F L L L P L K A Y A L C T L S N S
2341  TGATTGGCTTTCACGCAAGACTGCCACCTGCGCAACGCGACAAAAGCAGATCATGTCGCAACCCGATTGCCGGAGTTGGTACAGG 2430
      D W L S R K T A T L P N A D K K Q I I V A N P I A G V G T G
2431  CAGTTCGGGAAGTGCCTGAGGCAATTGGAAGAACGGATCTTCCGCGCGATTTCTTCAAAGTTGGTGAACCGGACACGCTTTGCACGCGTGA 2520
      S S G S A E A I R R T D L P R D S S K L V N A D S V C S A E
2521  GTGATCGAGTGCCTTGGCTGGATGGACACGCTTGAGCTTGGGCAACATCCATCAATTGTCAGCACGGCGATCGATTGCATCTCGACGCT 2610
      *
2611  AATCCGTCGGTGAGAGCGACACACGCTTGGCTTCGGCTGTGACTATCCAAAGGGCCGCTCACTATGCGATGGCCGGTTCTGCCAAGAAAT 2700
2701  ATTCGCAAGTAAGCTACCGGCCCGGCTGATGGCTCTTAAGACTUAGATAGACACATGTCCAAAGTAGCAATCGACCTTCCCGCGTGAA 2790
      M S K V A I D L A G V K
      nodI
2791  GAAGTCCTTCGGCGACAAGCTTGTGTGAACGGGCTGTGTTTCCCGTTCGCTCGGGAGAGTGTCTCGGTTTGTCTGGGCGCAACGGTGC 2880
      K S F G D K L V V N G L S F T V A S G E C F G L L G P N G A
2881  GGGCAAGAGCAGGATTCGGCTATGCTCCTTGGCATGACAGTGCCTGATCGGGCAAGATCACGGTCTTGGAGAGCCAGTGGGTGCCGG 2970
      G K S T I A R M L L G M T V P D A G K I T V L E A G E P V I G A R
2971  GAGTCGCTTGGCAGCGAAGGACATCGGCGTGGTTCGCACTTCGCAACCTTGACCAGGAATTCACCGTACGAGAGAACCCTGTTGGTGT 3060
      S R L A P K S I G V V P Q F D N L D Q E F T V R E N L L V F
3061  CGGCGCTACTTTCGGCATGAGCACAGCAAGATCAAGAGGTCATCCCGTCGCTCCTCGAGTTCGCGCCCTCTTGAGAGCAAGCGGATGC 3150
      G R Y F G R M S T R K I K E V I P S L L E S L E S K A D A
3151  ACGTTCGGCGAACTATCCGCGCGCATGAAACGGCTTTCACACTGGCACGTGCGCTGATCAACGACCCCACTACTCGTATGATGATGA 3240
      R V G E L S G G M K R R L T L A R A L I N D P Q L L V M D E
3241  ACCGACGACCGGCTCGACCCGACGCGCGACCTGATTTGGGAGCGTCTGCGTTTCTTGTGCGCGCGGTAAGACGATCATTTTGAC 3330
      P T T G L D P M I Y L F L E W E R L R F L L A R G K T I L T
3331  TACCCATTTTATGGAAGAGGCGGACGCTTATCGCATCGGCTTTCGCTTGGAGCATGGACGCAAACCTCGCGAAGCGAGCCCTCATGC 3420
      T H F M E E A E R L C D R L C V L E H G R K L A E G S P H A
3421  CCTGATTGAGGAACACATCGGCTGCGAGGTATCGAGATCTTCCGCGGAATCCCGAGGAGTGGTTTTCGCTGATCAGGCPATACGTGCA 3510
      L I E E H I G C Q V I E F G N P Q L E L V S L I R P V I G A R
3511  CGCGCTCGAGGTGACCGCGAGACGCTCTTTTGTCTATACGGCGATCCGGAGCAGGTTCCGCTACAACCTCGCGCGCGCGCGGCTCGG 3600
      R V E V S G E T L F C Y T A D P E Q V P V Q L R G R A G L R
      nodJ
3601  CTTCTGGAGCGTCCACCCAGTCTGGAGGACGTTTCTTCCGCGCTACCGGACGCGAGATGSAAGTGAACGATGGTAAAGGTTTTC 3690
      L L E R P P S L E D V F L F L T G R E M E K * M G K G F S
3691  GCGGCTCTACCGCCCAACGCTTGGAACTGGATTGCGGATGCGCGCAACTATCGCATGGACGAAGTCCGCTCGCGCTCGATTCT 3780
      A A L P A N A W N W I A V W R R N Y L A W T K V A L A S I L
3781  CGTAACCTCGCGCATTCATGATCTACCTGTCGGACTCGGACTGCTCGGAATGATGGTAGGTAGCTCGCTTGAAGACGCGCTCGTACCC 3870
      G N L A D P M I Y L F G L T G L G M M V G F V E D A S Y P
3871  TGCTTTTTTGGCAGCGCGCATGGTCGCGACAAGTGCATGACCCGCTCCACCTTCGAAACAATTTATGCGACTTCCCTCGCATGAACGA 3960
      A F L A A G M V A T S A M T A S T P E T I Y A T F P R M N E
3961  ACAGCGACCTGGGAAGCGATCTGCACACACAACCTTACCCCTCGCGACATCGTCTCGGTGAGTTGGTGGGCAACCACAAGGCGCTT 4050
      Q R T I W E L H T Q L T L G D I V L G E L V W A T T R A F
4051  TCTGGCCGTAACGGCAATTCGCGTGGCCGCTATAGCGGCTACTCAGCATGGTCGTCGCTCCCTCTATGTGCTACCGATCATCGCTCT 4140
      L A G T A I A M V A V I A G Y S A W S S V L Y V L P V I A L
4141  CACTGGGTTGGCCTTTCGGAGCCCTGGCGATGATCGTAACCGGCTTGGCCCGAGTTACCACTACTTTCATATTCAGCAGACGCTCTTCT 4230
      T G L A F A S L A M I V T A L A P S Y H Y F I F Y Q T R F L
4231  CACACCCATGTTGTTCTGCTCGCGCTGTCTTCCCGTCACTCAACTGCCAAGCACCTTTCAGCACATAGCGGGCATCTTACCGCTGGC 4320
      T P M L F L S G A V F P V T Q L P S T F Q H I A G I L P L A
4321  GCATTGATCGACCTGATTCGTCGGGACGCTGTGATCGCCCGGCTGGGACATCGCCCTGCACATTTGGTGGCGTTTGCATATACCGGCT 4410
      H S I D L I R F P A G D I A L H I G A L C I Y A V
4411  AGTGGCTTCTTCTCTCGATGGTGTCTTCCGCGCGCTGCTGCTGATGCTACTTACGAATACAAGAAGGAGAACGAGAGTACGC 4500
      V P P F L S M V L L R R K L L R *
4501  ATCGCAAGCGCTACAGCGCGAAGCTGCTTGGCCACGCACTGTGAGTTGGGACAGCAGAACAACAAGCTGAGGCGCACACCGGACC 4590
4591  GGACGACGCTTGCACCGGCTTGGGATA 4620

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from *R. meliloti* (Török et al. 1984; Egelhoff et al. 1985), *R. loti* (Collins-Emerson et al. 1990), *R. fredii* (Krishnan and Pueppke 1991), *R. loti* bv. *viciae* (Rossen et al. 1984), *R. l.* bv. *trifolii* (Schofield and Watson 1986), *Rhizobium* sp. NGR234 (Relić et al. 1994), *R. loti* bv. *phaseoli* (Vázquez et al. 1991) and *Bradyrhizobium* sp. Parasponia (Scott 1986). A comparison between the deduced protein sequences of *nodI* and *nodJ*

in strain N33 and sequences from other *Rhizobium* species showed 69 to 82% amino acid identity with the corresponding proteins from *R. loti* bv. *viciae* (Evans and Downie 1986), *R. l.* bv. *trifolii* (Surin et al. 1990), *R. loti* (Young et al. 1990), *R. etli* (Vázquez et al. 1993) and *B. japonicum* (Göttfert et al. 1990). The *nodBCIJ* proteins from *Azorhizobium caulinodans* (Goethals et al. 1989) are more distantly related to with those

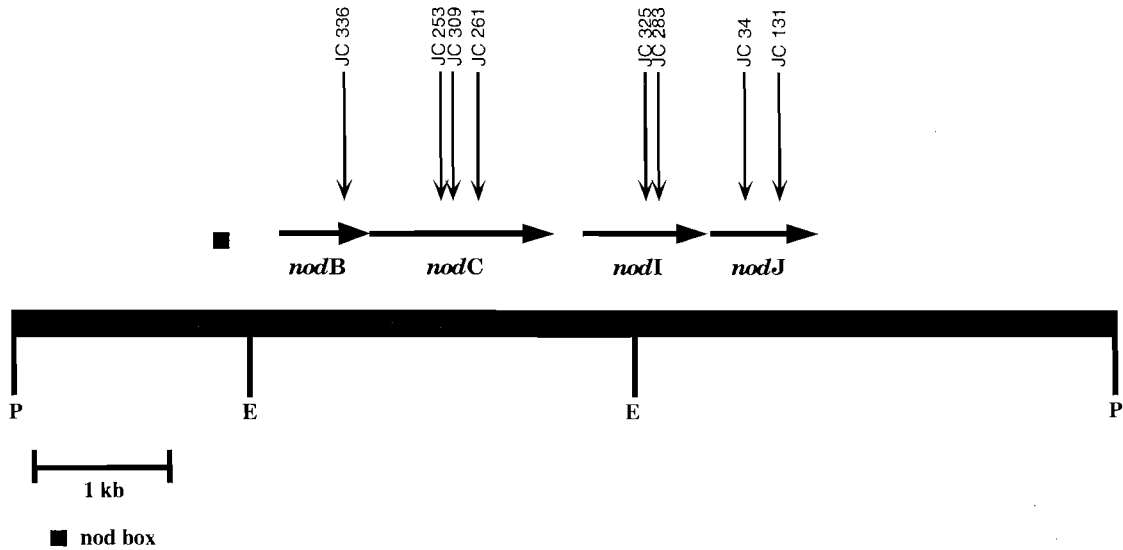


Fig. 2. Physical and genetic map of the common nodulation genes (*nodBCIJ*) of *Rhizobium* sp. strain N33. Vertical arrows indicated the position of various Tn5 insertions in the common *nod* region. The *nod* box and the positions and direction of transcription of *nodB*, *nodC*, *nodI*, and *nodJ* are indicated. Restriction sites for mapping are indicated: E, *EcoRI*; P, *PstI*

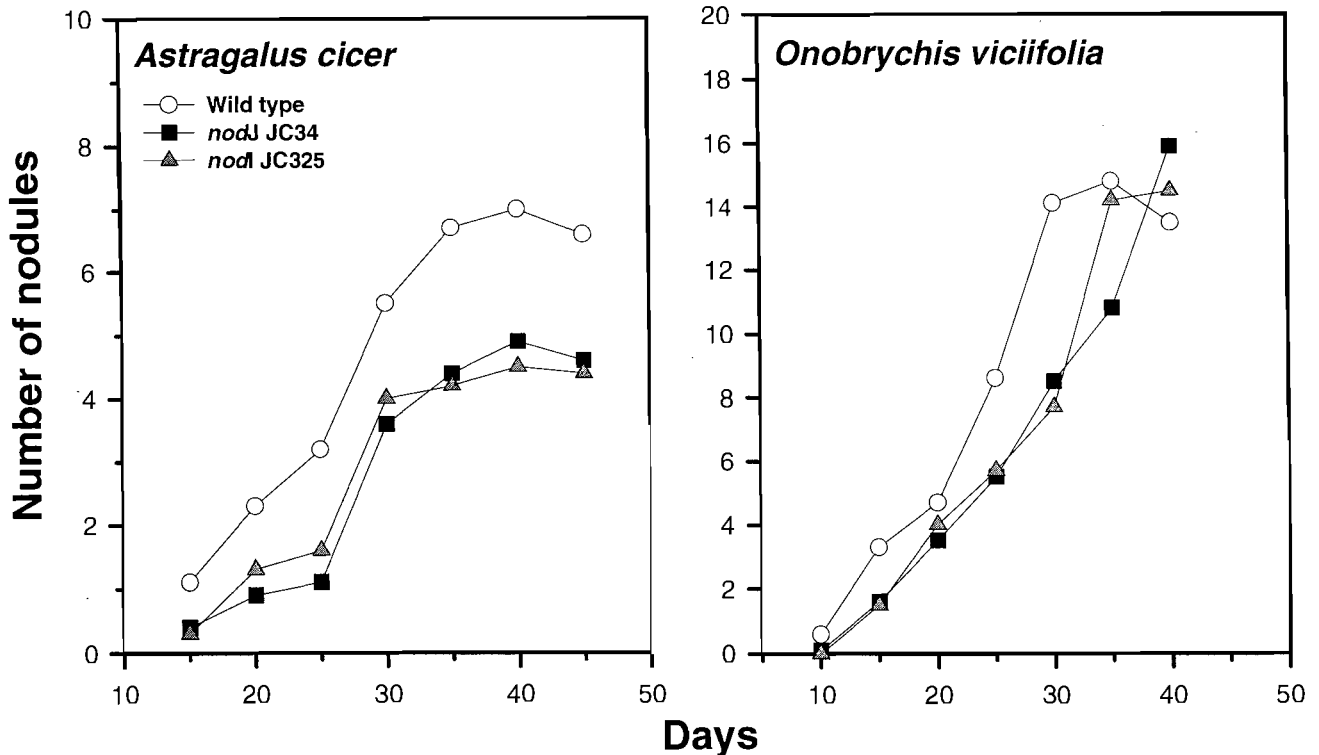


Fig. 3. Nodulation kinetics of *Rhizobium* sp. strain N33 and mutants carrying Tn5 insertions in the *nodIJ* genes. The host plants tested are indicated. Each value represents the mean of 10 plants. The standard deviation is  $\leq 34\%$  for *Onobrychis viciifolia* and  $\leq 36\%$  for *Astragalus cicer*.

from *Rhizobium* sp. strain N33. The amino acids identity are 39% for NodB, 52% for NodC, 31% for NodI, and 33% for NodJ. The C-terminus of NodC protein from strain N33 is longer than all other rhizobia except for *B. japonicum* (data not shown).

#### Nodulation kinetics of *nodBCIJ* mutants.

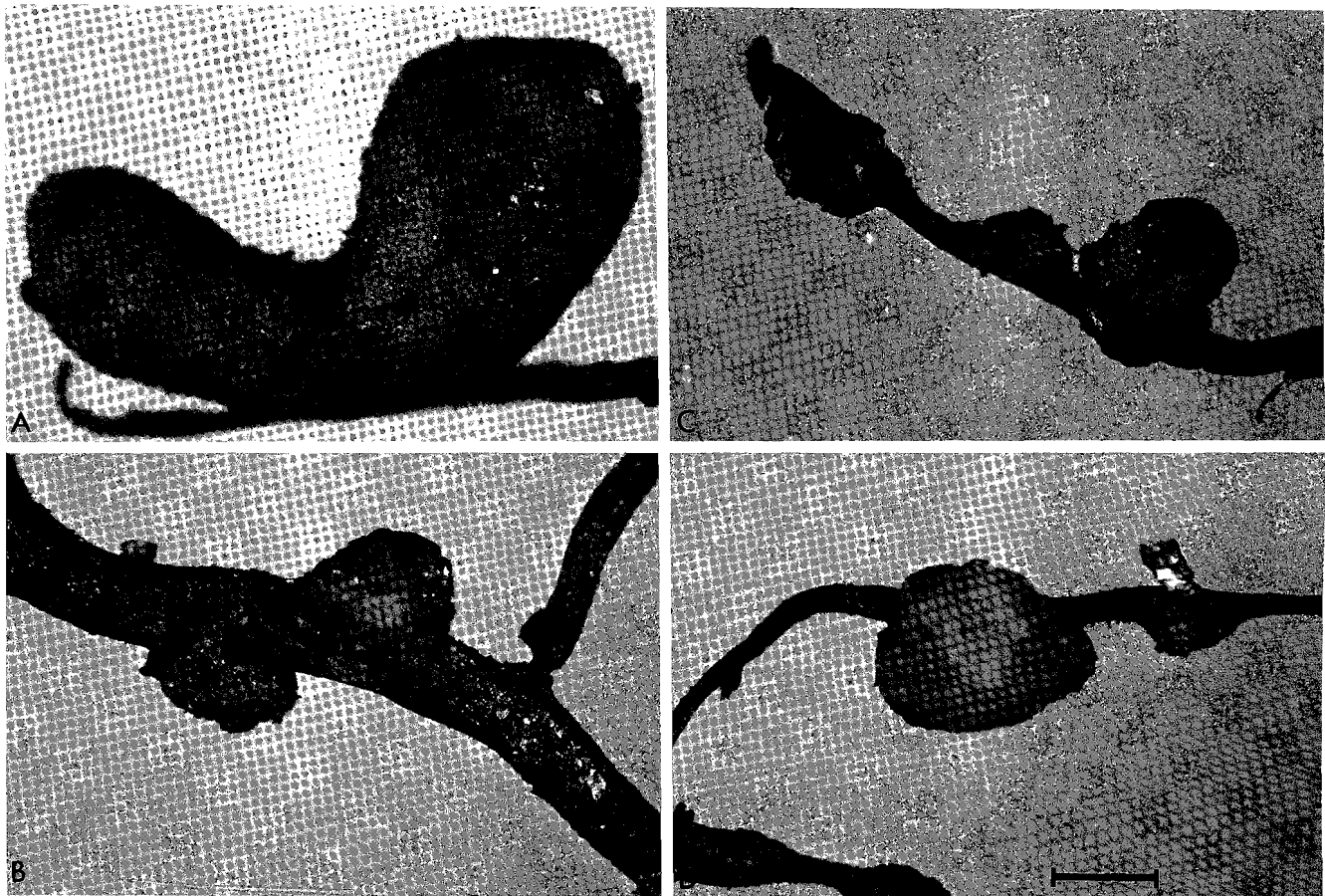
Many mutants in the *nodBCIJ* region were obtained following Tn5 mutagenesis (Fig. 2). Derivative of *Rhizobium* sp. strain N33 harboring mutations in the *nodB* (JC336) or *nodC* (JC253, JC261 and JC309) genes had a Nod<sup>-</sup> phenotype on their temperate hosts *Astragalus cicer* and *Onobrychis viciifolia*. However mutations in the *nodI* (JC325) and the *nodJ* (JC34) genes showed a reduced number of nodules on their temperate hosts compared to the wild-type strain (Fig. 3): The *nodI* mutant (JC283) and the *nodJ* mutant (JC131) showed the same nodulation phenotype (data not shown). The decrease in the number of nodules per plant induced on *Astragalus cicer* by *nodI* or *nodJ* mutants was more drastic and obvious over the whole nodulation period compared to what was observed on *Onobrychis viciifolia*. On the latter host, the number of nodules formed by both *nodI* and *nodJ* mutants was the same as the wild-type strain at the end of the nodulation period (35 to 40 days, Fig. 3), but the size of nodules induced by these mutants was about 10 times smaller in volume than the nodules formed by the

wild-type strain (Fig. 4). This size reduction was not observed on plants of *Astragalus cicer*.

We have only rarely been able to recover bacteria from crushed nodules induced by the *nodIJ* mutants on *Onobrychis viciifolia* (10%), when the nodules were observed to be slightly bigger (Fig. 4). The recovery of bacteria from nodules formed by the wild type strain was 100%. By contrast, no bacteria from crushed nodules induced by *nodI* or *nodJ* mutants on *Astragalus cicer* were recoverable, although a 40% recovery of the wild-type strain was obtained. This rather low level of recovery of the wild-type strain correlates with the observed low nitrogen fixation efficiency reported for strain N33 by Prévost (personal communication). We have not used the arctic legumes to test the nodulation phenotype of mutants in the *nodBCIJ* genes because it is very difficult to obtain enough seeds in order to perform statistically significant nodulation kinetics.

#### DISCUSSION

In comparison to temperate *Rhizobium* species, the *Rhizobium* sp. strain N33 is well adapted for growth and nitrogen fixation at low temperature (Prévost et al. 1987b). A knowledge of its nodulation genes should be useful in attempts to take advantage of the adaptation to low temperature which could be a desirable trait in agronomically important crop such



**Fig. 4.** Typical nodules induced on *Onobrychis viciifolia* by *Rhizobium* sp. strain N33 (A) and mutants of strain N33 carrying Tn5 insertion in the *nodI* (JC325) and *nodJ* (JC34) genes (B, C and D). All types of nodules in panel B, C and D are formed by both *nodI* and *nodJ* mutants. The scale bar correspond to 0.25cm.

as alfalfa in Canada. As a first step toward this goal, we have shown that strain N33, like all other rhizobia, possesses the so-called common nodulation genes *nodBCIJ*.

Tn5 insertions in the *nodB* and *nodC* genes totally blocked nodulation by strain N33 as is the case for other rhizobia (Long 1989). Mutations in the *nodIJ* genes causes reduction in the number of nodules on *Onobrychis viciifolia* and *Astragalus cicer*. This nodulation phenotype is similar to that observed for *A. caulinodans nodJ* mutant (Geelen et al. 1993), except that they also observed a nodulation delay. Mutations in the *nodI* and *nodJ* genes resulted in a nodulation delay in *R. leguminosarum* bv. *viciae* (Evans and Downie 1986), poor or no nodulation in *R. leguminosarum* bv. *trifolii* (Djordjevic et al. 1985; Canter-Cramers et al. 1988) but had no significant effect in *B. japonicum* (Nieuwkoop et al. 1987; Göttfert et al. 1989). It has been suggested by Vázquez et al. (1993) on the basis of similarity that NodIJ proteins of *Bradyrhizobium japonicum* and *R. l.* bv. *viciae* are similar to capsular polysaccharide secretion proteins from Gram negative bacteria and could be involved in the transport of the lipo-oligosaccharide (Nod factor) in the Rhizobiaceae family. Mutations in the *nodIJ* genes of *R. l.* bv. *trifolii* were shown to be essential for the Nod factor secretion after overnight induction (McKay and Djordjevic 1993). These results may explain why *nodIJ* mutants of *R. l.* bv. *trifolii* nodulated poorly their homologous hosts. Our results showed fewer

nodules were induced by *nodI* and *nodJ* mutants of strain N33 on its two temperate hosts than by the wild type: on *Onobrychis viciifolia*, the nodule size was also reduced. This observation requires further investigation to determine if the *nodI* and *nodJ* genes are responsible for the phenotype observed since it could be caused by a polar effect on downstream gene(s).

We have not been able to find a *nodA* gene within 0.5-kb region upstream of the *nodB* gene. Since a *nod* box is present 376 nucleotides before the *nodB* gene, it seems that the *nodA* gene of strain N33 is not located beside the *nodB* gene as found for many rhizobia. An examination of the sequence upstream of *nodB* gene indicated that a peptide of 24 amino acids shares homology with the C-terminus of NodA proteins from various rhizobia (Fig. 1). Also, the sequence ATGA between the truncated *nodA* gene and *nodB* gene are overlapping stop and start codons as is the case in many rhizobia. These results indicate that most of the *nodA* gene has been deleted except for the 24 amino acids that abut *nodB*. Vázquez et al. (1991) reported that *nodA* in *R. etli* is separated from *nodBC* by 20 kb. It was also shown by DNA sequencing that *nodB*-like DNA fragments were adjacent to the *nodA* gene and that a *nodA*-like DNA fragment was beside the *nodB* gene, thus indicating a complex genomic rearrangement had occur which involved *nodA* and *nodB* genes.

Preliminary sequence data revealed that the *nodA* gene in the *Rhizobium* sp. strain N33 is present 4.1-kb upstream of the *nodB* gene and that it is located on a 4.1-kb *EcoRI* fragment. Future work will include the characterization of this region in order to identify other nodulation genes which may be characteristic of *Rhizobium* sp. strain N33.

**Table 1.** Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<b>Strains</b>		
<i>Escherichia coli</i>		
DH5 $\alpha$	<i>endA1 hsdR17 supe44 thi-1 recA gyrA96 relA1 <math>\Delta</math>(argF<sup>-</sup> lacZYA)U169 <math>\Phi</math>80dlacZ<math>\Delta</math>M15</i>	Bethesda Research Lab.
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<sup>r</sup></i>	T. M. Finan
MT614	MT607 $\Omega$ tn5	T. M. Finan
MT616	MT607 containing pRK600, mobilizer	Finan et al. 1986
<b>Arctic rhizobia</b>		
N33	Wild type	Prévost et al. 1987a
N33	Wild type, Sm <sup>r</sup>	This study
JC34	<i>nodJ::Tn5, Sm<sup>r</sup>, Nm<sup>r</sup></i>	This study
JC131	<i>nodJ::Tn5, Sm<sup>r</sup>, Nm<sup>r</sup></i>	This study
JC253	<i>nodC::Tn5, Sm<sup>r</sup>, Nm<sup>r</sup></i>	This study
JC261	<i>nodC::Tn5, Sm<sup>r</sup>, Nm<sup>r</sup></i>	This study
JC283	<i>nodI::Tn5, Sm<sup>r</sup>, Nm<sup>r</sup></i>	This study
JC309	<i>nodC::Tn5, Sm<sup>r</sup>, Nm<sup>r</sup></i>	This study
JC325	<i>nodI::Tn5, Sm<sup>r</sup>, Nm<sup>r</sup></i>	This study
JC336	<i>nodB::Tn5, Sm<sup>r</sup>, Nm<sup>r</sup></i>	This study
<b>Plasmids</b>		
pPH1J1	pRK2 derivative (IncP), Cm <sup>r</sup> , Gm <sup>r</sup> , Sp <sup>r</sup>	Beringer et al. 1978
pRK600	pRK2013 Nm <sup>r</sup> ::Tn9, Cm <sup>r</sup> , Nm <sup>s</sup>	Finan et al. 1986
pRK7813	pRK2 derivative (IncP) carrying <i>cos</i> site, pUC9 polylinker, Tc <sup>r</sup>	Jones et al. 1987
pUC18/19	Cloning vector, ColE1 oriV bla, Ap <sup>r</sup>	Yanisch-Perron et al. 1985
pJC2	2.8-kb <i>EcoRI</i> fragment containing <i>nodBC</i> genes and part of <i>nodI</i> gene cloned into pUC18	This study
pJC3	5.7-kb <i>EcoRI</i> fragment containing <i>nodIJ</i> genes cloned into pUC18	This study
pJC5	8.2-kb <i>PstI</i> fragment containing <i>nodBCIJ</i> genes cloned into pUC18	This study

## MATERIALS AND METHODS

### Bacterial strains and plasmids.

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

### Media, antibiotics, and growth conditions.

The *Rhizobium* sp. strain N33 was grown on yeast mannitol broth (YMB) at 25°C (Vincent 1970). *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. Antibiotics were used at the following concentrations (micrograms per milliliter) for *Escherichia coli*: ampicillin, 80; chloramphenicol, 20; neomycin, 20; spectinomycin, 50; and tetracycline, 10.

### DNA manipulations.

Standard molecular biology techniques for DNA cloning, transformation, restriction endonuclease digestion analysis, agarose gel electrophoresis, Southern transfer, and hybridization were carried out as described by Sambrook et al. (1989). Hybridizations were done at 68°C using 2 $\times$  SSC containing 0.5% sodium dodecyl sulfate (SDS) and 0.25% w/v low fat milk powder. DNA probes were labeled with  $\alpha$ -<sup>32</sup>P-dCTP (3000 mCi/ml, Amersham) using the oligolabeling procedure (Feinberg and Vogelstein 1984).

Total genomic DNA from *Rhizobium* sp. strain N33 was isolated as described by Laberge et al. (1989). Plasmid DNA isolation was done according to Brun et al. (1991). Lambda phage DNA isolation was done according to Davis et al. (1986). Megaplasmid content of strain N33 was determined by the procedure of Wheatcroft et al. (1990).

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

Total genomic DNA from *Rhizobium* sp. strain N33 was partially digested using the restriction endonuclease *Sau3A*. DNA within the range of 15- to 20-kb was isolated by centrifugation (26000 rpm, Beckman rotor SW28, 16 h, 10°C) on a sucrose gradient (10 to 40% w/v), dialyzed to remove sucrose and concentrated by ethanol precipitation. DNA was ligated into the compatible *Bam*HI sites of EMBL3 phage arms (Promega), packaged in vitro into lambda heads (Promega) using the method suggested by the supplier. Lysate plaques (4,000 in total) were transferred on Hybond-N membranes (Amersham) and screened by hybridization with a DNA probe containing the *nodABC* genes and/or the *nodIJ* genes from *Bradyrhizobium japonicum* (Göttfert et al. 1990). Positive clones were selected and multiplied, their DNA isolated and digested with *Eco*RI or *Pst*I. Following agarose gel electrophoresis, Southern hybridizations was performed using the *nodABC* and *nodIJ* probes. *Eco*RI and *Pst*I positive fragments were cloned into pUC18.

#### DNA sequencing and computer analysis.

The complete double-stranded sequence of DNA fragments was done using the Nested Deletion Kit procedure from Pharmacia. DNA sequencing was done using the dideoxy chain termination method (Sanger et al. 1977; Sambrook et al. 1989).

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

#### Transposon mutagenesis.

Tn5 insertion in the 5.7-kb *Eco*RI and 8.2-kb *Pst*I fragments previously cloned into pRK7813 were generated in the Tn5 carrying *E. coli* strain MT614. Transposition onto the plasmid was identified by mobilizing the resulting Km<sup>r</sup> plasmids out of the strain into *E. coli* MT609 (*polA*) using *E. coli* MT616 containing the helper plasmid pRK600. The position of Tn5 insertions were determined following single and double digestions with various restriction enzymes: *Bam*HI, *Eco*RI, *Hind*III, *Pst*I, and *Sma*I. Plasmids containing Tn5 insertion were transformed into *E. coli* DH5 $\alpha$  and introduced into *Rhizobium* sp. strain N33 by triparental mating using the helper strain *E. coli* MT616. The transconjugants were selected on YMB media containing neomycin, tetracycline, and streptomycin (Yarosh et al. 1989).

#### Homogenization of *nodBCIJ* genes containing Tn5 insertions.

Marker exchange was performed by biparental mating using

*E. coli* strain J53 containing the IncP plasmid pPH1J1. Homogenotes were selected which had retained the transposon (Nm<sup>r</sup>) and the incoming plasmid pPH1J1 (Cm<sup>r</sup>) and which had lost tetracycline resistance. Homogenotes of strain N33 were cured of plasmid pPH1J1 by repeated subculturing in the absence of antibiotic selection (Ruvkun and Ausubel 1981). The structure of homogenotes DNA was confirmed by Southern blot analysis.

#### Nodulation assays and isolation of bacteria from nodules.

Seeds of *Onobrychis viciifolia* cv. Nova and *Astragalus cicer* cv. Oxley were obtained from Agriculture Canada research branch at Saskatoon, Saskatchewan. Seeds were surface sterilized by soaking in ETOH 95% for 30 s and twice in sodium hypochlorite (3.0%, v/v) 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% w/v agar petri dishes and incubated in darkness. Germinated seedlings were aseptically transferred and grown in vermiculite tubes (25 × 200 mm) supplemented with 20 ml of nitrogen free Hoagland's solution containing 0.1% w/v calcium carbonate (Vincent 1970). One milliliter of bacterial suspension containing at least 10<sup>8</sup> bacteria was added to each tube. Plants were observed at 5 days intervals and scored for i) number of plants showing nodules and ii) number of nodules on each plant. Ten tubes were observed at each interval. Plants were grown at 20°C under fluorescent light on a 16 h day, 8 h night cycle for 45 days. Ten nodules formed by each mutant and the wild-type strain N33 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.

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