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The Common Nodulation Genes of *Astragalus sinicus* Rhizobia Are Conserved despite Chromosomal Diversity

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The nodulation genes of *Mesorhizobium* sp. (*Astragalus sinicus*) strain 7653R were cloned by functional complementation of *Sinorhizobium meliloti nod* mutants. The common *nod* genes, *nodD*, *nodA*, and *nodBC*, were identified by heterologous hybridization and sequence analysis. The *nodA* gene was found to be separated from *nodBC* by approximately 22 kb and was divergently transcribed. The 2.0-kb *nodDBC* region was amplified by PCR from 24 rhizobial strains nodulating *A. sinicus*, which represented different chromosomal genotypes and geographic origins. No polymorphism was found in the size of PCR products, suggesting that the separation of *nodA* from *nodBC* is a common feature of *A. sinicus* rhizobia. Sequence analysis of the PCR-amplified *nodA* gene indicated that seven strains representing different 16S and 23S ribosomal DNA genotypes had identical *nodA* sequences. These data indicate that, whereas microsymbionts of *A. sinicus* exhibit chromosomal diversity, their nodulation genes are conserved, supporting the hypothesis of horizontal transfer of *nod* genes among diverse recipient bacteria.

Rhizobia are soil bacteria that can form nodules, in which they fix nitrogen, on leguminous plants in a host-specific manner. Nodulation (*nod*) genes have been identified that control the specific infection and nodulation of the plant hosts. The initial infection event is regulated by a NodD protein or proteins which activate the transcription of other *nod* genes in the presence of host-produced flavonoids (12, 25, 37). The *nodABC* genes are called common *nod* genes because they are present in all rhizobia. Other *nod* genes, such as *nodFE*, *nodH*, *nodSU*, and *nodZ* (12, 25, 37), are present in various combinations in rhizobial species and are called host-specific *nod* genes.

Expression of common and host-specific *nod* genes results in the production of lipochitooligosaccharides (Nod factors) that act as morphogenic signal molecules on specific legume hosts (12, 37). All Nod factors have a β-1,4-linked *N*-acetyl glucosamine oligosaccharide backbone ranging in length from 3 to 5 residues and substituted for by an N-acyl chain at the nonreducing end and other chemical groups on the glucosamine residues. The common *nodABC* gene products are involved in the synthesis of the N-acylated oligosaccharide core, while the host-specific *nod* gene products are involved in the decoration of this backbone with substitutions that confer plant specificity. The *nodABC* genes encode an acyltransferase, a chitin oligosaccharide deacetylase, and a chitin oligosaccharide synthase, respectively (3, 33). The common *nod* genes are also involved in determining host range specificity to some extent. For example, different NodA proteins recognize and transfer different fatty acid chains to the chitooligosaccharide chain, the length of which is determined by NodC (11, 27, 32). The common *nodABC* genes are essential for nodule formation. Mutation in any of them abolishes the ability to produce Nod factors and results in a nonnodulating (Nod^-) phenotype (12).

Astragalus sinicus L. (Chinese milk vetch) is an important winter-growing green manure, traditionally grown in the rice fields of southern China, Japan, and Korea. *A. sinicus* is a very specific host and usually forms nodules only with rhizobia isolated from itself (4), the only reported case of cross-inoculation being with a rhizobial strain isolated from *Astragalus ciceri* (24). Chen et al. (5) undertook a taxonomic study of nine *A. sinicus* isolates and proposed a new species, *Rhizobium huakuii*, for the rhizobia isolated from this host. On the basis of 16S ribosomal DNA (rDNA) sequence data and other taxonomic criteria, Jarvis et al. (21) proposed a new genus, *Mesorhizobium*, to which *R. huakuii* was transferred. Our previous work has shown that *A. sinicus* rhizobia are diverse (17, 42): of 204 strains analyzed, all are *Mesorhizobium*, but they belong to four different 16S rDNA genotypes. These four genotypes can be subdivided into seven genotypes when 16S and 23S rDNA data are combined. *Mesorhizobium* sp. (*A. sinicus*) strain 7653R belongs to the dominant 16S rDNA genotype, genotype 3, which is different from that of the *M. huakuii* type strain, CCBAU2609. Strain 7653R contains two large plasmids, and the *nod* genes are carried by the larger plasmid (43). The structures of the Nod factors produced by three *Mesorhizobium* sp. (*A. sinicus*) strains, including 7653R, have been determined: the three strains produce identical pentameric lipochitooligosaccharides that are O sulfated and partially N glycolylated at the reducing end and N acylated at the nonreducing end by a $C_{18:4}$ fatty acid (40). The aims of this study were to isolate and characterize the *nod* genes of *Mesorhizobium* sp. (*A. sinicus*) strain 7653R, to compare the common *nod* genes with those from other species of rhizobia, and to look at *nod* gene variation among *A. sinicus* nodule isolates representing the different chromosome types (17, 42).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Rhizobia were grown at 28°C on either tryptone-yeast (TY) medium (tryptone, 5 g/liter; yeast extract, 3 g/liter; $CaCl_2 \cdot 2H_2O$, 0.87 g/liter) or yeast extract-mannitol (YEM) medium (39). *Escherichia coli* strains were grown at 37°C on Luria-Bertani (LB) medium (28). Where appropriate, antibiotics were added to the following concentrations (mi-

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TABLE 1. Strains and genotypes of *A. sinicus* rhizobia used in this study

Strain	Geographic origin (China)	16S rDNA type[a]	16S–23S rDNA IGS type (23S rDNA genotype)[b]
nodA sequencing			
ZJ15B7	Deqin, Zhejing	a3	9 (1)
HB5A4	Wuhan, Hubei	c7	? (10)
HN14A16	Nan, Hunan	c8	? (10)
HN8A1	Henshan, Hunan	b4	5 (1)
JX2B5	Nanchang, Jiangxiu	a3	8 (5)
JS5A15	Wujin, Jiangsu	a3	1 (3)
HN15B23	Nan, Hunan	c7	? (11)
nodDC intergenic region PCR			
7653R	Nanjing, Jiangsu	a3	3
ZJ9A16	Nanxi, Zhejiang	a	2
ZJ10B11	Nanxi, Zhejiang	a	3
ZJ13A7	Shaoxin, Zhejiang	b	7
ZJ13A4	Shaoxin, Zhejiang	b	7
ZJ13B16	Shaoxin, Zhejiang	c	7
GX8A7	Guilin, Guangxi	a	3
GX8B23	Guilin, Guangxi	a	3
HB7A6	Wuhan, Hubei	d	3
HB7B4	Wuhan, Hubei	a	6
JS6A16	Wujin, Jiangsu	a	10
JS6A11	Wujin, Jiangsu	a	10
HN2A1	Changsha, Hunan	a	9
HN6A4	Henshan, Hunan	b	5
HN6B13	Henshan, Hunan	b	5
HN8A3	Henshan, Hunan	a	3
HN15A6	Nan, Hunan	c	?
HN15B23	Nan, Hunan	c	?
HN15B4	Nan, Hunan	a	3
JX5B2	Yujiang, Jiangxi	c	?
JX6A2	Xinyu, Jiangxi	d	?
JX6A4	Xinyu, Jiangxi	a	7
JX6A18	Xinyu, Jiangxi	b	5
JX6A19	Xinyu, Jiangxi	b	7
JX6B8	Xinyu, Jiangxi	d	?

[a] Letters refer to the 16S rDNA genotype obtained by RFLP analysis with four enzymes, and numbers refer to the 16S rDNA genotype obtained with nine enzymes (42).

[b] Question marks indicate that more than one band was produced in the 16S–23S rDNA IGS PCR amplification (17). The number in parentheses is the 23S rDNA genotype (42).

crograms per milliliter): streptomycin, 200; ampicillin and kanamycin, 100; phleomycin, 20; and tetracycline, 10. The rhizobial strains from *A. sinicus* are described in Table 1. The other rhizobial strains and plasmids used in this study are shown in Table 2.

Construction of a cosmid gene library. Total DNA of *Mesorhizobium* sp. (*A. sinicus*) strain 7653R was prepared according to the method of Ma et al. (23) and partially digested with the restriction enzyme *Eco*RI. Fragments of 20 to 30 kb were isolated by centrifugation (25,000 rpm on a Beckman SW28 rotor; 20°C, 24 h) through a sucrose density gradient (10 to 50% [wt/vol]), dialyzed to remove the sucrose, and then concentrated to a suitable volume by precipitation with 2 volumes of ethanol by resuspension in distilled water. DNA was then ligated with a sevenfold excess of *Eco*RI-digested and phosphatase-treated cosmid vector pLAFR3 (33). The ligated DNA was packaged into lambda bacteriophage particles and introduced into *E. coli* LE392 by transfection (28). Three thousand single colonies were picked and stored separately in 20% glycerol at −70°C by using 96-well plates. The colonies were mixed before use.

Conjugation and plant experiment. The cosmid gene library was introduced into *Sinorhizobium meliloti* mutant strains through triparental mating with the help of plasmid pRK2013 (13); crosses were carried out on TY medium by the method described by Christensen and Schubert (6). *Medicago sativa* seeds and nodules were surface sterilized by soaking in 95% ethanol for 5 min and in 0.2% acidified $HgCl_2$ for 3 min and then being rinsed 10 times in sterile water. The surface-sterilized seeds were germinated at 22°C in darkness overnight, and the seedlings were aseptically grown in test tubes on Jensen nitrogen-free agar slants (9). Two seedlings were put into each tube.

DNA manipulations and sequence analyses. Southern blots were carried out on Hybond-N^+ membranes (Amersham) according to the manufacturer's instructions. The *nod* gene probes were labeled with the Promega Multiprimer Labeling kit. The *nodD* and *nodC* gene probes were retrieved from pRmSL42, which contains the *nod* genes of *S. meliloti* (15). The *nodA* gene of *Mesorhizobium loti* cloned in plasmid pPN25 (31) was used for *nodA* gene hybridization. A 0.6-kb *Sal*I fragment from pGMI174, internal to the *S. meliloti* 2011 *nodE*, was used as a *nodE* probe (8). Sequencing of the common *nod* genes of *Mesorhizobium* sp. strain 7653R was achieved by further subcloning the 4.2-kb *Eco*RI and 10-kb *Hin*dIII fragments as several smaller fragments (about 1 to 2 kb) into the sequencing vector pBluescript KS+ (28). Sequencing reactions were performed by Sanger's dideoxy chain termination method, and the extension products were separated and detected on an Automated Laser Fluorescent DNA Sequencer (Pharmacia). Primers were synthesized for sequencing in order to get the whole sequences of both strands.

The DNA sequences were analyzed by using the GCG software version 7.1 (Genetics Computer Group, University of Wisconsin, Madison). The sequences used for phylogenetic studies were first aligned by using PILEUP, and phylogenetic trees were constructed by using ClustalW (36), which uses the neighbor-joining algorithm with the K2P distance correction. The resultant trees were displayed with TreeView (26). The *nodABC* gene sequences of the following species or strains used in phylogenetic analyses were obtained from the EMBL database (accession numbers in parentheses): *Azorhizobium caulinodans* ORS571 (L18897), *Rhizobium leguminosarum* bv. viciae (Y00548), *Rhizobium galegae* (X87578), *S. meliloti* Rm1021 (M11268), *S. meliloti* Rm41 (X01649), *Rhizobium etli* CE-3 (M58625 and M58626), *Sinorhizobium* sp. strain NGR234 (X73362), *Sinorhizobium fredii* USDA257 (M73699), *M. loti* NZP2037 (X52958), *Mesorhizobium* sp. strain N33 (U53327), and *Rhizobium tropici* CFN299 (X98514).

PCR amplifications. The *nodD*-to-*nodC* region of *A. sinicus* rhizobia was amplified by using primers DC1 (5′-GTA CAG GAG GGC ATC GCG AA-3′) and DC2 (5′-CTG CAG CTG CAG CGA ATC TG-3′). Primer DC1 corresponds to positions 1244 to 1225 in the *Mesorhizobium* sp. strain 7653R *nodDBC* sequence (in *nodD*), and primer DC2 corresponds to positions 3189 to 3170 (in the *nodC* gene). The PCR was performed in a 60-μl volume containing 1× reaction buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100), 0.5 μM each primer, 1.25 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (dNTP), 30 ng of template DNA, and 2 U of *Taq* polymerase (Sangon). The following temperature profile was used: initial denaturation at 94°C for 3 min; 30 cycles of 94°C

TABLE 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source[a] or reference
Strains		
Sinorhizobium meliloti		
Rm2011 = SU47	Wild type, Nod$^+$ on *Medicago sativa*	GMI
Rm1021	Str^r derivative of Rm2011	GMI
GMI5382	Rm2011 *nodA*::Tn*5* Str^r Ph^r Nm^r	10
GMI5383	Rm2011 *nodB*::Tn*5* Str^r Ph^r Nm^r	10
GMI5387	Rm2011 *nodC*::Tn*5* Str^r Ph^r Nm^r	10
GMI6227	Rm1021 *nodD1*::Tn*5* *nodD2*::tm *nodD3*::sp/g-1	20
GMI5619	Rm2011 Δ(*nodH*)8 Nm^r Ph^r	9
GMI6348	Rm1021 *nodQ1*::Tn*5-233* *nodQ2*::Tn*5*	30
GMI6628	Rm2011 Δ(*nodF*)*13* *nodL*::Tn*5*	2
Mesorhizobium (type strains)		
M. loti	LMG6125, Nod$^+$ on *Lotus tenuis*	LMG
M. ciceri	USDA3383, Nod$^+$ on *Cicer arietinum*	USDA
M. tianshanense	USDA3592, Nod$^+$ on *Glycyrrhiza pallidiflora*	USDA
M. mediterraneum	USDA3392, Nod$^+$ on *Cicer arietinum*	USDA
M. huakuii	CCBAU2609, Nod$^+$ on *Astragalus sinicus*	5
M. plurifarium	HAMBI193, Nod$^+$ on *Acacia senegal*	HAMBI
Plasmids		
pLAFR3	IncP, cosmid derived from pRK290, Tc^r	33
pRK2013	Helper plasmid, Km^r	13
pBluescript KS+	Sequencing vector, Ap^r	28
pHN50	pLAFR3 containing a 30-kb *nod* gene region *Mesorhizobium* sp. strain 7653R	This work
pHN71	pLAFR3 containing a 25-kb *nod* gene region of *Mesorhizobium* sp. strain 7653R	This work
pHN72	pLAFR3 containing a 21-kb *nod* gene region of *Mesorhizobium* sp. strain 7653R	This work
pHN51	4.2-kb *Eco*RI fragment of pHN50 cloned in pBluescript KS+	This work
pHN61b	2.8-kb *Eco*RI fragment of pHN72 cloned in pBluescript KS+	This work
pHN64	10-kb *Hin*dIII fragment of pHN50 cloned in pBluescript KS+	This work
pRmSL42	pBR325 carrying the common *nod* genes of *S. meliloti*	15
pPN25	pLAFR1 carrying 7.1-kb *nod* genes of *M. loti*	31
pGMI174	pBR322 derivative carrying *S. meliloti* 2011 *nodFEGH*	8

[a] USDA, U.S. Department of Agriculture, Beltsville, Md.; HAMBI, University of Helsinki, Helsinki, Finland; GMI, LBMRPM INRA-CNRS, Castanet-Tolosan, France; LMG, Collection of Bacteria of Laboratorium voor Microbiologie, University of Gent, Ghent, Belgium; CCBAU, Culture Collection of Beijing Agricultural University, Beijing, China.

for 1 min, 56°C for 1 min, and 72°C for 2 min; and final extension at 72°C for 10 min.

Amplification of *nodA* sequences with nodA-1 and nodA-2 used the protocol of Haukka et al. (18). Primers nodA-3 (5′-TCA TAG CTC YGR ACC GTT CCG-3′) and nodA-4 (5′-ATC ATC KYN CCG GNN GGC CA-3′), corresponding to positions 980 to 960 and 955 to 936 of the *Mesorhizobium* sp. strain 7653R *nodA* sequence, were designed with this sequence and those present in the databases. Where necessary, these primers were used in a nested PCR, involving two rounds of amplification, with the product of the first amplification, with primers nodA-1 and nodA-3, being used as a template for the second amplification, with primers nodA-1 and nodA-4. The same amplification conditions were used for each primer pair in 50-μl volumes containing 1× reaction buffer, 50 pmol of each primer, 1.63 mM $MgCl_2$, 0.2 mM dNTP, and 1 U of *Taq* polymerase (Promega). The following temperature profile was used: initial denaturation at 97°C for 2 min; 25 cycles of 92°C for 40 s, 55°C for 1 min, and 72°C for 1.5 min; and final extension at 72°C for 5 min. Amplified *nodA* sequences were cleaned by using the PCR Purification kit (Qiagen) and eluted in 30 μl of H_2O before sequencing with the Dye Terminator Ready Reaction kit (Perkin-Elmer) and an ABI 377 automated sequencer.

Nucleotide sequence accession number. The DNA sequences of *nodDBC* and *nodA* are available in the EMBL database under accession no. AJ249393 and AJ249353, respectively. The *nodA* sequences are available in the EMBL database under accession no. AJ25040 to AJ25042.

RESULTS

Cloning of common and specific *nod* genes from *Mesorhizobium* sp. (*A. sinicus*) strain 7653R by functional complementation. Nod factors produced by *M. huakuii* strains have been shown to be similar to those from *S. meliloti* (40): both are O sulfated at C-6 of the reducing end and N acylated by α,β-polyunsaturated acyl chains at the nonreducing end. We hypothesized that both species possess a similar set of nodulation genes and that various *S. meliloti* Nod$^-$ mutants could be used to identify clones containing their *M. huakuii* counterparts by heterologous functional complementation by a genomic cosmid library of strain 7653R. The seven *S. meliloti* 2011 *nod* mutant strains listed in Table 2, which exhibit a clear Nod$^-$ phenotype on alfalfa, were used. The transconjugants of each cross were used to inoculate 200 seedlings of *Medicago sativa*, the macrosymbiont of *S. meliloti*. Efficient nodulation occurred with a high proportion (80 to 97%) of replicates for all crosses, except that of the *nodFL* mutant (GMI6628), for which nodulation was found in only 18% of tubes.

Cosmids carrying genes complementing the *S. meliloti nod* mutants were isolated from the surface-sterilized nodules and characterized by restriction analysis. A physical map of the nodulation region was constructed (Fig. 1). Cosmids identical to pHN50 were isolated from Nod$^+$ transconjugants of the *S. meliloti nodA*, *nodB*, *nodC*, and $nodD_1D_2D_3$ mutants, indicating that pHN50 contains all of these genes. Southern hybridizations using the *nodD*, *nodA*, and *nodC* probes located the *nodC* and *nodD* genes to a 4.2-kb *Eco*RI fragment and the *nodA* gene to a 20-kb *Eco*RI fragment and a 10-kb *Hin*dIII fragment. These results suggest that *nodA* is separated from the *nodBC* genes, in contrast to the arrangement seen in most rhizobia. Cosmid pHN71, isolated from a Nod$^+$ transconju-

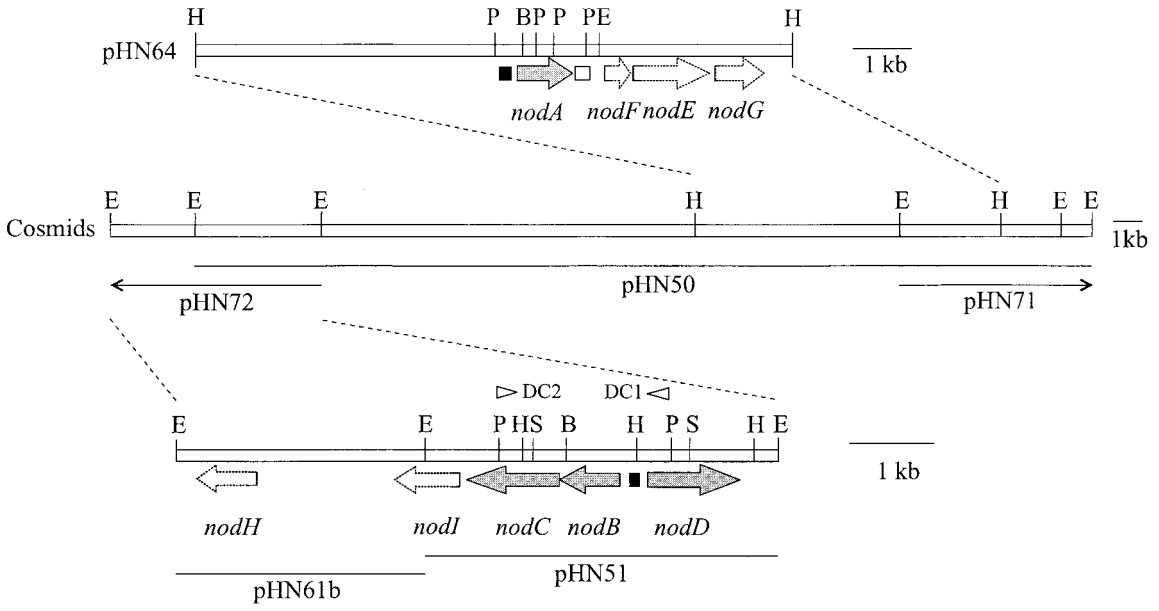

FIG. 1. Physical and genetic map of the nodulation (*nod*) gene region of *Mesorhizobium* sp. strain 7653R. Black boxes indicate possible *nod* box sequences, shaded arrows indicate sequenced genes, open arrows represent partially sequenced genes, and the white box represents the truncated *nodB*-like sequence downstream of *nodA*. The positions of primers DC1 and DC2 are indicated by triangles. Only the regions of cosmids pHN71 and pHN72 relevant to this study are shown (→). E, *Eco*RI; S, *Sal*I; B, *Bgl*II; P, *Pst*I; H, *Hin*dIII.

gant of the *S. meliloti nodF nodL* mutant (GMI6628), and pHN50 share 5.0- and 0.8-kb *Eco*RI fragments adjacent to the 20-kb *Eco*RI fragment carrying *nodA*. Southern hybridization using the *nodE* probe indicated that the 5-kb fragment contains a *nodE* homolog. The pHN72 cosmid, isolated from a Nod$^+$ transconjugant of the *nodH* mutant (GMI5619), overlaps pHN50 by the 4.2-kb *Eco*RI fragment carrying the *nodDBC* homologs. Partial sequencing of the adjacent 2.8-kb *Eco*RI fragment of pHN72 indicated that it contains a *nodH* homolog and a partial *nodI* homolog.

Sequence analysis of the common *nod* genes of strain 7653R. The 4.2-kb *Eco*RI fragment that hybridized with the *nodD* and *nodC* probes was subcloned into pBluescript KS+ (pHN51) and sequenced (accession no. AJ249393). This revealed four open reading frames, which correspond to the full-length *nodD*, *nodB*, and *nodC* genes and a partial *nodI* gene (Fig. 1). There is a 47-bp *nod* box (positions 1484 to 1530) in the *nodDB* intergenic region (Fig. 2), but a full-length *nodA* gene is not present in this region. Alignment of *nodB* gene sequences from 7653R and other rhizobia indicated that the 7653R *nodB* gene has an extra 54 bp at its 5′ end, which shows 68.3% identity with the 5′ end of the 7653R *nodA* gene (Fig. 2).

The 10-kb *Hin*dIII fragment carrying *nodA* was subcloned into pBluescript KS+ (pHN64). Southern hybridization with the *nodA* probe and subcloning localized the gene to a 1.15-kb *Pst*I-*Eco*RI DNA fragment which was sequenced (accession no. AJ249353). This region contains a full-length *nodA* gene which has a recognizable *nod* box upstream (positions 242 to 288) (Fig. 3). These results confirm that *nodA* is separated

```
1451  TTGAAACGCA TGTCCGCCAT CCACCGAGCG GCCATCCATC GAGTGGATAC
                 nodD ←                  YATCCAY. .YRYRGATG.
1501  CTCTCATCGA AACAAAGAAT TTTACCAGCT TTGCTGAACG TCGCATAGGG
      .....ATCYA AACAATCRAT TTTACCAATC Y......... ....ATAG
1551  ACCTTGCAAG GATGTAAGCC TGAAATGAAA CTTGGTGTTT ACGTTGGTGA
1601  CGAGCAGCAT TGCCGGTGAC GCCTTACGGC GTCATCCGGA TGTTTATAGC
1651  GGTTAAGCTT CTCCGGTAAT CTACGTCAAC CACAACGCGT CGGCTCGACG
1701  CGACAAGCTC CCTATCATTA GGCGCTTACC TCAAGAAAAC ACGAACAAAT

                 →nodB
1751  ACGAGGTCTT TCCATGCGTT CTGATGTGCG GTGGACGTTG TGCAGGGGCA
                 ***** * * *  ****  *****  *** *** *** *
      7653R nodA    ATGCGCT CAGCCGTGCA GTGGAGATTG TGCTGGGAAA

1801  ATGAGTCGCA ACATGCTCAG CATGTAGAAC TCTCGGGCTT TAAACAGG..
      ****   *** **   *  *  ***** **** **** * ***
      ATGACCTGCA ACTGACCGAC CATGTCGAAC TCTCCGACTT ..
```

FIG. 2. Organization of the 7653R *nodDB* intergenic region. The 5′ end of *nodB* is similar to the 5′ end of 7653R *nodA* (sequence written in italic letters). The nucleotide sequence of the *nodDB* intergenic region is numbered as in accession no. AJ249393. Arrows indicate start codons of the *nodD* and *nodB* genes. Boldface letters indicate the consensus *nod* box (29).

```
201  GCTGCTTGAA ATGCATGTCC GCGGTCGTCC ATCGAGTGGC CATCCATCGA
                                                YATCCAY..Y
251  GTGGATACCT TACATCGAAA CAAAGGATTT TATTGTCGTT CTGGAATCTT
     RYRGATG... ...ATCYAAA CAATCRATTT TACCAATCY. ..........
301  GCATGGACAA ACACCTGACG CGCCGATCAC CGCTGCCCTC TATGCCGTGA
     ..ATAG
                                                  nodA
351  TGAGCGTCTT ACGGACAGCC CGAAAGGAGA AATTCTTCCA TGCGCTCA..
     .......... .......... .......... .......... ..........

951  ..GATCGACC GGAACGGTTC GGAGCTATGA AACTTCTGAA CTGCAGGTGC

1001 GAGCCTGGAG TGACTGGGGT TGTGGCGCAG AAGATCGCAG CGTGTAT--G
                           * * * * **** *****  ** ***  *
     7653R nodB            ..GTCACCG AAGAGCGCAG TGTTTATCTG

1049 ACCTTTGACG AT-CTCCCAA TCCACTTTGC A--------- -CCTCGA-TT
     ** ******* *   *** **  **  ****  *          ****** **
     ACATTTGACG ACGGTCCGAA CCCGATTTGG ACACCGGAGG TCCTCGATTT

1087 GTTGGCGCAA CATTGGATGC CGGCGACGTT C-TCGTAGAC GGAGCCTG..
     * ******** *** ** * * ******* ** * ****   * ** ***
     GCTGGCGCAA CATCGGGTAC CGGCGACATT CTTCGTGATC GGTGCC..
```

FIG. 3. Organization of the 7653R DNA sequence upstream and downstream of the *nodA* gene. DNA downstream *nodA* shows similarity to a fragment of the *nodB* gene (sequence written in italic letters). The nucleotide sequence is numbered as in accession no. AJ249353. The start and stop codons of the *nodA* gene are underlined. The consensus *nod* box (29) is indicated by boldface letters.

from *nodBC* by a distance of approximately 22 kb. A 107-bp truncated *nodB*-like sequence (Δ*nodB*) was identified downstream of *nodA*, which shares 50.5% sequence identity with the corresponding part of the full-length *nodB* gene (Fig. 3). The Δ*nodB* start codon overlaps the *nodA* stop codon (ATGA). This kind of junction between *nodA* and *nodB* has been found in many rhizobia. The presence of Δ*nodB* immediately downstream of *nodA* and the presence of a *nodA*-like extension at the 5′ end of *nodB* are molecular evidence that genome rearrangements occurred at the *nodA-nodB* junction in an ancestor of the nodulation region now carried by strain 7653R.

The organization of common *nod* genes varies among *Mesorhizobium* species but is conserved in *Mesorhizobium* isolated from *A. sinicus*. To determine whether the separation of *nodA* from *nodBC* was unique to strain 7653R or was present in other *A. sinicus* isolates, primers DC1 and DC2 were designed to amplify the *nodDC* region, which corresponds to the 2.0-kb *Pst*I fragment of pHN51 (Fig. 1). Twenty-four representative strains were selected from a collection of field isolates isolated from six southern provinces of China. These strains had previously been characterized by restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rRNA genes and 16S-23S rDNA intergenic spacers (IGS) (Table 1) (17, 42). PCR amplification of each of the 24 strains, using primers DC1 and DC2, produced a single band of 2.0 kb. This is the same size as obtained from 7653R and indicates that the separation of *nodA* from *nodBC* is a general feature of *A. sinicus* rhizobia.

Additional evidence that *nodA* is separated from *nodBC* was provided by using primers nodA-1 and nodA-2. These primers had been used previously to amplify *nodA* sequences from tropical tree rhizobia and correspond to residues 14 to 37 of *nodA* and 65 to 43 of *nodB* of the *S. meliloti* 1021 sequence (M112684) (18). Although a *nodB*-like sequence follows the *nodA* gene of *Mesorhizobium* sp. strain 7653R, the nodA-2 primer site in this sequence is different, and amplification would be unlikely. PCR using the nodA-1 and -2 primer pair did not produce visible products with any of the seven *A. sinicus* isolates tested (Table 1). This finding is further evidence that *nodA* is separated from *nodB* in the *Mesorhizobium* strains of *A. sinicus*.

In contrast, the *M. ciceri*, *M. mediterraneum*, *M. plurifarium*, and *M. tianshanense* type strains produced an amplification product with the nodA-1–nodA-2 primer combination, indicating that, in these species, the *nodB* gene is located immediately downstream of *nodA*. The nodA-1–nodA-2 primer pair did not amplify *nodA* in the related species *M. loti*. This is in agreement with the previous report showing that, in *M. loti*, the *nodB* gene is separated from the *nodAC* genes (31). The fact that the rearrangements in *M. huakuii* and *M. loti* are different suggests that these rearrangements occurred independently.

Identity of *nodA* sequences in *A. sinicus* rhizobia of various chromosomal types. Having shown that the general organization of the common nodulation genes was conserved among *A. sinicus* rhizobia, we wanted to know whether the common *nod* genes were also conserved at the DNA sequence level in these rhizobia. For this work, we focused on the *nodA* gene and used only seven strains that represent each of the seven genotypes identified in an earlier study (42) (Table 1). Since the nodA-2 primer could not be used to amplify *nodA* genes from *A. sinicus* rhizobia, new reverse *nodA* PCR primers, nodA-3 and nodA-4, were designed to match the 3′ region of aligned *nodA* sequences. PCR using the nodA-1–nodA-3 primer pair produced weak amplification products with most of the *A. sinicus* isolates. However, a nested PCR using the nodA-1–nodA-4 primer pair and the nodA-1–nodA-3 PCR product as a template gave clean amplification products for all seven *A. sinicus* isolates, and each PCR product was sequenced on both strands. All seven *nodA* sequences were identical to that of strain 7653R, showing total conservation in spite of the diversity of geographical origins and chromosome backgrounds.

Phylogenetic studies. Phylogenetic relationships between the *nodA* genes of *A. sinicus* rhizobia and the *nodA* sequences available in the GenBank/EMBL database were studied at both DNA and deduced protein levels. The results indicated that the closest relatives of 7653R *nodA* genes are those of two other *Mesorhizobium* strains, *Mesorhizobium* sp. (*Oxytropis arctobia*) strain N33 and *M. loti*. In order to assess whether the *nodA* genes of all described *Mesorhizobium* species clustered together, we sequenced the *nodA* genes from type strains of *M. tianshanense*, *M. ciceri*, *M. mediterraneum*, and *M. plurifarium*, after PCR amplification of *nodA* by using the nodA-1 and nodA-2 primers. The dendrograms based on *nodA* nucleotide sequence are shown in Fig. 4. All *Mesorhizobium nodA* sequences, except the *M. plurifarium* sequence, lie in the same clade with 97% bootstrap support. The *M. plurifarium* type strain was isolated from *Acacia senegal*, a tropical leguminous tree, which is distantly related to the legume hosts of the other type strains surveyed in this work (14). The *nodA* sequences of *M. ciceri* and *M. mediterraneum*, two species isolated from the same host plant (chickpea), differ at a single position.

Comparison of the 7653R *nodDBC* gene sequences with the corresponding sequences available in databases also indicated that the closest relatives of 7653R genes are those of *Mesorhizobium* sp. (*Oxytropis arctobia*) strain N33 and *M. loti*. The dendrogram based on *nodC* nucleotide sequences is shown in Fig. 4.

DISCUSSION

The *nod* gene organization in *A. sinicus* rhizobia is unusual. The common nodulation genes in most rhizobial species are organized in a single operon, *nodABC*, with a copy of *nodD* upstream and divergently transcribed (15, 37). The exceptions described previously are *R. etli* (38), *M. loti* (31), and *Mesorhizobium* sp. strain N33 (7). *M. loti* strains NZP2037 and NZP2213 have *nodB* apart from *nodAC* (Fig. 5). The *nodA* gene is separated from *nodBC* in *R. etli* and *Mesorhizobium* sp. strain N33. However, in all cases studied so far, the *nodA*, *-B*, and *-C* genes are in the same orientation. Physical mapping and sequence analysis of the common nodulation gene region of *Mesorhizobium* sp. (*A. sinicus*) strain 7653R revealed that *nodA* is separated from *nodBC* by about 22 kb, and the two operons are divergently transcribed. Like *R. etli*, *M. loti*, and *Mesorhizobium* sp. strain N33, strain 7653R has a truncated *nodB*-like sequence immediately downstream of the *nodA* gene (Fig. 5). The complete 7653R *nodB* gene has extra sequence at the 5′ end that shows homology with the full-length *nodA* gene. These two observations support the hypothesis that *nodABC* was the ancestral operon organization and that genome rearrangements have occurred.

Horizontal transfer of nodulation genes. Genetically dissimilar microsymbionts have frequently been isolated from a single legume species (25). This is well documented in the case of the symbionts of soybeans, which belong to two different genera, *Bradyrhizobium* and *Sinorhizobium* (25), and of beans which belong to five different species of *Rhizobium* (*R. leguminosarum*, *R. etli*, *R. tropici*, *R. gallicum*, and *R. giardinii*) (1). Our previous work indicated that rhizobia belonging to four different 16S rDNA types nodulate *A. sinicus* (42). The high genetic diversity of *A. sinicus* rhizobia has been confirmed by the analysis of other marker genes, such as the 23S rRNA gene (42), *recA*, *glnA*, and *glnII* (our work [unpublished data]). How-

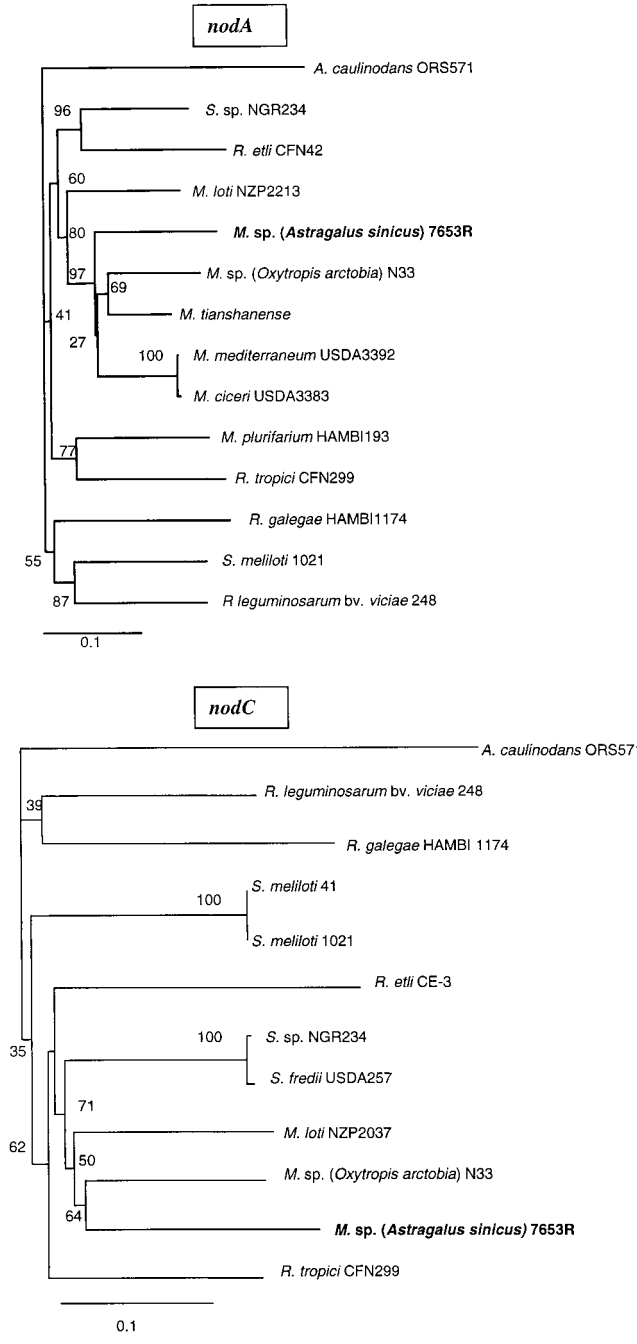

FIG. 4. Phylogenetic position of the *Mesorhizobium* sp. (*A. sinicus*) strain 7653R *nodA* and *nodC* sequences, calculated by using the neighbor-joining algorithm and the Kimura two-parameter model. Percentage bootstrap values obtained after 1,000 trials are shown, and the scale bars refer to the number of substitutions per site. A., *Azorhizobium*; S., *Sinorhizobium*; M., *Mesorhizobium*.

ever, the results presented here show that representatives of the different rhizobial rDNA types have the same common *nod* gene organization and identical *nodA* gene sequences. The chemical structures of Nod factors produced by *Mesorhizobium* sp. (*A. sinicus*) strain 7653R and two *M. huakuii* strains, Ra5 and Ra98, have been shown to be the same (40). These findings suggest that the *nod* genes of *A. sinicus* rhizobia are conserved in contrast to their chromosomal diversity.

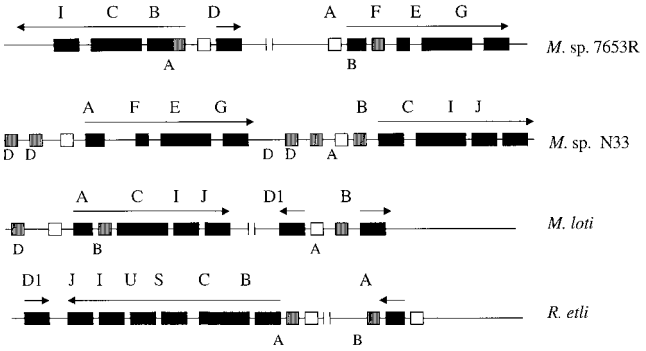

FIG. 5. Schematic map of selected nodulation genes in rhizobial species where rearrangements of the *nodABC* genes have been detected (7, 31, 37). Black boxes indicate functional *nod* genes. Shaded boxes indicate DNA fragments similar to *nod* genes, while white boxes indicate *nod* boxes. Arrows indicate the direction of transcription of the *nod* genes. M., *Mesorhizobium*.

The phylogenetic positions of different 16S rDNA genotypes within *Mesorhizobium* have been shown to be independent of host plant origin (22). Dendrograms based on both 16S and 23S rDNA indicate that *Lotus* rhizobia, which are known to be diverse, are the closest relatives of the different rDNA types of *A. sinicus* rhizobia studied in this paper (42). Sullivan et al. (34) observed transfer of the ability to form effective nodules with *Lotus corniculatus* between different species of mesorhizobia within soil communities. Although the symbiotic genes of *Lotus* rhizobia are on the chromosome, they can be transferred to nonsymbiotic mesorhizobia in the field environment because they are carried on a symbiotic island (35). Some *Lotus* rhizobia and *A. sinicus* rhizobia have the same chromosomal background but different host ranges. For example, *Mesorhizobium* sp. (*A. sinicus*) strain 7653R and *M. loti* NZP2037 belong to the same 16S rDNA genotype, which is different from those of both the *M. loti* type strain, NZP2213, and the *M. huakuii* type strain, CCBAU2609. This situation is equivalent to that known for the three biovars of *R. leguminosarum*, which have the same chromosomal type, but have different symbiotic genes and host ranges. Like *R. leguminosarum*, the nodulation genes of *A. sinicus* rhizobia are carried on a large plasmid, which could spread within field populations by horizontal plasmid transfer (17). Rapid horizontal transfer would explain why identical symbiotic genotypes are associated with different chromosomal backgrounds. This work has also revealed that the *nodA* sequences of the *M. mediterraneum* and *M. ciceri* type strains are almost identical. This finding parallels the results for the *A. sinicus* isolates: both the *M. ciceri* and *M. mediterraneum* type strains were isolated from the same host plant, *Cicer arietinum* (chickpea), again suggesting symbiotic gene transfer between chromosomal types that are sufficiently divergent to be considered separate species.

These findings suggest that symbiotic transfers can occur between different species of *Mesorhizobium*, in agreement with the findings of Sullivan et al. (34) for *Lotus* rhizobia. Interspecies transfer has also been documented for *Rhizobium* species: the same symbiotic genotypes have been isolated from *R. leguminosarum* bv. phaseoli, *R. etli*, *R. gallicum*, and *R. giardini* (1). If different species of fast-growing rhizobia are exchanging symbiotic genes at detectable frequencies, other genes might also be exchanged. If this were the case, it would bring into question the reliability and usefulness of species designations. However, we have recently sequenced internal fragments of the *recA* and *atpD* genes of the type strains of most rhizobium species, and these data support the 16S phylogeny (unpub-

lished data). The three genera *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium* are separated in all three phylogenies, and there is no evidence for intergenus gene exchange. Analysis of the glutamine synthetase genes (*glnA* and *glnII*) of rhizobial type strains may indicate an ancient exchange of genes between a *Rhizobium* species and *M. huakuii* but does not suggest that intergenus transfers of chromosomal genes are common (41).

We have shown that the functional *nodA* sequences of *A. sinicus* rhizobia are totally conserved between strains with different chromosomal backgrounds. While the amino acid sequence of *nodA* is undoubtedly constrained by functional considerations, the redundancy of the genetic code means that many base substitutions in the DNA sequence will not alter the protein. Such "silent" substitutions usually have little selective effect and accumulate relatively rapidly over evolutionary time. Complete identity at the DNA level is therefore strong evidence for a recent common ancestry of the *nodA* genes. The nonfunctional Δ*nodB* sequence is presumably not under selective pressure and will accumulate mutations more rapidly than a protein-coding gene. The Δ*nodB* sequence could serve as a molecular marker to further investigate symbiotic gene transfers among *A. sinicus* rhizobia for population dynamic and evolutionary studies. If symbiotic gene transfers are rare, the phylogeny of Δ*nodB* would be more similar to the 16S rDNA (chromosomal) phylogeny, since both symbiotic and chromosomal genes will have had a shared history. If, on the other hand, there has been a recent rapid spread of the *A. sinicus* symbiotic genes, sequence variation within Δ*nodB* will not reflect the chromosomal background. The Δ*nodB* sequence might therefore give an insight into how common horizontal gene transfer is in the genus *Mesorhizobium*.

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