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Diversity and Evolution of Hydrogenase Systems in Rhizobia

Cecilia Baginsky,[1] Belén Brito,[1] Juan Imperial,[2] José-Manuel Palacios,[1] and Tomás Ruiz-Argüeso[1]*

Laboratorio de Microbiología, E.T.S. Ingenieros Agrónomos, Universidad Politécnica de Madrid,[1] and Consejo Superior de Investigaciones Científicas (CSIC), Ciudad Universitaria s/n,[2] 28040 Madrid, Spain

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Uptake hydrogenases allow rhizobia to recycle the hydrogen generated in the nitrogen fixation process within the legume nodule. Hydrogenase (*hup*) systems in *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bv. viciae show highly conserved sequence and gene organization, but important differences exist in regulation and in the presence of specific genes. We have undertaken the characterization of *hup* gene clusters from *Bradyrhizobium* sp. (*Lupinus*), *Bradyrhizobium* sp. (*Vigna*), and *Rhizobium tropici* and *Azorhizobium caulinodans* strains with the aim of defining the extent of diversity in *hup* gene composition and regulation in endosymbiotic bacteria. Genomic DNA hybridizations using *hupS*, *hupE*, *hupUV*, *hypB*, and *hoxA* probes showed a diversity of intraspecific *hup* profiles within *Bradyrhizobium* sp. (*Lupinus*) and *Bradyrhizobium* sp. (*Vigna*) strains and homogeneous intraspecific patterns within *R. tropici* and *A. caulinodans* strains. The analysis also revealed differences regarding the possession of hydrogenase regulatory genes. Phylogenetic analyses using partial sequences of *hupS* and *hupL* clustered *R. leguminosarum* and *R. tropici hup* sequences together with those from *B. japonicum* and *Bradyrhizobium* sp. (*Lupinus*) strains, suggesting a common origin. In contrast, *Bradyrhizobium* sp. (*Vigna*) *hup* sequences diverged from the rest of rhizobial sequences, which might indicate that those organisms have evolved independently and possibly have acquired the sequences by horizontal transfer from an unidentified source.

A large amount of hydrogen is released from legume root nodules during the nitrogen fixation process. This hydrogen production has been described as one of the major factors that affect the efficiency of symbiotic nitrogen fixation (39). Uptake hydrogenases allow endosymbiotic bacteria to oxidize the hydrogen produced by nitrogenase. This symbiotic hydrogen oxidation has been shown to reduce the energy losses associated with nitrogen fixation and to enhance productivity in certain legume hosts (1, 14).

A detailed characterization of the hydrogen uptake (*hup*) system has been carried out in *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bv. viciae (for a review, see reference 35). In both genera, the first component of this system is a membrane-bound, dimeric [NiFe] hydrogenase composed by two polypeptides of 35 and 65 kDa. These polypeptides are synthesized as precursors, which are proteolytically processed after metal cluster insertion. The *hup* genetic determinants are clustered in large DNA regions (20, 21), whose sequence analysis has revealed the presence of at least 17 common genes (*hupSLCDFGHIJKhypABFCDEX*) arranged in at least three operons with conserved gene composition and organization (35). Hydrogenase structural subunits are encoded by the *hupS* and *hupL* genes, whereas the remaining *hup* and *hyp* gene products are involved in the recruitment and incorporation of nickel and other metallic groups into the hydrogenase active site (for reviews, see references 12 and 35). Although the *R. leguminosarum* and *B. japonicum* hydrogenase systems are highly homologous, they show important differences in regulation and in the presence of specific genes. The *hupE* gene is specific for the *R. leguminosarum* UPM791 *hup* gene cluster. The function of its predicted product is unknown, but it has been proposed that it might act as a nickel transporter (35). In contrast, this strain lacks the *hupNOP* genes, whose gene products are involved in nickel metabolism in *B. japonicum* (16). Two completely different regulatory circuits control hydrogenase gene expression in these bacteria (36). *Bradyrhizobium japonicum* expresses *hup* genes in symbiosis as well as in microaerobic free-living cells. Four proteins are involved in regulation in this latter condition: those of the regulatory hydrogenase formed by HupU and HupV, the HupT repressor, and the transcriptional activator HoxA (5, 44, 45). In contrast, *R. leguminosarum hup* genes are only induced in symbiotic conditions (29). Analysis of the *hupSL* promoter expression showed that *hup* gene transcription is activated by NifA, the key regulator of the nitrogen fixation process (11). No genes homologous to *hupUV* and *hupT* have been found in this bacterium (10), and genetic analysis has determined that the *hoxA* gene present in *R. leguminosarum* is truncated and inactive (11). This may explain why vegetative cells of *R. leguminosarum* express no hydrogenase activity in the same cultural conditions that induce hydrogen uptake in *B. japonicum* (29).

Analysis of legume nodules for the presence of hydrogenase-positive strains has been carried out for several rhizobia-legume systems (for a review, see 2). These studies revealed that hydrogen oxidation capability is not a common trait in endosymbiotic bacteria. Hydrogenases are common among *Bradyrhizobium* species but rare in *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium*. In addition to *Bradyrhizobium japonicum*, hydrogenase systems have been described for *Bradyrhizobium* sp.

* Corresponding author. Mailing address: Laboratorio de Microbiología, E.T.S. Ingenieros Agrónomos, Universidad Politécnica de Madrid, 28040 Madrid, Spain. Phone: 34-913365759. Fax: 34-913365757. E-mail: ruizargueso@bit.etsia.upm.es.

(*Lupinus*) (25) and *Bradyrhizobium* sp. (*Vigna*) (7, 31, 38), the microsymbionts of lupines and cowpeas, respectively. The Hup trait is widely represented among strains of these two species. On the basis of their hybridization patterns, several groups of Hup^+ strains have been identified in *Bradyrhizobium* sp. (*Lupinus*) and *Bradyrhizobium* sp. (*Vigna*) (25, 31). In contrast, the presence of a hydrogenase system has been reported in a few strains of *Rhizobium leguminosarum* bv. viciae (33) and it has never been described for *R. leguminosarum* bv. phaseoli (7), *R. leguminosarum* bv. trifolii (34), and *Mesorhizobium* sp. (*Cicer*) (24). As an exception, a high number of *Rhizobium tropici* strains possess the Hup trait but the hydrogenase activity displayed is not sufficient to eliminate the hydrogen evolved from nodules (23, 26, 43). Also, hydrogenase activity has been described for free-living cultures under nitrogen fixation conditions for *Azorhizobium caulinodans* ORS571 as well as for *Sesbania rostrata* bacteroids (40, 41).

Besides the identification of Hup^+ strains, little information is available on *hup* gene composition for rhizobia other than *B. japonicum* USDA110 or *R. leguminosarum* UPM791. It is possible that different organizations of *hup* gene clusters exist, since differences in gene composition and regulation have already been observed in the two systems analyzed. In this work, we have characterized *hup* genetic determinants from strains belonging to *Bradyrhizobium* sp. (*Lupinus*), *Bradyrhizobium* sp. (*Vigna*), *Azorhizobium caulinodans*, and *Rhizobium tropici* to define the range of diversity and differential characteristics of *hup* gene clusters in endosymbiotic bacteria. In addition, the relatedness of *hup* genes in these genera has been estimated from phylogenetic analysis carried out with partial *hupS* and *hupL* sequences.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains used in this work are listed in Table 1. *Rhizobium leguminosarum*, *Bradyrhizobium japonicum*, *Bradyrhizobium* sp. (*Lupinus*), *Bradyrhizobium* sp. (*Vigna*), and *Rhizobium tropici* strains were routinely grown in tryptone-yeast extract (4), yeast-mannitol (46), or *Rhizobium* minimal (27) medium at 28°C. The *Azorhizobium* sp. and *A. caulinodans* strains were cultivated in YEB medium (17).

DNA manipulation techniques. Genomic DNA of *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* strains was extracted as previously described (22). Restriction enzyme digestions, PCR amplifications, agarose gel electrophoresis, and Southern blot transfers were carried out by standard protocols (37). For Southern hybridizations, *hupS*, *hupE*, *hupUV*, *hypB*, and *hoxA* DNA probes (Fig. 1) were labeled by PCR with digoxigenin DIG-11-dUTP (Roche Molecular Biochemicals, Mannheim, Germany) at a 40 μM final concentration. The *R. leguminosarum* UPM791 gene probes were generated using plasmid pRL618 as the template (3), except in the case of *hoxA*, where genomic DNA was used as the template. Primers used were DH1-PHO1 for *hupS*, hupE1–hupE2 for *hupE*, U69-L588 for *hypB*, and PC1–PC2 for *hoxA*. The sequences of the primers are listed in Table 2. A 250-bp DNA fragment of the *B. japonicum hypB* gene was used as probe after PCR amplification and labeling with the degenerate primer pair hypB1–hypB2. These primers were also used to investigate the presence of the *hypB* gene in different strains by PCR amplification. Two *B. japonicum hoxA* probes of 436 and 994 bp were obtained using the AD1–AD2 and PC1–PC2 primers, respectively, and plasmid pHU52 as template (19). To generate the *B. japonicum hupUV* probe, we cloned a 1,555-bp *Pst*I/*Hin*dIII DNA region, containing the 3′ end of *hupU* and the 5′ half of *hupV* from plasmid pRY12 (5), into the pBluescript SK vector (Stratagene). The *hupUV* region was amplified and labeled by PCR with the T7 and Reverse primers. The hybridizing bands were visualized using a chemiluminescent DIG detection kit as described by the manufacturer (Roche Molecular Biochemicals). DNA sequencing was carried out using the BigDye Terminator Cycle-Sequencing Ready Reaction kit and an ABI377 automatic sequencer (PE Biosystems, Foster City, Calif.).

Plasmid profiles were resolved by following the procedure of Eckhardt (13) with some modifications. Cultures of *R. tropici* were grown on HP medium (18) for 16 h, diluted in tryptone-yeast extract medium, and incubated until the optical density at 600 nm was 0.2. A volume of 1.5 ml was centrifuged, washed with 0.3% Sarkosyl, and resuspended in 10% Ficoll–1 mg of lysozyme $liter^{-1}$–1 mg of RNase $liter^{-1}$–0.1% bromophenol blue in Tris-borate–EDTA buffer. Samples were loaded into a 0.6% agarose gel containing 1% sodium dodecyl sulfate. The gel was run at 10 mA for 2 h and 70 mA for 14 h at 4°C. For plasmid visualization, gels were stained with ethidium bromide. Plasmid DNA was transferred to nylon membranes by the Southern blotting technique. The *R. leguminosarum nifH* probe was generated and labeled by PCR, using primers nifHU1 and nifHL1 (Table 2) and UPM791 genomic DNA as the template.

Construction of *hupS*, *hupL*, and 16S ribosomal DNA (rDNA) phylogenetic trees from rhizobial sequences. Partial *hupS* and *hupL* sequences were obtained by PCR amplification using genomic DNA from each strain and the degenerate primers hupSL1 and hupSL2, which amplify a ca. 1.5-kb DNA fragment con-

TABLE 1. Strains used in this work

Strain	UPM strain number[a]	Source or reference[b]
R. leguminosarum		
UPM791	791	22
PRE	1025	J. Hontelez (Wageningen, The Netherlands)
B. japonicum		
122DES	804	H.J. Evans (Corvallis, Oregon)
Bradyrhizobium sp. (*Lupinus*)		
UPM860	860	25
624	873	C. Rydin (DMAC, Uppsala, Sweden)
466	878	D.C. Jordan (DMG, Guelph, Canada)
Z89	1029	N. Lissova (UAAN, Lvic-Obroshyn, Ukraine)
IM43B	939	M. Chamber (SIA, Sevilla, Spain)
A. caulinodans		
ORS571	1143	B. Dreyfus (Montpellier, France)
ORS591	1160	6
Azorhizobium sp.		
ORS552	1161	6
SG05	1162	32
SD02	1163	32
Bradyrhizobium sp. (Vigna)		
M2	1166	This laboratory
M5	1167	This laboratory
M18	1168	This laboratory
M21	1169	This laboratory
M43	1170	This laboratory
B78	1171	This laboratory
B96	1172	This laboratory
B97	1173	This laboratory
32HI	938	39
R. tropici		
USDA 2738	1144	P. van Berkum (USDA, Beltsville, Md.)
USDA 2822	1145	P. van Berkum (USDA, Beltsville, Md.)
USDA 9030	1146	P. van Berkum (USDA, Beltsville, Md.)
USDA 2801	1147	P. van Berkum (USDA, Beltsville, Md.)
USDA 2786	1148	P. van Berkum (USDA, Beltsville, Md.)
USDA 2840	1149	P. van Berkum (USDA, Beltsville, Md.)
USDA 2787	1150	P. van Berkum (USDA, Beltsville, Md.)
USDA 2813	1151	P. van Berkum (USDA, Beltsville, Md.)
USDA 2734	1152	P. van Berkum (USDA, Beltsville, Md.)
USDA 2793	1153	P. van Berkum (USDA, Beltsville, Md.)
USDA 2838	1154	P. van Berkum (USDA, Beltsville, Md.)

[a] UPM, Universidad Politécnica de Madrid, Madrid, Spain.
[b] USDA, U.S. Department of Agriculture.

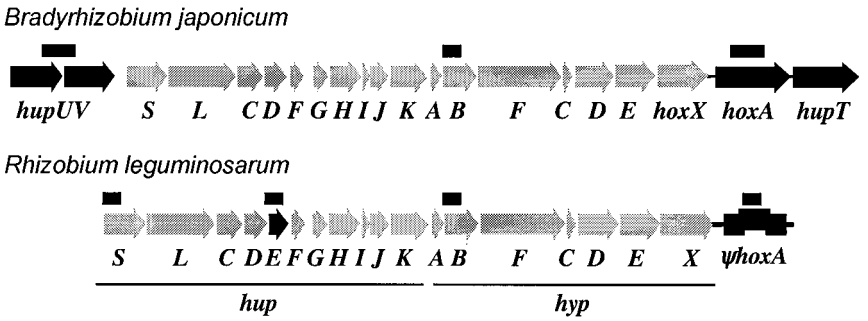


FIG. 1. Genetic organization of hydrogenase clusters in *Bradyrhizobium japonicum* 122DES and *Rhizobium leguminosarum* bv. viciae UPM791. Grey arrows indicate *hup* and *hyp* genes common to both species. Black arrows show genes present and functional in only one microorganism. Thick lines above genes show the positions of DNA probes used in Southern hybridizations.

taining the *hupSL* genes (Table 2). The temperature program was 180 s at 94°C; 35 cycles of 45 s at 95°C, 45 s at 48 or 51.7°C, and 90 s at 68°C; and 420 s at 72°C. Each PCR product was cloned in the PCR2.1-TOPO vector (Invitrogen BV, Groningen, The Netherlands) and sequenced using the T7 and Reverse primers. For the 16S rDNA sequences of rhizobial strains, a DNA region corresponding to nucleotides (nt) 20 to 338 of the *Escherichia coli* 16S rDNA was amplified from each strain using the Y1 and Y2 primers (Table 2) and the PCR amplification conditions described by Young et al. (51). The resulting fragments were cloned in PCR2.1-TOPO vector and sequenced. DNA sequences were optimally aligned using the CLUSTALX program (42) and visual refining. Neighbor-joining matrixes and trees were generated by CLUSTALX after bootstrapping (15) with 1,000 reiterations. Trees were drawn using TreeView software (28)

Nucleotide sequence accession number. The nucleotide sequences obtained in this study have been deposited in GenBank. Accession numbers for each strain are as follows: for the *hupS* region, AF466154 (Z89), AF466155 (UPM860), AF466156 (M2), AF466157 (M5), AF466158 (B78), AF466159 (B96), AF466160 (ORS571), AF466161 (ORS552), AF466162 (ORS591), AF466163 (USDA 2734), AF466164 (USDA 2838), and AF466165 (USDA 2787); for the *hupL* region, AF466753 (Z89), AF466754 (UPM860), AF466755 (466), AF466756 (M2), AF466757 (M5), AF466758 (B78), AF466759 (ORS571), AF466760 (ORS552), AF466761 (ORS591), AF466762 (USDA 2734), AF466763 (USDA 2838), and AF466764 (USDA 2787); and for the 16S rDNA region, AY072787 (UPM791), AF466166 (Z89), AF466167 (UPM860), AF466168 (IM43B), AF466169 (M5), AF466170 (B78), AF466171 (B96), AF4661672 (ORS552), AF466173 (ORS591), AF466174 (USDA 2734), AF466175 (USDA 2838), and AF466176 (USDA 2787).

TABLE 2. List of primers used in this work

Primer	Sequence (5′–3′)[b]	Target gene and position[a]
hupSL1	GGNYTNGARTGYACNTGYTG	*hupS* 178–197
hupSL2	CCCCARTANCCRTTY**TT**RAA	*hupL* 559–540
DH1	**CAT**ATGGCAACTGCCGAGAC	*hupS* 1–17
PHO1	**TCT**AGAGTCGGGCCCTTGCAGCCC	*hupS* 813–795
hupE1	CTCGATCATATCCTGGCGAT	*hupE* 57–76
hupE2	CGATGCACATGACGCTCTAT	*hupE* 618–599
hypB1	ATHGARGGNGAYCARCARAC	*hypB* 408–430
hypB2	GCRAACATRTCNGGRTAYTT	*hypB* 691–672
U69	CCACGGCCATCATCATCACG	*hypB* 68–87
L588	AGGCGGCGGGACAGACGAGA	*hypB* 606–587
PC1	CGGCATCTACCAATATATCACC	*hoxA* 305–325
PC2	CGGTATAGGCGCCCTTCT	*hoxA* 741–723
AD1	ATYCTSTGCGAYCAGCGSATG	*hoxA* 150–170
AD2	TCSCGVAGRTTNCCSGGCCAA	*hoxA* 1144–1125
nifHU1	CACTACGTCCCAAAACACG	*nifH* 53–71
nifHL2	AGCATRTCYTCVAGYTCYTC	*nifH* 808–789
Y1	TGGCTCAGAACGAACGCTGCGGC	*rm* 20–43
Y2	CCCACTGCTGCCTCCCGTAGGAGT	*rm* 361–338

[a] Primer positions are given from the first nucleotide of the start codon in the corresponding gene.

[b] Boldface letters correspond to bases added to create restriction sites in the amplified product.

RESULTS

Analysis of *hup* gene clusters in *Bradyrhizobium* sp. (*Lupinus*), *Bradyrhizobium* sp. (*Vigna*), *Azorhizobium caulinodans*, and *Rhizobium tropici*. In this work, we have characterized *hup* gene clusters of *Bradyrhizobium* sp. (*Lupinus*), *Bradyrhizobium* sp. (*Vigna*), *R. tropici*, *Azorhizobium caulinodans*, and *Azorhizobium* sp. strains by DNA hybridization, using probes of the *hupS*, *hupE*, *hypB*, and *hoxA* genes from *R. leguminosarum* bv. viciae UPM791 and the *hypB*, *hupUV*, and *hoxA* genes from *B. japonicum* 122DES. For these assays, we have used *R. leguminosarum* bv. viciae UPM791 (22) and *B. japonicum* 122DES (34) as positive control strains and *R. leguminosarum* bv. viciae PRE (3) as the negative control. The results obtained are separately described for each group and are summarized in Table 3.

***Bradyrhizobium* sp. (*Lupinus*).** Southern blot experiments using a *hupS* gene probe from *R. leguminosarum* revealed hybridizing bands ranging from 10 to 23 kb in all *Bradyrhizobium* sp. (*Lupinus*) strains (Fig. 2A). This experiment showed the presence of *hup* homologous DNA in strain Z89, which had never been subjected to this test before, and in strain IM43B, in which previous hybridization assays did not reveal *hup* homologous sequences (25). No hybridization signals were observed in any strain with the *hupE* gene probe (data not shown). In contrast, using the *R. leguminosarum hypB* probe, we detected a hybridizing band in all strains except for IM43B (Fig. 2B). In an attempt to improve DNA hybridization, we used a *B. japonicum hypB* probe but similar results were obtained (data not shown). As *hypB* is an essential constituent of all *hup* gene clusters characterized to date, we further investigated whether *hypB* was present in IM43B. This goal was addressed by PCR amplification using degenerate primers hypB1-hypB2 and genomic DNA from this strain. A 250-bp DNA fragment was obtained whose sequence revealed an 85% identity with *B. japonicum hypB* at the nucleotide level. This DNA fragment was used as probe in Southern experiments, and a hybridizing band of ca. 20 kb was observed for IM43B. For the remaining strains, we detected bands of sizes similar to those observed with the *B. japonicum* probe (data not shown). Using the *hupUV* probe, specific hybridization signals were

TABLE 3. Summary of hydrogen oxidation gene composition in the rhizobial strains tested

Strain	Detection of hybridization signal[a] (PCR amplification)				
	hupS	*hupE*	*hypB*	*hoxA*	*hupUV*
R. leguminosarum					
UPM791	+	+	+	+	−
PRE	−	−	−	−	−
B. japonicum					
122DES	+	−	+	+	+
Bradyrhizobium sp. (Lupinus)					
UPM860	+	−	+	+	+
624	+	−	+	+	+
466	+	−	+	+	+
Z89	+	−	+	+	+
IM43B	+	−	− (+)	+	−
A. caulinodans					
ORS571	+	−	+	−	+
ORS591	+	−	+	−	+
Azorhizobium sp.		−			
ORS552	+	−	+	−	+
SG05	− (−)	−	−	−	−
SD02	− (−)	−	−	−	−
Bradyrhizobium sp. (Vigna)					
M2	+	−	+	+	−
M5	+	−	+	+	−
M18	+	−	+	+	−
M21	+	−	+	+	−
M43	+	−	+	+	−
B78	+	−	+	+	−
B96	+	−	+	+	−
B97	+	−	+	+	−
32H1	+	−	− (+)	+	−
R. tropici					
USDA2738	+	−	+	−	−
USDA2822	+	−	+	−	−
USDA9030	+	−	+	−	−
USDA2801	+	−	+	−	−
USDA2786	+	−	+	−	−
USDA2840	+	−	+	−	−
USDA2787	+	−	+	−	−
USDA2813	+	−	+	−	−
USDA2734	+	−	+	−	−
USDA2793	+	−	+	−	−
USDA2838	+	−	+	−	−

[a] The symbols + and − indicate presence and absence of hybridization signal in Southern blot experiments using the corresponding DNA probe. Symbols in parentheses indicate detection of the corresponding gene by PCR amplification and sequencing of the corresponding DNA fragment. PCR amplification were carried out using genomic DNA of the corresponding strains and primers hupSL1-hupSL2 and hypB1-hypB2 for *hupS* and *hypB*, respectively, as described in Materials and Methods.

identified in strains UPM860, 624, 466, and Z89 but not IM43B (Fig. 2C). In this filter, the *hupUV*-hybridizing bands had apparently the same size as those detected with the *hupS* probe (compare Fig. 2A and 2C), suggesting that *hupUV* and *hupS* might be adjacent genes in the genome of these strains, as is the case in the *B. japonicum hup* gene cluster (5). Finally, two *hoxA* gene probes constructed with primers PC1-PC2 and genomic DNA from either *R. leguminosarum* or *B. japonicum*

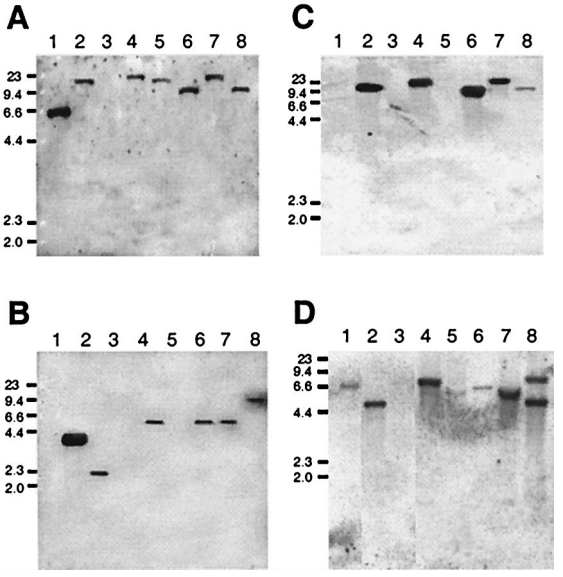


FIG. 2. Genomic DNA hybridizations of *Bradyrhizobium* sp. (*Lupinus*) strains with *hup*, *hyp*, and *hox* DNA probes. Panels A and B and panels C and D show Southern hybridizations using *hupS* and *hypB* probes from *R. leguminosarum* and *hupUV* and *hoxA* probes from *B. japonicum,* respectively. Genomic DNA was restricted with *Eco*RI enzyme. Strains: *R. leguminosarum* UPM791 (lane 1), *B. japonicum* 122DES (lane 2), *R. leguminosarum* PRE (lane 3), *Bradyrhizobium* sp. (*Lupinus*) 624 (lane 4), IM43B (lane 5), Z89 (lane 6), 466 (lane 7), and UPM860 (lane 8). Numbers on the left indicate molecular sizes of markers (in kilobases).

were used to identify this gene in *Bradyrhizobium* sp. (*Lupinus*) strains. The *R. leguminosarum hoxA* probe did not reveal any hybridization band (data not shown). In contrast, using the *B. japonicum* probe, signals corresponding to *hoxA* were detected in all strains (Fig. 2D).

The analysis of hybridizing bands obtained with the *hup*, *hyp*, and *hox* probes revealed four different profiles in *Bradyrhizobium* sp. (*Lupinus*) strains, one apparently shared by strains 624 and 466 and three additional profiles corresponding to strains Z89, UPM860, and IM43B. In addition, the presence of *hoxA* and *hupUV* genes suggests that *Bradyrhizobium* sp. (*Lupinus*) strains present a *hup* gene composition and regulation profile similar to that found in *B. japonicum*.

***Bradyrhizobium* sp. (*Vigna*).** DNA hybridization using the *R. leguminosarum hupS* probe showed different profiles of *hup*-specific bands among the *Bradyrhizobium* sp. (*Vigna*) strains (Fig. 3A). Analysis of the *hypB* gene in these strains was carried out with *R. leguminosarum* and *B. japonicum hypB* gene probes. Similar results were obtained using both *hypB* gene probes. Hybridizing bands were detected in all strains except 32H1 (Fig. 3B). Following an approach similar to that used with *Bradyrhizobium* sp. (*Lupinus*), we used degenerate primers to check for the presence of the *hypB* gene in this strain. PCR amplification and subsequent sequence analysis of the DNA fragment revealed a sequence 78% identical to that of the *B. japonicum hypB* gene, thus indicating the presence of *hypB* in this strain. No hybridization signals were obtained with the *R. leguminosarum hupE* gene probe (data not shown). In the search for the *hoxA* regulatory gene, we used two different

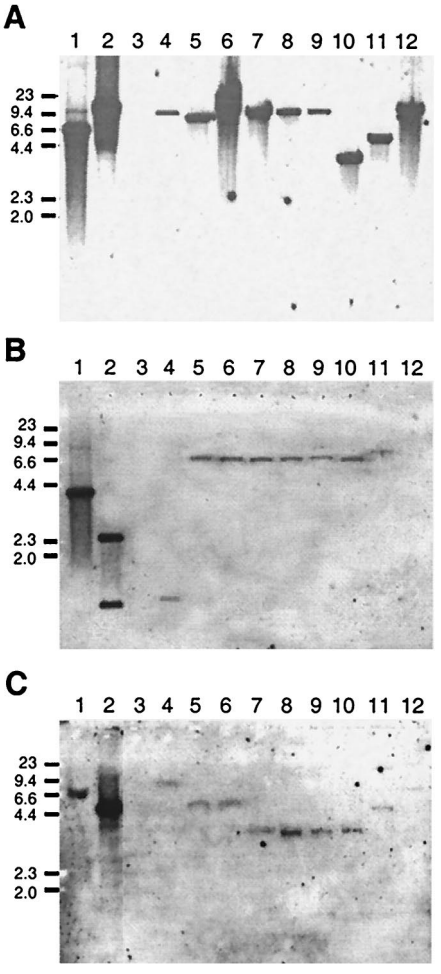


FIG. 3. Hybridization profiles of *hup* genes in *Bradyrhizobium* sp. (*Vigna*) strains. *Eco*RI-digested genomic DNAs were hybridized with *Rhizobium leguminosarum hupS* (A) and *hypB* (B) probes and with a *hoxA* probe from *B. japonicum* (C). Strains: *R. leguminosarum* UPM791 (lane 1), *B. japonicum* 122DES (lane 2), *R. leguminosarum* PRE (lane 3), *Bradyrhizobium* sp. (*Vigna*) M2 (lane 4), M5 (lane 5), M18 (lane 6), M21 (lane 7), M43 (lane 8), B78 (lane 9), B96 (lane 10), B97 (lane 11), and 32H1 (lane 12). Numbers on the left indicate molecular sizes of markers (in kilobases).

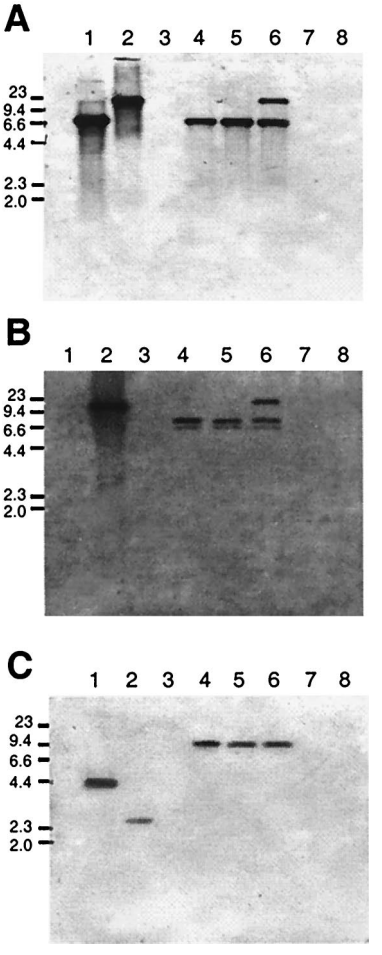


FIG. 4. Southern hybridizations of genomic DNA from *Azorhizobium* sp. and *A. caulinodans* strains with *hup* and *hyp* DNA probes. Panels A and C show Southern hybridizations using *R. leguminosarum hupS* and *hypB* probes, respectively. Panel B shows the hybridization signals obtained with a *hupUV* probe from *B. japonicum*. In all cases, genomic DNAs were restricted with *Eco*RI enzyme. Strains: *R. leguminosarum* UPM791 (lane 1), *B. japonicum* 122DES (lane 2), *R. leguminosarum* PRE (lane 3), *Azorhizobium caulinodans* ORS571 (lane 4), *Azorhizobium* sp. ORS552 (lane 5), *Azorhizobium caulinodans* ORS591 (lane 6), *Azorhizobium* sp. SD02 (lane 7), and *Azorhizobium* sp. SG05 (lane 8). Numbers on the left indicate molecular size of markers (in kilobases).

B. japonicum probes constructed with PC1-PC2 and AD1-AD2 primers. Both *hoxA* probes showed similar results, which were visualized as faint hybridization bands in all strains (Fig. 3C). In contrast, *hupUV* genes were not detected using the corresponding *B. japonicum* probe (data not shown). Overall, our results show at least seven different *Eco*RI restriction patterns of *hup* hybridizing bands in the *Bradyrhizobium* sp. (*Vigna*) strains tested and, more important, that these strains apparently differ from those of *B. japonicum* in the possession of *hup* regulatory genes.

***Azorhizobium* sp. and *Azorhizobium caulinodans*.** Hybridization assays with the *R. leguminosarum hupS* probe revealed DNA bands of similar sizes (ca. 7 kb) in *A. caulinodans* strains ORS571 and ORS591 (Fig. 4A). An additional, upper band was observed in ORS591. In *Azorhizobium* sp. strain ORS552, a ca. 7-kb band was also observed, whereas no hybridizing signals were detected in strains SD02 and SG05. Since *hupS* is essential for hydrogenase activity and no studies in this regard had been carried out in these two strains, we further analyzed the presence of the *hupS* gene in these two latter strains by PCR amplification using genomic DNA and the degenerate primers hupSL1-hupSL2, designed to amplify an internal DNA region of the *hupSL* genes. No DNA product of the expected size (ca 1.5 kb) was obtained under any PCR condition tested (data not shown), suggesting that these strains are indeed Hup$^-$. DNA bands hybridizing with the *hupUV* probe were also observed in strains ORS571, ORS552, and ORS591 (Fig. 4B). These bands had sizes similar to those hybridizing with the *hupS* gene probe, but an additional 6.6-kb band was also present that might have been due either to the presence of a second, less-conserved copy of the *hupUV* genes or of an *Eco*RI restriction site in the genomic DNA homologous to the

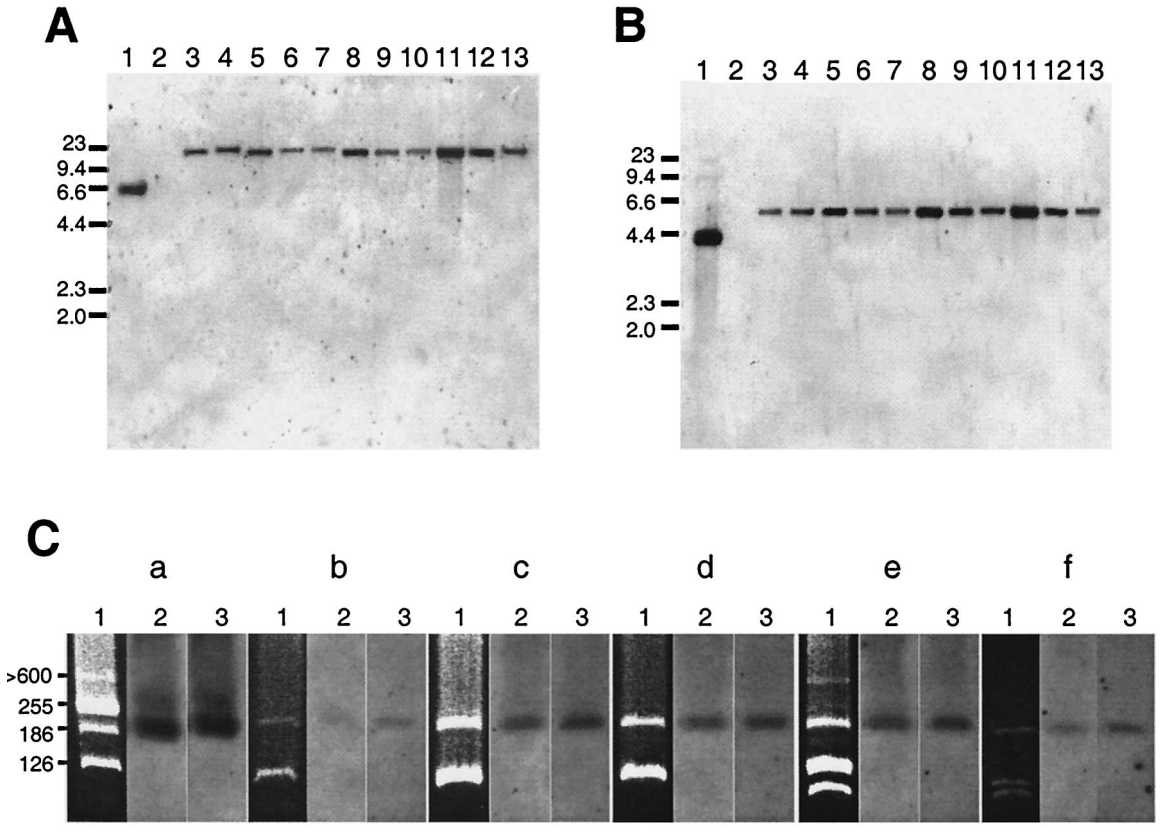


FIG. 5. DNA hybridization with *hup* and *hyp* probes and plasmid profiles of *R. tropici* Hup$^+$ strains. (A and B) *Eco*RI-digested genomic DNAs were hybridized with the *R. leguminosarum hupS* (A) and *hypB* (B) probes. Strains for panels A and B: *R. leguminosarum* UPM791 (lane 1) and PRE (lane 2), *Rhizobium tropici* USDA 2734 (lane 3), USDA 2786 (lane 4), USDA 2738 (lane 5), USDA 2787 (lane 6), USDA 2793 (lane 7), USDA 9030 (lane 8), USDA 2801 (lane 9), USDA 2840 (lane 10), USDA 2838 (lane 11), USDA 2813 (lane 12), and USDA 2822 (lane 13). Numbers on the right indicate molecular sizes, in kilobases. (C) Plasmids were resolved by the Eckhardt procedure (see Materials and Methods) (lanes 1), transferred to a membrane, and hybridized to *R. leguminosarum hupS* (lanes 2) or *nifH* (lanes 3) gene probes. Subpanels: (a) *R. leguminosarum* UPM791 (control strain); (b to f) *R. tropici* strains USDA 9030 (b), USDA 2840 (c), USDA 2838 (d), USDA 2813 (e), and USDA 2822 (f). Numbers on the left indicate molecular sizes (in megadaltons) of *R. leguminosarum* UPM791 plasmids.

probe. An extra upper band was again detected in ORS591, which may correspond to a second copy of the *hupS* and *hupUV* genes in the genome of this strain. Analysis with the *R. leguminosarum hypB* probe revealed a 9-kb band in strains ORS571, ORS552, and ORS591 (Fig. 4C). Finally, no hybridization signals were observed with either the *R. leguminosarum hupE* probe or the *R. leguminosarum* or *B. japonicum hoxA* gene probes for any strain tested (data not shown), suggesting that these genes are not present in *Azorhizobium* sp. and *A. caulinodans* Hup$^+$ strains.

***Rhizobium tropici*.** Previous studies on *R. tropici* using a *B. japonicum* structural gene probe showed a conserved *hup* hybridization pattern in all strains tested (26). To further investigate *hup* gene composition and variability within this species, we hybridized genomic DNA from those strains (USDA 2734, USDA 2786, USDA 2738, USDA 2793, USDA 9030, and USDA 2838), as well as that from strains USDA 2787, USDA 2801, USDA 2813, USDA 2822, and USDA 2840, with the hydrogen oxidation gene probes. All strains showed the same hybridization pattern with the *R. leguminosarum hupS* and *hypB* probes, containing 20- and 6-kb hybridizing bands, respectively (Fig. 5A and B), but no signal was detected with the *hupE*, *hupUV*, or *hoxA* gene probes. The conserved sizes of the *hup* hybridizing bands in all strains were further confirmed by analysis of genomic DNA digested with different restriction enzymes (*Hin*dIII, *Xho*I, *Pst*I, and *Sal*I), using a probe of the whole *hup* gene cluster of *R. leguminosarum*. This hybridization assay demonstrated that all *R. tropici* strains display the same *hup* hybridizing DNA bands, regardless of the enzyme used for DNA restriction (data not shown). The results described above show a remarkable conservation of the *hup* gene sequences in all *R. tropici* strains. Also, they reveal an apparent lack of homologues to the *B. japonicum* hydrogenase regulatory genes in this species.

In addition, we studied the putative plasmid localization of *hup* genes in *R. tropici* strains by running Eckhardt gels and subsequent hybridization with *R. leguminosarum hupS* and *nifH* probes (Fig. 5C). For strain USDA 9030, *hup* genes have been previously localized in the symbiotic plasmid (26). In our study, at least three different plasmid profiles were observed by the Eckhardt method: profile a (strains USDA 9030, USDA 2840, and USDA 2838), profile b (strain USDA 2813), and profile c (strain USDA 2822) (Fig. 5C, lanes 1). The resolved plasmid DNA was transferred to filters and hybridized with the

R. leguminosarum hup and *nifH* probes (Fig. 5C, lanes 2 and 3, respectively). In all strains, *hup* and *nif* hybridization signals were colocalized in the same plasmid, indicating that *R. tropici hup* genes are always located in the symbiotic plasmid.

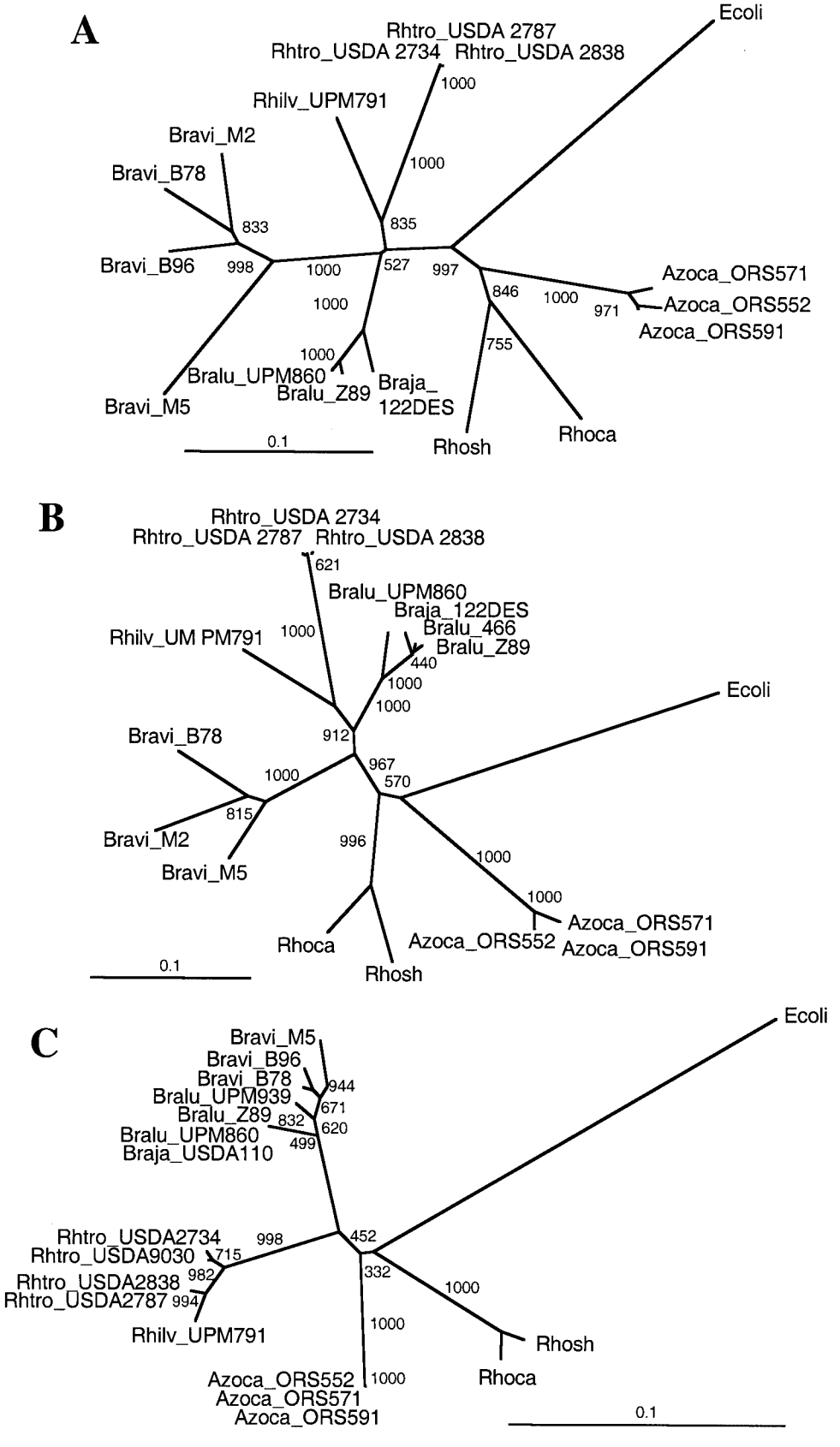
Phylogenetic analysis of rhizobial *hup* sequences. Partial *hupS* and *hupL* sequences were obtained from DNA fragments amplified from genomic DNA using the degenerate primer pair hupSL1-hupSL2. DNA sequences of 475 nt were obtained for *hupS*, covering 44% of the whole gene length. These DNA sequences encode 158 C-terminal amino acid residues of HupS, containing residues critical for hydrogenase activity (47). For *hupL*, DNA sequences were 453 nt long, spanning 25% of the *hupL* gene length. These sequences correspond to 151 N-terminal amino acid residues of HupL, including a conserved motif involved in metal center ligation (47). These *hupS* and *hupL* nucleotide sequences, along with corresponding data bank sequences from related α-proteobacteria (*Rhodobacter capsulatus* and *R. sphaeroides*) and from *Escherichia coli* hydrogenase 1, were optimally aligned, and phylogenetic trees were constructed by using the neighbor-joining method and the *E. coli* sequence as the outgroup (Fig. 6A and B). The *hupS*- and *hupL*-based trees were very similar, but a higher level of variability was observed for the *hupS* sequences. *Azorhizobium* sequences clustered together as a separate group, and they were closer to *Rhodobacter* than to rhizobial sequences. *Bradyrhizobium* sp. (*Lupinus*) sequences clustered together with *Bradyrhizobium japonicum*, and *R. tropici*, although showing some differences, appeared close to *R. leguminosarum. Bradyrhizobium* sp. (*Vigna*) sequences clustered together as a highly heterogeneous group, especially those for *hupS*, and were clearly separated from the other rhizobia. These results were surprising, since a priori it was expected that *Bradyrhizobium* and *Rhizobium* sequences would form respectively homogeneous groups.

Since the taxonomical characteristics of species within the genus *Bradyrhizobium* are not well defined (48, 49, 50), it was possible that the observed discrepancies were the result of differences in the genomic backgrounds of the analyzed strains. For that reason, we obtained and compared partial 16S rDNA sequences. A fragment corresponding to the region between positions 20 and 338 in the *E. coli* 16S rDNA was amplified by PCR from genomic DNA with the primer pair Y1-Y2 (51), cloned, and sequenced. The DNA sequences were aligned, and the most likely phylogenetic tree was derived as described above (Fig. 6C). The results obtained in the comparison of the 16S rDNA sequences were consistent with the taxonomic placement of the different strains. *Azorhizobium* strains clustered together as a separate group, equally distant from the rhizobial and the *Rhodobacter* strains. *Bradyrhizobium* and *Rhizobium* strains clustered into two well-differentiated groups. Each group was quite homogeneous, especially the *Bradyrhizobium* group, which showed branches shared by strains nodulating *Vigna* and *Lupinus*.

DISCUSSION

This work represents the first attempt to study the genetic composition and organization of *hup* gene clusters in a wide range of rhizobia. It was promoted by three independent observations: (i) the Hup trait is rare among rhizobia; (ii) when present and functional, *hup* genes can contribute to an increase in the energy efficiency of rhizobia-legume symbiosis by recycling the hydrogen evolved from the nitrogenase reaction; and (iii) comparison of the sequenced *hup* clusters from *R. leguminosarum* bv. viciae UPM791 and *B. japonicum* 122DES shows very high sequence and genetic organization conservation but also substantial differences in regulatory genes and in the presence or absence of specific ancillary genes. We reasoned that a comparative study of the different rhizobial *hup* systems might help clarify the evolution of such systems and also suggest reasons for the paucity of Hup^+ strains. Furthermore, efforts from our laboratory to engineer rhizobia for high symbiotic energy efficiency by incorporating the *R. leguminosarum hup* cluster (3) might benefit from a better understanding of existing rhizobial Hup systems, especially in view of factors limiting symbiotic hydrogenase activity (8, 9) and of the regulatory requirements for expression (11).

In this work, three types of gene probes were used: (i) *hupS* and *hypB*, genes necessary for hydrogenase activity (the *hupS* gene must be present by definition, whereas for *hypB*, there is room for variability, especially regarding the long histidine-rich tract at the N terminus [30]); (ii) *hupE*, a gene presently believed to be characteristic of *R. leguminosarum* alone; and (iii) regulatory genes *hoxA* and *hupUV*. Of the two model systems, *R. leguminosarum* UPM791 lacks *hupUV* and its *hoxA* is a pseudogene (11), and *B. japonicum* 122DES lacks *hupE*. Genes *hupS* and *hypB* were present in all Hup^+ strains, although in the case of some *Bradyrhizobium* strains, such as 32H1 and IM43B, evidence for the presence of a *hypB* gene could only be obtained by PCR amplification and DNA sequencing (Table 3). Sequence conservation of these *hypB* DNA fragments with the corresponding regions in *B. japonicum* ranged from 78 to 85% of identity. These values were significantly lower than the 94% of DNA sequence identity observed in strains that hybridized with the *B. japonicum* probe. These results emphasize the fact that bradyrhizobial strains very often exhibit high levels of heterogeneity at the nucleic acid level, even though they appear as closely related by most other taxonomic criteria (48, 49, 50), and question the reliability of negative results obtained in Southern blot hybridization experiments with *Bradyrhizobium* strains. Gene *hupE* could not be identified in any of the tested strains other than *R. leguminosarum*, not even in any of the eleven *R. tropici* Hup^+ strains (Table 3). This fact emphasizes the specificity of *hupE* for the *R. leguminosarum hup* cluster and the function encoded by this gene for hydrogenase activity in this species. In contrast, different situations were found in the search for regulatory genes *hoxA* and *hupUV* (Table 3). Both were present in *Bradyrhizobium* sp. (*Lupinus*) but absent in *R. tropici*. In *Azorhizobium,* the Hup^+ strains showed the *hupUV* genes, but not *hoxA*, whereas the opposite situation was found for *Bradyrhizobium* sp. (*Vigna*): *hoxA* could be identified in all strains but not *hupUV*. Since the *hoxA* and *hupUV* genes are involved in the same regulatory pathway, their presence may indicate a mechanism of *hup* gene activation like that of *B. japonicum*, whereas in their absence one might speculate that a mode of regulation exists that is similar to that of *R. leguminosarum*. For the intermediate situations, several circumstances must be considered. We have already discussed the reliability of negative results in the hybridization assays. On the other hand, faint *hoxA* hybridizing bands might also corre-


A
Ecoli
Rhtro_USDA 2787
Rhtro_USDA 2734 Rhtro_USDA 2838
1000
Rhilv_UPM791
Bravi_M2
1000
Bravi_B78
833
835
Bravi_B96
998
1000
527
997
Azoca_ORS571
846
1000
Azoca_ORS552
971
Azoca_ORS591
1000
755
1000
Bralu_UPM860
Bralu_Z89
Braja_
122DES
Bravi_M5
0.1
Rhosh
Rhoca
B
Rhtro_USDA 2734
Rhtro_USDA 2787 Rhtro_USDA 2838
621
Bralu_UPM860
Braja_122DES
Bralu_466
Rhilv_UM PM791
1000
Bralu_Z89
440
1000
1000
Ecoli
Bravi_B78
912
967
1000
570
815
Bravi_M2
996
1000
Bravi_M5
1000
Azoca_ORS571
Rhoca
Azoca_ORS552
Azoca_ORS591
0.1
Rhosh
C
Ecoli
Bravi_M5
Bravi_B96
Bravi_B78
944
Bralu_UPM939
671
Bralu_Z89
832
620
Bralu_UPM860
499
Braja_USDA110
Rhtro_USDA2734
998
452
Rhtro_USDA9030
715
332
Rhtro_USDA2838
982
1000
Rhtro_USDA2787
994
1000
Rhosh
Rhilv_UPM791
Azoca_ORS552
1000
Rhoca
Azoca_ORS571
Azoca_ORS591
0.1

spond to cross-hybridization with regulatory genes of the NtrC family to which the *hoxA* gene belongs (45). In addition, detection of *hoxA* and *hupUV* gene sequences does not mean that they are functional; they might correspond to nonfunctional genes, as it is the case for the *R. leguminosarum hoxA* pseudogene (11). At this point of the investigation, it is difficult to determine the actual explanation of these results and their biological significance. However, the different gene compositions might indicate the presence of *hup* regulatory pathways alternative to those described for *B. japonicum* and *R. leguminosarum*, which would imply a wide range of variation within Hup^+ rhizobia with regard to the mechanism of *hup* gene regulation. The study of these different regulatory adaptations is presently under way in our laboratory and might represent a contribution to efforts aimed at spreading the Hup trait among rhizobial strains of agricultural significance.

It is interesting that the *hup* sequence divergence within the *R. tropici* strains was minimal and much lower than that of their 16S rDNAs, despite the fact that the *hup* genes are encoded in the symbiotic plasmid. This situation is very similar to that observed within *R. leguminosarum* bv. viciae Hup^+ strains, where the *hup* genes are always present in the symbiotic plasmid (22) and where an extremely high conservation of *hup* cluster sequences has been documented (D. Fernández, A. Toffanin, J. M. Palacios, T. Ruiz-Argüeso, and J. Imperial, submitted for publication). This contrasts sharply with the variability found for *Bradyrhizobium* sp. (*Lupinus*) and *Bradyrhizobium* sp. (*Vigna*), where *hup* sequences are probably encoded in the chromosome, since no plasmids could be detected in these strains (31). We know very little regarding the mechanisms for gene evolution in rhizobia, but these results suggest that *hup* genes evolved differently in *Rhizobium* and *Bradyrhizobium* strains. In addition, *Bradyrhizobium* sp. (*Vigna*) *hup* sequences clustered apart from those of *Bradyrhizobium japonicum* and *Bradyrhizobium* sp. (*Lupinus*) strains in the phylogenetic studies. This anomalous high divergence shown by *Bradyrhizobium* sp. (*Vigna*) *hup* sequences might reflect the occurrence of independent events of gene acquisition from other soil bacteria.

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FIG. 6. Phylogenetic trees derived from *hup* and 16S rDNA sequences of rhizobia. Partial *hupS* and *hupL* sequences from *Bradyrhizobium* sp. (*Lupinus*), *Bradyrhizobium* sp. (*Vigna*), *Rhizobium tropici*, *Azorhizobium* sp., and *Azorhizobium caulinodans* strains were obtained and aligned with the corresponding sequences from *Rhizobium leguminosarum* bv. viciae UPM791, *Bradyrhizobium japonicum* 122DES, two other α-proteobacteria (*Rhodobacter capsulatus* and *Rhodobacter sphaeroides*), and *hyaA* and *hyaB* (hydrogenase 1 structural genes) from *E. coli* (used as the outgroup). Minimum-distance trees were generated for *hupS* (A) and *hupL* (B) by using CLUSTALX and TREEVIEW software. A similar tree was constructed from 16S rDNA sequences of the rhizobial strains mentioned above or database 16S rDNA sequences from strains belonging to the same taxa (C). Tree scales are indicated as per site substitutions. Figures at nodes indicate bootstrap values (per 1,000). The accession numbers of the sequences obtained from databases are as follows: *R. leguminosarum* bv. viciae UPM791 (*hupS* and *hupL*, gi:1167855; 16S rDNA, AY072787), *B. japonicum* USDA110 (16S rDNA, gi:534881), 122DES (*hupS* and *hupL*, gi:152100), *E. coli* (*hyaA* and *hyaB*, gi:146419; 16S rDNA, gi:174375), *R. capsulatus* (*hupS* and *hupL*, gi:46032; 16S rDNA, gi:1944502), *R. sphaeroides* (*hupS* and *hupL*, gi:4539150; 16S rDNA, gi:303817), *R. tropici* USDA9030 (16S rDNA, gi:1895079), *A. caulinodans* ORS571 (16S rDNA, gi:870816). Abbreviations: Azoca, *Azorhizobium caulinodans*; Braja, *Bradyrhizobium japonicum*; Bralu, *Bradyrhizobium* sp. (*Lupinus*); Bravi, *Bradyrhizobium* sp. (*Vigna*); Ecoli, *Escherichia coli*; Rhilv, *Rhizobium leguminosarum* bv. viciae; Rhtro, *Rhizobium tropici*; Rhoca, *Rhodobacter capsulatus*; Rhosh, *Rhodobacter sphaeroides*.

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