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Glutamine Synthetase II Constitutes a Novel Taxonomic Marker in *Rhizobium etli* and Other *Rhizobium* Species

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Different *Rhizobium* species may be identified by using polymorphisms in their glutamine synthetases (GSII) but not by their GSI profiles. We analyzed the GSs of various *Rhizobium tropici* and *Rhizobium etli* strains (which are capable of nodulating and fixing nitrogen in *Phaseolus vulgaris* beans), as well as strains of other species included for comparison. The GS polymorphisms were determined by identifying variations in native enzyme mobility (revealed by GS activity staining) and in the isoelectric points of the monomers (revealed by immunodetection with antibodies against the GS proteins) by using gel electrophoresis. Restriction fragment length polymorphism patterns obtained by hybridizing an internal fragment of the GSII gene obtained from *R. etli* with total fragmented DNAs from different strains clearly distinguished the different groups. GSII is a novel and useful marker for *Rhizobium* groups and species, and GSII data support *R. tropici* and *R. etli* as bona fide species.

Glutamine synthetases (GSs), which are key enzymes in nitrogen metabolism, are ubiquitous and well-conserved enzymes. Different GSs (e.g., GSI and GSII) which differ in their primary and tertiary structures have been described. GSI and GSII seem to have resulted from a duplication event early in evolution (56) that preceded the split of the *Archaeobacteria* and *Eubacteria* (46) or the split of the prokaryotes and eukaryotes (30). Very few prokaryotes have two GS isoenzymes; two prokaryote genera whose members have two GS isoenzymes are the genera *Rhizobium* (12, 14, 52, 56) and *Agrobacterium* (22).

Rhizobium species have attracted a great deal of research interest because of their capacity to fix nitrogen in legume root nodules. In *Rhizobium* strains, GSs are central enzymes in nitrogen metabolism and, together with glutamate synthase, are responsible for ammonium assimilation (10). GSI and GSII have different roles in symbiotic and free-living bacteria (reviewed in reference 21). Only GSI activity has been detected in symbiotic bacteria (42, 57). GSI is present in bacteria growing in rich medium, and GSII is present in minimal medium cultures. We have also found that GSI activity is related to a pseudofermentative metabolism and that GSII activity is found mainly during aerobic metabolism in *Rhizobium* strains (19, 20). The individual contributions of GSI and GSII to glutamine synthesis depend on fine regulatory mechanisms that affect the expression of the respective genes (1, 37, 44) or the enzymatic activities (6, 26, 34, 35, 49, 57).

The genus *Rhizobium* is a heterogeneous group of bacteria, and recently our knowledge of the scope of the diversity of *Rhizobium* species has increased because many strains isolated from different hosts have been analyzed (33). At the present time the most reliable method for characterizing and identifying strains is analysis of ribosomal gene sequences. These sequences have become the universal reference criteria for defining bacterial phylogenies (61), and data obtained from partial or complete 16S rRNA sequences have been essential in proposing new *Rhizobium* species, such as *Rhizobium etli* (55) and *Rhizobium tropici* (40). *R. etli* has been isolated from *Phaseolus vulgaris* bean nodules in Mesoamerica, and *R. tropici* has been isolated from bean and *Leucaena* nodules obtained from South American acid soils. Both of these species are broad-host-range rhizobia that nodulate many hosts in addition to bean and *Leucaena* species (25). Originally, *R. etli* was classified as *Rhizobium leguminosarum* bv. phaseoli (type I), but on the basis of the sequence of fragments of ribosomal genes, *R. etli* was recognized as a chromosome lineage that was distinct from *R. leguminosarum*.

Recent results challenge the validity of single-gene-based phylogenies in the genus *Rhizobium* (17) and in other bacteria (8) and point out the need to consider additional markers. It has been recognized that polyphasic characterization would contribute to validation of the present *Rhizobium* taxonomy (24, 33, 39). The results of polyacrylamide gel electrophoresis of total proteins (15) and repetitive extragenic PCR characterization (13) allow workers to group *Rhizobium* strains. The identification of additional useful markers would be valuable in *Rhizobium* systematics. GSs are good candidates for this since they behave as ideal molecular clocks (45) to trace evolution, and very reliable genetic distances among organisms have been estimated with the data obtained (30, 46).

The aim of this study was to characterize GSI and GSII in different *Rhizobium* species, with special emphasis on *R. etli* and *R. tropici* strains, to further validate the taxonomic position of these organisms. We found that GSII provides a novel marker for groups and species belonging to the genus *Rhizobium*, while GSI is conserved in all of the species tested.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains which we used are listed in Table 1. *Rhizobium* strains were grown in PY medium or minimal medium (7). Minimal medium contained 10 mM succinate and 10 mM potassium nitrate supplemented as described previously (7). Extracts of the different *Rhizobium* species were obtained from 150 ml of PY medium or minimal medium batch cultures grown in 250-ml Erlenmeyer flasks. *Escherichia coli* was grown in Luria broth.

GS purification. GSI and GSII were purified from cultures of *R. etli* CFN42^T

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TABLE 1. Bacterial strains

Strain	Original host plant and/or relevant characteristics	Source or reference[a]
R. etli strains		
CFN42^T	*P. vulgaris* L. from Guanajuato, Mexico, wild-type reference strain	55
CFN277	*P. vulgaris* L. from Jalisco, Mexico	38
CFN279	*P. vulgaris* L. from Hidalgo, Mexico	38
CFN403	*P. vulgaris* L. from Tepoztlán, Morelos, Mexico	Martínez
Nitragin 8251	*P. vulgaris* L.	47
Viking 1	*P. vulgaris* L. from Belize	47
Bra-5	*P. vulgaris* L. from Brazil	47
F-17	*P. vulgaris* L. from Tepoztlán, Morelos, Mexico	47
CFN3	*P. vulgaris* L. from Guanajuato, Mexico	47
R. leguminosarum bv. viciae VF39	*Vicia faba* from Bielefeld, Germany	48
R. leguminosarum bv. trifolii strains		
TA1	*Trifolium repens* from Australia	53
ANU843	Wild type, Nod$^+$ Fix$^+$ on white and subterranean clovers	51
R. leguminosarum bv. phaseoli strains		
Sp18	*P. vulgaris* L. from England	Young
M1928	*P. vulgaris* L. from England	Young
248	*P. vulgaris* L. from England	Brewin
R. fredii USDA 193	*Glycine max*	USDA
R. meliloti strains		
2011	Alfalfa, wild type	INRA
1021	Streptomycin resistant, from 2011	41
104A14	Alfalfa, wild type	57
R.me2	Alfalfa from Guanajuato, Mexico	Martínez
R.me8	Alfalfa from Guanajuato, Mexico	Martínez
R. tropici type A strains		
CFN299	*P. vulgaris* L., wild-type reference strain	40
BR845	*Leucaena leucocephala* from Brazil	40
BR846	*L. leucocephala* from Brazil	40
BR10042	*P. vulgaris* L. from Brazil	40
BR10043	*P. vulgaris* L. from Brazil	40
C-05-1	*P. vulgaris* L. from Brazil	40
R. tropici type B strains		
CIAT899^T	*P. vulgaris* L., wild-type reference strain	40
BR852	*L. leucocephala* from Brazil	40
BR853	*L. leucocephala* from Brazil	40
BR857	*L. leucocephala* from Brazil	40
BR863	*L. leucocephala* from Brazil	40
Rhizobium sp. strain OR191	Alfalfa, ineffective nodules from Oregon	18
B. japonicum strains		
CJ1	Soybean	36
USDA110	Soybean	USDA
E. coli MX727		4

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(T = type strain). To isolate GSII, bacteria were grown for 10 h in shaken minimal medium cultures at 30°C. Pellets were obtained from 5 liters of culture by centrifugation, washed, and suspended in 400 ml of 10 mM imidazole–1 mM manganese chloride buffer (pH 7) (buffer A). Extracts were obtained by sonicating preparations with a Soniprep 150 apparatus (MSE) in an ice water bath by using 10 1-min pulses interrupted by 3-min cooling periods, using a macrotip. The extracts were centrifuged at 12,000 × *g* for 15 min at 5°C. The clear supernatants obtained were precipitated by adding solid ammonium sulfate to a concentration that was 35% of the saturation concentration. After centrifugation, the supernatant was recovered, and ammonium sulfate was added to a concentration that was 55% of the saturation concentration. The pellet recovered after centrifugation was dissolved in 10 mM imidazole–5 mM manganese chloride buffer (pH 6.8) (buffer B), dialyzed against the same buffer, and chromatographed on a Reactive Blue 2–Sepharose CL-6B (Sigma) column. The column was eluted with 5.3 mM ADP in buffer B, and fractions were assayed for GS transferase activity (5). The fractions with the highest activities were pooled, loaded onto a QMA/ACCEL ion-exchange high-performance liquid chromatography (HPLC) column, and eluted with a linear 0 to 1 M KCl gradient for 30 min. The fractions with the highest activity were loaded onto a preparative Laemmli protein gel (31). A prestained molecular weight standard mixture (molecular weight range, 34,000 to 191,000) obtained from Sigma was used as a reference. A 40-kDa band corresponding to GSII was recovered from the gel.

GSI was purified from 5 liters of a CFN42^T culture growing in PY medium. Clear extracts were obtained by the procedure described above. Before the ammonium sulfate precipitation step, the supernatants were warmed for 1 h at 50°C followed by centrifugation. Protein was precipitated with ammonium sulfate to a final concentration that was 70% of the saturation concentration, and the pellet was dialyzed against buffer A and chromatographed with a Reactive Blue 2–Sepharose CL-6B column as described above, except that washing and elution were with buffer A instead of buffer B. HPLC purification and Laemmli polyacrylamide electrophoresis were performed as described above for GSII purification, but a 50-kDa band corresponding to GSI was isolated.

Anti-GS antibodies. GSII and GSI were recovered from Laemmli gels in a sterile saline (0.85% NaCl) solution, mixed with Freund adjuvant, and used for subcutaneous rabbit immunization every week for 6 weeks. The antibody titer was determined by serially diluting the serum. The optimal dilutions were 1:8,000 with anti-GSI serum and 1:6,000 with anti-GSII serum when we used 10 μg of total protein extract in Western blots (immunoblots) (see below). No cross-reactivity was observed with anti-GSII serum and purified GSI. Anti-GSI serum did not react with purified GSII. Only specific reactions in which single spots

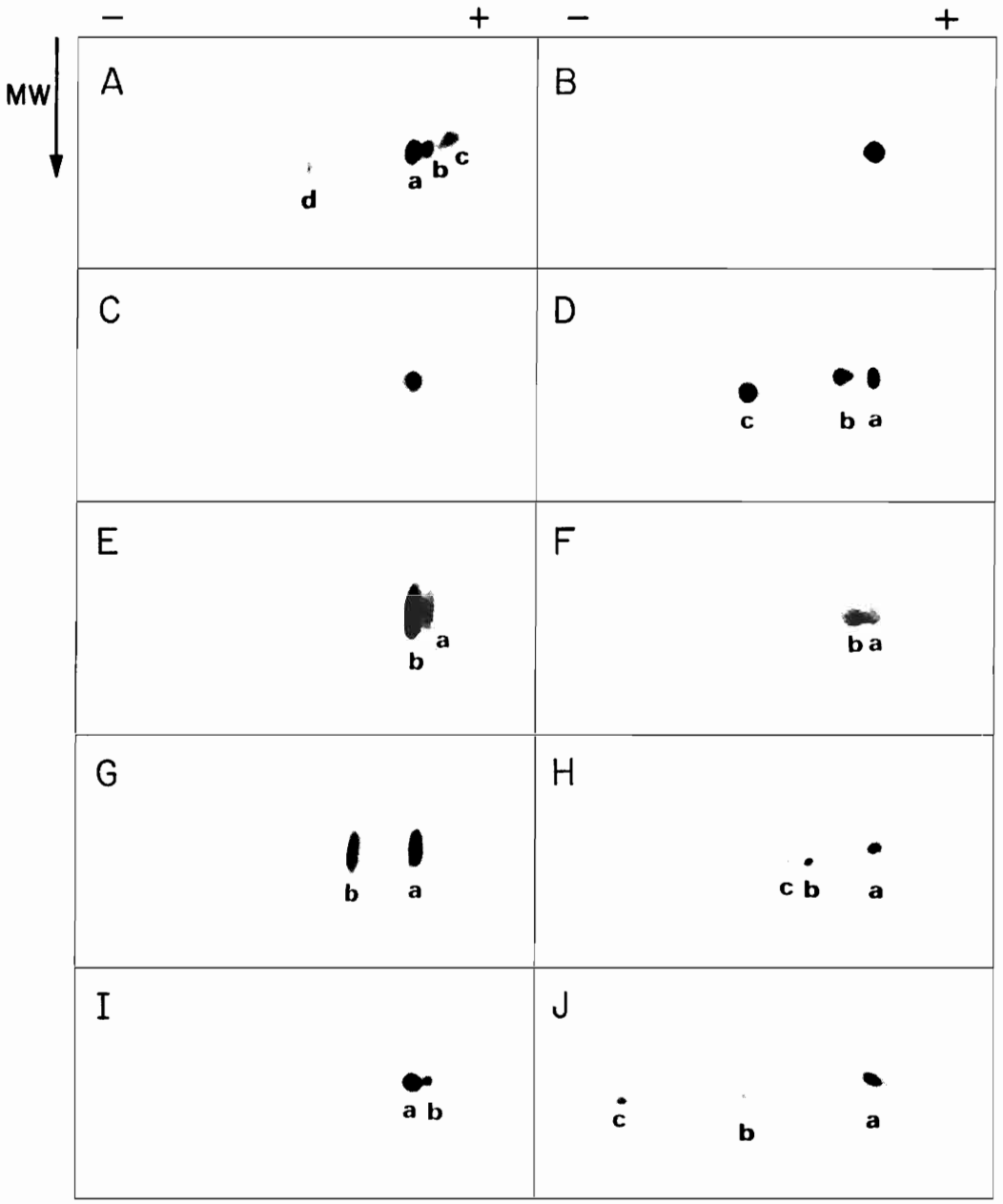

FIG. 1. Immunodetection of GSII in blots of two-dimensional protein gels prepared with different *Rhizobium* strains. (A) *R. etli* CFN42^T (spot a), *R. tropici* BR846 (spot b), *R. tropici* BR852 (spot c), and *R. meliloti* R.me8 (spot d). (B) *R. etli* CFN42^T, Nitragin 8251, and CFN403. (C) *R. etli* CFN42^T and *Rhizobium* sp. strain OR191. (D) *R. etli* Bra5 (spot a), *R. leguminosarum* bv. trifolii TA1 and ANU 843 (spot b), and *R. meliloti* R.me8 (spot c). (E) *R. etli* CFN42^T (spot a) and *R. leguminosarum* bv. phaseoli Sp18 (spot b). (F) *R. etli* CFN42^T (spot a) and *R. leguminosarum* bv. phaseoli M1928 (spot b). (G) *R. etli* CFN42^T (spot a) and *R. leguminosarum* bv. phaseoli 248 (spot b). (H) *R. etli* CFN42^T (spot a), *R. meliloti* 104A14 (spot b), and *R. meliloti* 1021 (spot c). (I) *R. meliloti* 1021 (spot a) and *R. fredii* USDA193 (spot b). (J) *R. etli* Bra5 (spot a), *R. meliloti* R.me2 (spot b), and *B. japonicum* CJ1 (spot c). MW, molecular weight.

were observed were obtained. The negative control used consisted of serum obtained before the immunization procedure was performed.

GS electrophoresis. GS activity was detected by the transferase assay (5) in nondenaturating polyacrylamide (4 to 8%) continuous gels. To determine the isoelectric points, bacterial extracts were electrophoresed in two-dimensional gels as described by O'Farrell (43). Isoelectric focusing was performed in the first dimension with 2% (vol/vol) ampholines (Pharmacia LKB); the pH ranges used were pH 3.5 to 10 and pH 5 to 7 (in cylindrical acrylamide gels). Isoelectric focusing markers (pH range, 3.6 to 9.3) were obtained from Sigma. Proteins were separated in the second dimension mainly by their molecular weights.

GS immunodetection. O'Farrell protein gels were blotted onto Schleicher & Schuell nitrocellulose membranes (59), and the membranes were processed as described by Walker and Gaastra (60). Enzymatic detection of membrane-bound antigens was performed by using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma). Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolylphosphate were used as the substrates.

DNA-DNA hybridization. pCV002 is pBR322 (9) containing a 7-kb cloned fragment from *R. etli* CFN42^T isolated from a library of *Sau*3A fragments obtained by partial digestion. pCV002 was selected from a pool of clones that hybridized to a 2.1-kb insertion obtained from pBJ196A, which contains GSII gene from *Bradyrhizobium japonicum* (11). pCV002 was identified by its ability to complement *E. coli* GS mutant MX727 (*glnA21*::Tn*5* [4]) for growth in minimal medium because the GS enzymatic activity is restored. A 0.5-kb *Sal*I-*Bgl*III fragment (in the GSII gene) was identified from pCV002 by hybridization with a 30-bp oligonucleotide (5′-CCGACCTCCGGTTATCCGGCGCCGCAGGGC-3′) on the basis of the *B. japonicum* GSII gene sequence, labelled with [γ-^{32}P]ATP as described previously (3). The 0.5-kb fragment was purified, labelled with [^{32}P]dCTP by nick translation (50), and used as a probe. Total DNAs

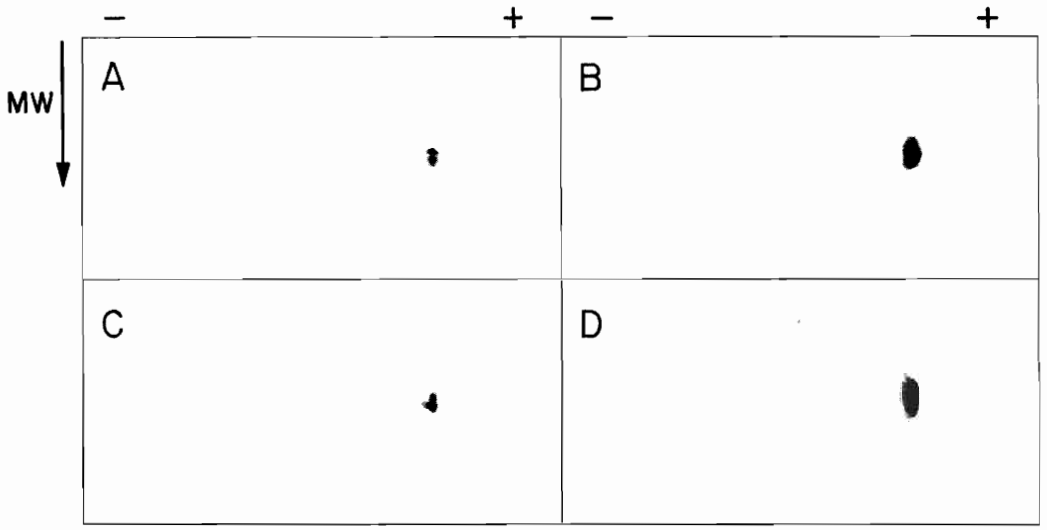

FIG. 2. Immunodetection of GSI as described in the legend to Fig. 1. (A) *R. etli* CFN42^T and *R. tropici* C-05-1 and BR852. (B) *R. etli* CFN42^T and *R. meliloti* 1021. (C) *R. etli* CFN42^T and *R. leguminosarum* bv. trifolii ANU843. (D) *R. etli* CFN42^T and *B. japonicum* USDA110. MW, molecular weight.

from different *Rhizobium* strains were purified as described previously (3). *Sal*I or *Xho*I digests were electrophoresed in agarose gels, and blot transfers were hybridized under high-stringency conditions to the 0.5-kb fragment obtained from pCV002, which was prepared by the alkaline lysis method (3). Hybridization signals were detected by autoradiography.

RESULTS

GS enzyme electrophoresis. As a first approach to characterizing *Rhizobium* GSII, the mobility variants of the native enzymes obtained from several *R. etli*, *R. tropici*, and *R. meliloti* strains were identified by the GS transferase assay on one-dimensional gels. A single band was produced by all of the *R. etli* strains tested (CFN42^T, CFN3, F-17, Viking 1, Nitragin 8251), another band with a different mobility was produced by all of the *R. tropici* strains analyzed (CIAT899^T, CFN299, C-05-I, C-05-II), and a third mobility variant was produced by the *Rhizobium meliloti* strains (2011, R.me2, R.me8). Therefore, GSII was monomorphic in each of the species tested, and these species could be distinguished by the mobilities of their native GSII enzymes. These first results obtained with only three species were considered the basis for a more complete analysis of GSs in which we used the molecular weights and isoelectric points of the GSs of a larger number of *R. etli* and *R. tropici* strains and strains of other *Rhizobium* species.

GSII isoelectric points determined by Western blotting. GSs are oligomeric enzymes that consist of identical monomers or subunits. A very fine method for exploring enzyme polymorphism is determining the isoelectric points of the monomers, and this was accomplished by performing an immunodetection analysis of GSII in two-dimensional O'Farrell gels (43). To compare the GSs of different strains, we coelectrophoresed extracts of the strains, and the results of some of our experiments are shown in Fig. 1. The results obtained with *R. tropici*, *R. etli*, and *R. meliloti* GSII isoforms are shown in Fig. 1A. Each species has a distinct monomer. Even though the GSII mobility variants of all *R. tropici* strains were identical on one-dimensional native gels, the GSII isoenzymes of *R. tropici* type A strains CFN299, BR845, BR846, and BR10043 were different from the GSII isoenzymes of *R. tropici* type B strains CIAT899^T, BR852, BR857, and BR863 on the two-dimensional gels. The *R. etli* strains tested (CFN42^T, Viking 1, CFN279, Nitragin 8251, Bra5, and CFN403) all had identical isoforms (the results obtained with some of these strains are shown in Fig. 1B), which were also identical to the GSII isoenzyme of *Rhizobium* sp. strain OR191 (Fig. 1C).

Our results showed that *R. etli* has a different GSII isoform than *R. leguminosarum* bv. phaseoli SP18 (Fig. 1E), M1928 (Fig. 1F), and 248 (Fig. 1G), *R. leguminosarium* bv. trifolii ANU843 and TA1 (Fig. 1D), and *R. leguminosarum* bv. viciae VF39 (data not shown).

Two different types of *R. meliloti* have been identified (16). When the *glnII* (GSII gene) hybridization patterns obtained for some *R. meliloti* strains were analyzed, *R. meliloti* 104A14 was the only one of five *R. meliloti* strains that had a distinctive profile (57). We also found that *R. meliloti* 104A14 had a different GSII monomer than *R. meliloti* 1021 (Fig. 1H). The GSIIs of *R. meliloti* 1021, 2011, R.me8, and R.me2 had identical isoelectric points (data not shown). *Rhizobium fredii* USDA193 had a GSII isoform that was similar but not identical to the GSII isoforms of the *R. meliloti* strains (Fig. 1I).

The most different GSII monomer was the GSII monomer of *Bradyrhizobium japonicum* CJ1, and this finding is consistent with the separation of the genera *Rhizobium* and *Bradyrhizobium* (reviewed in reference 39).

GSI isoelectric point. In contrast to the results obtained with GSII, GSI was conserved in all of the *Rhizobium* species tested, and these organisms could not be distinguished by their GSI isoenzymes. *R. etli*, *R. tropici*, *R. meliloti*, and *B. japonicum* strains had GSIs with identical or very similar isoelectric points (Fig. 2).

Restriction fragment length polymorphism of GSII genes. To complement our GS characterization of different *Rhizobium* strains, we analyzed GSII gene restriction fragment length polymorphism patterns. Each of the *Rhizobium* species tested produced a characteristic pattern (Fig. 3), and the patterns obtained from *R. tropici* type A strains were different from the patterns obtained from *R. tropici* type B strains.

DISCUSSION

We characterized the GSIs and GSIIs of a number of *Rhizobium* strains, and we found that GSII is a novel marker that can be used for identification of *Rhizobium* species. We gave special emphasis to the species that nodulate beans, *R. etli* (55)

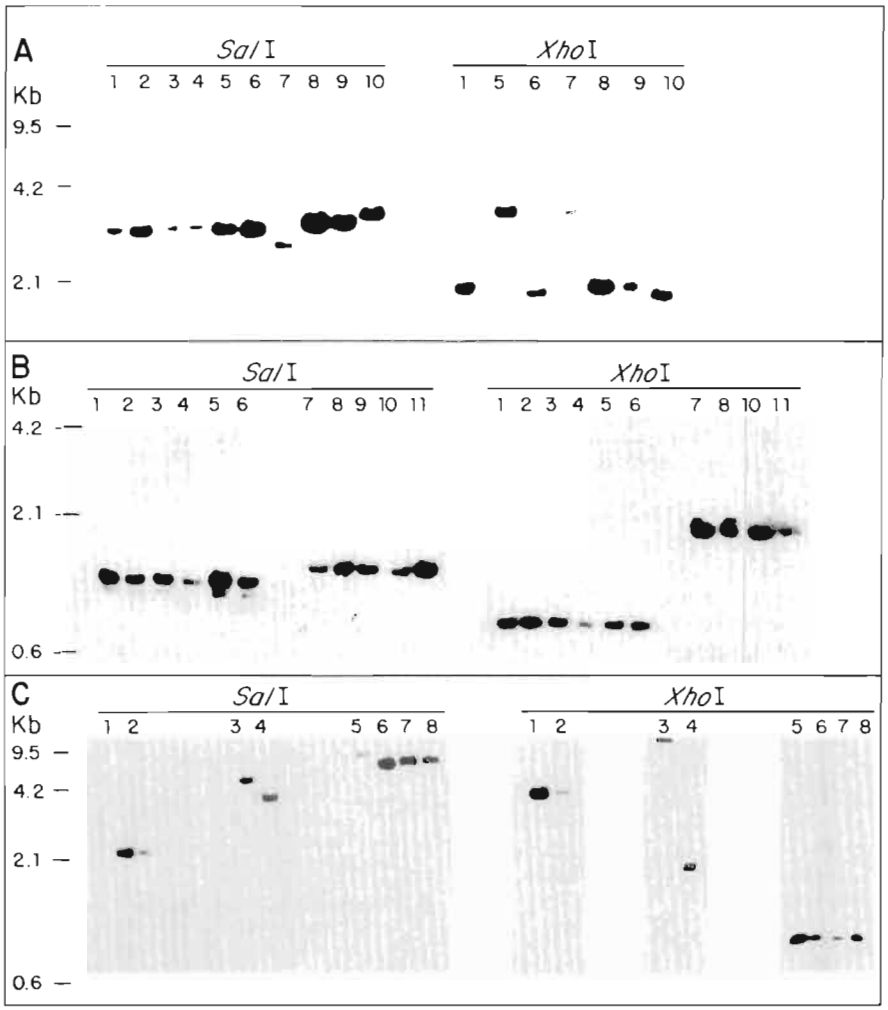

FIG. 3. Southern blots of total genomic DNAs of several *Rhizobium* species hybridized with a 0.5-kb *glnII* (GSII gene) internal fragment. The endonucleases used in each experiment are indicated on the figure. (A) *R. etli* strains. Lanes 1 and 2, CFN42^T; lane 3, CFN279; lane 4, F-17; lanes 5, Bra5; lanes 6, Viking 1; lanes 7, CFN3; lanes 8, CFN277; lanes 9, CFN403; lanes 10, Nitragin 8251. (B) Lanes 1 through 6, *R. tropici* type A strains BR845, CFN299, BR846, BR10043, BR10042, and C-05-1, respectively; lanes 7 through 11, *R. tropici* type B strains BR852, BR857, BR863, BR853, and CIAT899^T, respectively. (C) Lanes 1 and 2, *B. japonicum* USDA 110 and CJ1, respectively; lanes 3 and 4, *R. leguminosarum* bv. trifolii ANU843 and TA1, respectively; lanes 5 through 8, *R. meliloti* R.me2, R.me8, 2011, and 1021, respectively.

and *R. tropici* (40), and our results support the recognition of these taxa as bona fide *Rhizobium* species.

Each of the *Rhizobium* species which we analyzed could be clearly distinguished by its GSII isoforms. One interpretation of our results is that each species is monophyletic, meaning that the strains of each *Rhizobium* species have a common ancestor. It has been proposed that differences in isoelectric points may make one isoenzyme better suited to a particular substrate or intracellular environment (27). Since different GSII isoenzymes are encountered in the distinct *Rhizobium* groups, we believe that GSIIs may reflect a functional and metabolic compromise that tightly links them to a whole set of other genes ("core *Rhizobium* genetic information") that determine the overall metabolic features of the divergent bacterial lineages.

The isoelectric points that we observed under our experimental conditions (data not shown) correspond to the following theoretical isoelectric points that we estimated from the previously reported GSII gene sequences (11, 56): 5.19 for *R. meliloti* GSII enzymes and 6.51 for *B. japonicum* GSII enzymes. The amino acid compositions of the GSIIs of *R. meliloti* and *B. japonicum* are 91% identical, and only one-third of the different amino acids were responsible for the isoelectric points.

Our results are consistent with previously reported results. For example, *Rhizobium* sp. strain OR191 was isolated from ineffective alfalfa nodules in the United States and is capable of nodulating *P. vulgaris*, which is the normal symbiont of *R. etli*. On the basis of a partial sequence of its ribosomal genes, strain OR191 is closely related to *R. etli* (level of homology, 99%) (18). The GSIIs of *Rhizobium* sp. strain OR191 and *R. etli* could not be distinguished by the results of isoelectric focusing (Fig. 1C). It has been observed previously that the differences between the two *R. tropici* types could be enough to reclassify them as different subspecies or even as different species (23). Our results, which show that the type A strain GSII is different from the GSII found in type B strains, support the subdivision of *R. tropici*. Although partial sequences of the 16S rRNA genes of *R. fredii* and *R. meliloti* are identical (28), these organisms can be distinguished both by the complete sequences of their 16S rRNA genes and by their GSII isoforms.

Several workers have described *R. etli* strains as very diverse (17, 47, 54). *R. etli* and *R. tropici* have been clearly separated by multilocus enzyme electrophoresis data (40, 55), by their fatty acid compositions (29), and on the basis of restriction digests of PCR-synthesized rRNA genes (2, 32). Recently, some *R. etli* strains have been found to have an allele of the 16S rRNA gene corresponding to the allele found in *R. leguminosarum* (17). This finding could be explained by a lateral transfer of rRNA genes from *R. leguminosarum* to *R. etli* (17). We analyzed one of the anomalous *R. etli* strains, Nitragin 8251, and found that it produces a GSII pattern corresponding to the *R. etli* pattern. Recent results also suggest that there may be recombination of chromosomal markers in *R. etli* strains in local populations (58), which probably indicates that *Rhizobium* strains are not strictly clonal, as has been suggested previously (47). Thus, it is necessary to sample a broader portion of the genome to get a good image of it, and identification of additional markers linked to core genetic information in the bacteria is essential.

Although the procedure for determining isoelectric points is very laborious and not easily adapted for everyday laboratory screening of bacteria, it provides a way to pursue analyses of GSII genes in different *Rhizobium* species with the goal of developing gene-specific probes which would allow rapid screening of strains for taxonomic purposes.

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