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Rhizobium huautlense sp. nov., a symbiont of *Sesbania herbacea* that has a close phylogenetic relationship with *Rhizobium galegae*

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The nitrogen-fixing rhizobial symbionts of *Sesbania herbacea* growing in the nature reserve at the Sierra de Huautla, Mexico, were isolated and characterized. All 104 isolates together with the type strain for *Rhizobium galegae*, HAMBI 540^T, had similar 16S rRNA genes as revealed by PCR-RFLP analysis. Similarity in the sequences of the 16S rRNA genes placed the isolates on a phylogenetic branch shared with *R. galegae*. Among 66 randomly selected isolates, three closely related electrophoretic alloenzyme types (ETs) were identified, which were distinct from 10 ETs distinguished among 23 strains of *R. galegae*. A new species *Rhizobium huautlense*, represented by the *Sesbania* isolate S02^T, is proposed based upon low estimates of DNA relatedness between our chosen type strain and the type strains for the other species, the dissimilarity of the nucleotide sequence of the 16S rRNA genes, and their distinct ETs compared with *R. galegae*. The description of *R. huautlense* is significant because in the reconstruction of the phylogeny of *R. huautlense* there was a shift in the node of the branch of *Agrobacterium vitis* relative to that of *R. galegae*. The revised phylogenetic tree would tend to indicate common ancestry between *R. galegae* and *Rhizobium leguminosarum*.

Keywords: *Rhizobium huautlense* sp. nov., symbionts, *Sesbania herbacea*, nitrogen fixers

INTRODUCTION

There are 70 species within the legume genus *Sesbania*, which are widespread in the warmer latitudes of both hemispheres of the world (1). Many species are annual or short-lived perennials, which because of their fast growth may be useful to agriculture as green manure or fallow crops (1, 25). Besides biomass production, selected species also are useful to control soil erosion, as windbreaks, live fences and as shade in coffee and tea plantations. Several species have fibre that is useful for production of rope, fishing nets and paper. Leaves may be a source of ascorbic acid, diuretics and laxatives.

Since *Sesbania* species are leguminous plants they are able to form beneficial nitrogen-fixing symbioses with soil bacteria belonging to the family *Rhizobiaceae*. A symbiosis is evident from the presence of hypertrophies or nodules on the roots of the host plant. In addition to

Abbreviations: ET, electrophoretic type; I_A, index of association; MLEE, multilocus enzyme electrophoresis.

The GenBank accession numbers for the 16S rRNA gene sequences of strains S02^T and 59A2 are AF025852 and AF025853, respectively.

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Table 1. Isolates and strains used in this study and some of their characteristics

Isolate or strain	16S rRNA gene RFLP pattern*	MLEE analysis		Plasmid size (kb)†	Origin
		ET	Cluster		
Rhizobium huautlense					
S02^T, S04, S07, S08, S09, S12, S24, S25, S27, S28, S29, S30, S31, S42, S43, S77, S94, S101, S102, S104, S106, S110, S111, S113, S114, S115, S116, S119, S123, S131, S132, S151–S165, S167, S168, S169, S170	AAAA	1	3	900, 400	Mexico
S41	AAAA	1	3	900	Mexico
S172	AAAA	1	3	900, 400, 220	Mexico
S32, S182	AAAA	1	3	NO	Mexico
S03, S11, S22, S34, S35, S78, S93	AAAA	2	3	900, 400	Mexico
S103, S105, S124	AAAA	3	3	900, 400	Mexico
Rhizobium galegae					
HAMBI 1461	ND	4	1	> 1000	Russia
HAMBI 1460	ND	4	1	> 1000	Finland
HAMBI 540^T	AAAA	5	1	> 1000	Finland
HAMBI 1147	ND	5	1	> 1000	Finland
HAMBI 1155	ND	5	1	> 1000	Russia
HAMBI 1174	ND	5	1	> 1000	Finland
HAMBI 1207	ND	6	1	> 1000, 180	New Zealand
HAMBI 1208	ND	6	1	> 1000, 180	New Zealand
HAMBI 1428	ND	7	1	> 1000, 180	Russia
HAMBI 1143	ND	8	2	> 1000, 340	New Zealand
HAMBI 1144	ND	8	2	> 1000, 340	New Zealand
59A2	ND	9	2	> 1000	USA
HAMBI 490	ND	9	2	> 1000	Finland
HAMBI 1146	ND	10	2	> 1000, 180	New Zealand
HAMBI 1183	ND	11	2	> 1000, 340	England
HAMBI 1145	ND	11	2	> 1000, 340	New Zealand
HAMBI 1184	ND	11	2	> 1000, 340	England
HAMBI 1187	ND	12	2	> 1000	England
HAMBI 1189	ND	12	2	> 1000	England
HAMBI 1185	ND	12	2	> 1000	England
HAMBI 1186	ND	13	2	> 1000, 180	England
HAMBI 1190	ND	13	2	> 1000, 180	England
HAMBI 1191	ND	13	2	> 1000, 180	England
Other reference strains					
Sinorhizobium terangae ORS 1009^T	BBBA	14			Senegal
Sinorhizobium saheli ORS 609^T	BBBB	15			Senegal
Sinorhizobium meliloti USDA 1002^T	CBBB	ND			USA
Azorhizobium caulinodans ORS 571^T	DBCC	ND			Senegal
Rhizobium leguminosarum USDA 2370^T	EBDD	ND			USA
Rhizobium tropici A CFN 299	FBEE	ND		> 1000, 410, 225, 185	Brazil
Rhizobium tropici B CIAT 899^T	CBDD	ND			Columbia
Rhizobium etli CFN 42^T	EBFC	ND		630, 510, 390, 270, 150	Mexico
Mesorhizobium loti NZP 2213^T	GBGA	ND			New Zealand
Bradyrhizobium japonicum USDA 6^T	HCHF	ND			Japan
Agrobacterium tumefaciens IAM 13129^T	ND	ND		400, 220	Unknown
Agrobacterium rhizogenes IAM 13570^T	ND	ND			Unknown
Agrobacterium vitis IAM 14140^T	ND	ND			Unknown
Agrobacterium rubi IAM 13569^T	ND	ND			Unknown
Plasmid or cosmid					
pMR133	2·0 kb *Eco*RI–*Pst*I insert with *nodDA* and *nodB* from pSym of *R. tropici* CFN 299 cloned into pUC18 (unpublished)				
pEM15	pSUP205 derivative with *nifKDH* of *R. etli* CFN 42^T (34)				

ND, Not detected; NO, not observed.

* Four letters were arbitrarily assigned to represent specific fingerprint patterns of 16S rDNA RFLP digested with restriction endonuclease *Msp*I, *Hin*fI, *Hha*I and *Sau*3AI, respectively.

† Molecular sizes of plasmids were estimated from their mobilities in 0·7% agarose gels and the plasmids of *R. etli* CFN42^T were used as standards. The standard deviation was 6 kb. Strains used for identification of symbiotic plasmids were S02^T, S94, S03, S11, S103, S124 and HAMBI 540^T.

root nodules, *Sesbania rostrata* forms stem nodules (13), which has been explored for determining the potential of using this plant as a green manure crop in the cultivation of rice (3, 19).

The bacterial symbionts of legumes are highly diverse and are classified within the α-*Proteobacteria*. Several fundamental morphological and physiological characters separate *Rhizobium* and *Bradyrhizobium* (22), and their classification as separate genera is indisputable (16). Similarly, the stem-nodulating symbiont of *Sesbania rostrata*, *Azorhizobium caulinodans*, is distinct from the genera *Rhizobium* and *Bradyrhizobium* (15). Within the group which was classified as *Rhizobium*, three separate genera, *Rhizobium* (18), *Sinorhizobium* (8, 11) and *Mesorhizobium* (21), are now recognized.

Positive nodulation scores have been reported for 27 of the 70 species of *Sesbania* (10), but very few of these rhizobial isolates have undergone extensive analysis to determine their genetic relationships within the α-*Proteobacteria*. The most widely known symbiont is *Azorhizobium caulinodans*, which forms stem and root nodules on *Sesbania rostrata* (13) and is distinguishable by its ability to grow in pure culture on N_2 as sole nitrogen source (14). Although *Azorhizobium caulinodans* was predominant among the 191 isolations made in Senegal and the Philippines, an additional genomic species within this genus was discovered and a small proportion were indicated to belong to the genus *Rhizobium* (44). Subsequently, several *Sesbania* species growing in Senegal, including *Sesbania rostrata*, were reported to form symbioses with two newly described species within the genus *Sinorhizobium*, and two isolates from *Sesbania marginata* that originate from Brazil were classified within the genus *Rhizobium* (11). However, reconstruction of the phylogeny using 16S rRNA gene sequences of the *Sesbania* isolates within the genus *Rhizobium* were not reported. The reported phylogeny of isolates from *Sesbania aculeata*, originating from Japan (46) and from India (42), indicated a close affinity with *R. galegae* (28).

The molecular evolutionary relationships of bacterial symbionts isolated from the nodules of *Sesbania* species growing in the North American continent have not been reported. We selected *Sesbania herbacea*, native to Mexico and the Southern USA, growing in the undisturbed nature reserve at the Sierra de Huautla, Mexico, to avoid the possibility of isolating rhizobial symbionts which became established because of legume inoculation practices.

Our objective was to isolate *Sesbania* symbionts that are native to the North American continent, determine their genetic diversity and phylogenetic relationships within the α-*Proteobacteria*.

METHODS

Reference strains, isolations and nodulation assays. Reference strains and isolates used in genetic analyses are shown in Table 1. All the isolates from *Sesbania herbacea* originated from root nodules which were collected during August and October 1996 from about 20 plants growing in flooded areas at the Sierra de Huautla in Morelos, mountains which have a typical tropical climate in the middle part of Mexico. These plants were collected from two sites, a brook and a lake which are 1 km from each other and are separated by the mountains. Isolates S02^T–S43 were from the same plant collected from the brook and others were from both of the sites and from different plants. Isolations were made according to the methods described by Vincent (56) using PY (38) as growth medium. Plates were incubated aerobically for 3 d at 28 °C and the isolates were purified by streaking single colonies on fresh PY plates. Each isolate was verified as a symbiont of *Sesbania herbacea* by plant test using the method of Vincent (56). Seeds were surface sterilized with conc. H_2SO_4 for 20 min, or scarified with fine sandpaper before they were surface-sterilized with a 1% (w/v) solution of NaOCl. Treated seeds were germinated on the surface of 0·75% water-agar and after germination were sown in plastic cups filled with vermiculite moistened with the N-free nutrition solution described previously (53). Cultures grown overnight in PY broth were used to inoculate the seedlings which were grown for 4 weeks under natural sunlight before the ability of each culture to nodulate the trap host was scored. A Leonard jar assembly (27) was used to determine the ability of S02^T and HAMBI 540^T to nodulate *Galega orientalis*, *Glycine max*, *Macroptilium atropurpureum*, *Medicago sativa*, *Leucaena leucocephala*, *Pisum sativum*, *Phaseolus vulgaris*, *Sesbania rostrata*, *Sesbania herbacea*, *Trifolium repens* and *Vigna unguiculata*. These tests were done in duplicate and results were compared to plants which were not inoculated.

PCR-RFLP analysis and the determination of nucleotide sequences of the 16S rRNA genes. Techniques described previously (54) were used for cell lysis to extract the DNA of each culture for PCR analysis and the generation of the 16S rRNA gene fragments (approx. 1500 bp) by PCR using primers fD1 and rD1 corresponding to positions 8–27 and 1524–1540, respectively, of the *Escherichia coli* 16S rRNA gene (58). For sequencing analysis template DNA for the PCR reaction was prepared from 50 ml MAG (53) grown cells according to the protocol of Navarro *et al.* (36). The presence of amplification products in the PCR reactions was confirmed by horizontal agarose gel electrophoresis of 10 μl subsamples. Subsequently, 5 U each of *Hha*I, *Hin*fI, *Msp*I or *Sau*3AI were used to digest 10–15 μl subsamples of the PCR reactions (26). Restriction fragment lengths of the different 16S rRNA gene products were determined by their separation in 3% agarose gels (26). Full length sequences of the 16S rRNA genes of strains S02^T and 59A2 were determined from purified PCR products as described previously (53).

Analysis of the sequence data. The sequences were aligned using the PILEUP program in the Wisconsin package of the Genetics Computer Group (Madison, WI, USA). Aligned sequences were analysed using the DNADIST and the NEIGHBOR programs of the software package PHYLIP version 3.5c (17) and trees were constructed using RETREE and DRAWTREE. Additional analyses were made using the Molecular Evolutionary Genetics Analysis (MEGA) package version 1.01 (24) to derive the nucleotide sequence similarities of the small-subunit rRNA genes and to investigate how tree topology was affected by increasing the number of sequences in the analysis.

Multilocus enzyme electrophoresis (MLEE). Culture extracts for MLEE analysis were prepared according to the procedure of Caballero-Mellado & Martínez-Romero (7) from 40 ml PY broth cultures grown overnight at 28 °C. The extracts were stored at −20 °C.

Starch-gel electrophoresis and the selective staining for the 12 metabolic enzymes listed in the legend to Table 2 were according to the procedures described by Selander *et al.* (48). Relative electrophoretic mobilities of each of the enzymes were determined on three separate occasions. Distinctive mobility variants (electromorphs) of each enzyme, numbered in order of decreasing anodal mobility, were equated with alleles at the corresponding structural gene locus. Allele profiles or electrophoretic types (ETs) were equated with multilocus genotypes. The genetic distance between pairs of ETs was estimated as the proportion of loci at which dissimilar alleles (mismatches) occurred. Clustering of ETs from a matrix of pairwise genetic distances was by the method described by Nei & Li (37). Genetic diversity (h) at an enzyme locus was calculated as described by Selander *et al.* (48) $h = [1-\Sigma x_i^2]\,[n/(n-1)]$, where x_i is the frequency of the ith allele at the locus and n is the number of ETs in the population. The extent of linkage disequilibrium (non-random combinations of alleles) among ETs was estimated by comparing observed and expected moments of allelic mismatch frequency distributions (59). An 'index of association' or I_A, originally described by Brown *et al.* (6), was used to demonstrate a non-random association of alleles. Equations for the calculation of I_A and its associated error variance are described by Maynard Smith *et al.* (32). Programs for the analysis of genetic diversity, ET clustering and linkage disequilibrium were written by T. S. Whittam (Pennsylvania State University). A Monte Carlo procedure with 10000 iterations as described by Souza *et al.* (51) was also used to estimate the extent of linkage disequilibrium.

Determination of DNA base composition and DNA–DNA hybridization analysis. Cultures were grown in 5·0 ml PY broth for 12 h at 28 °C and were centrifuged at 12000 ***g*** for DNA preparation by using a DNA/RNA isolation kit (USB) as described by Chirgwin *et al.* (9). These samples were used to estimate DNA G+C content (T_m) by the method of De Ley (12) and to estimate DNA homologies by using the filter method as described by van Berkum *et al.* (54). Cultures grown in 50 ml MAG (53) were used to prepare DNA (36) for estimation of DNA homology by using the hydroxyapatite batch procedure to separate single- and double-stranded DNA (4, 5). The DNA concentrations in samples prepared by either procedure were determined spectrophotometrically by absorbance at 260 nm.

Determination of cellular plasmid content and identification of symbiotic plasmids. The plasmid contents of 66 isolates from *Sesbania herbacea* and 24 strains of *R. galegae* were visualized by using a modified Eckhardt procedure (20). Plasmid mobilities were determined in 0·7% agarose gels using plasmids of *R. etli* CFN 42^T (45) and of *R. tropici* CFN 299 (19, 30) as reference molecular size markers. The approximate molecular sizes of plasmids were estimated from the gels by using the computer program Seqaid II version 3.5 (43). Symbiotic plasmids were identified by Southern hybridization analysis using as probes a 2·0 kb *Eco*RI–*Pst*I fragment containing *nodDA* and *nodB* of *R. tropici* CFN 299 cloned in pUC18 and a 5·1 kb *Bam*HI restriction fragment containing *nifHDK* of *R. etli* CFN 42^T (34). The type strain of *R. etli* (47), CFN 42^T (45), and the type A strain of *R. tropici* (31), CFN 299 (19, 30), were used as positive controls.

Phenotypic characterization. The phenotypic characters of 15 isolates from *Sesbania herbacea* and nine strains of *R. galegae* were determined. The strains and isolates were selected according to their position in the clustering analysis of the MLEE data and according to their plasmid content. With the exception for the determination of growth in LB broth the phenotypic characters were examined by growth on solid media in Petri plates. Each Petri plate was used to determine the characters of six cultures and each character was examined in duplicate. Cultures were scored for growth after incubating the Petri plates for 4 d at 28 °C. Intrinsic antibiotic resistance was determined on PY medium amended with filter-sterilized solutions of the following antibiotics (final concns): 5, 50 and 100 μg ampicillin ml^{-1}; 5, 50, 100 and 300 μg chloromycin ml^{-1}; 5, 50, 100 and 300 μg erythromycin ml^{-1}; 5, 50, 100 and 300 μg gentamicin ml^{-1}; 5, 50 and 100 μg kanamycin ml^{-1}; 5, 50, 100 and 300 μg lincomycin ml^{-1}; 5, 50, 100 and 300 μg neomycin ml^{-1}; 5 and 50 μg streptomycin ml^{-1}; and 5, 50, 100 and 300 μg tetracycline ml^{-1}. A basal medium described by Novikova *et al.* (39), supplemented with filter-sterilized solutions of pyridoxine and thiamin at final concentrations of 0·025 mg ml^{-1} according to Oyaizu *et al.* (41), was used to determine carbon and nitrogen substrate utilization. Bromothymol blue at 0·0025% (w/v) was added as pH indicator. Filter sterilized carbon substrates were added at a final concentration of 0·1% (w/v) and included acetate, adonitol, D-arabinose, citrate, dulcitol, D-fructose, fumarate, D-galactose, D-gluconate, D-glucose, *meso*-inositol, lactate, lactose, DL-malate, melibiose, maltose, propionate, D-raffinose, rhamnose, D-sorbitol, succinate, sucrose and D-xylose. Mannitol was used as positive control, and no addition of carbon substrate was used as a negative control. The appearance of single colonies and production of acid or alkaline were recorded. The same basal medium was used to determine utilization of adenine, L-arginine, L-aspartic acid, anthranilic acid, L-cystine, *p*-fluoro-DL-phenylalanine, L-glutamic acid, glycine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-threonine and L-tryptophan at final concentrations of 10 mM. Basal medium containing NH_4NO_3 (0·01% w/v) and lacking a nitrogen source were used as positive and negative controls, respectively. All amino acids were prepared as filter-sterilized stock solutions. Colony morphology and acid production were examined on YMA (56) and PY after 2 and 4 d growth. Growth at 37 °C and at pH 5·0, 5·5 and 9·0 were evaluated on PY medium. Tolerance to NaCl at concentrations of 1·0, 1·5, 2·0 and 2·5% (w/v) was determined on YMA. Ability to grow in LB medium was determined with 5 ml broth. Growth rate was determined in YM broth by following the increase in culture optical density spectrophotometrically at 600 nm which was used to derive the generation times (56) of each of the cultures tested.

Plant trapping tests. Surface sterilized, germinated seeds of *Sesbania herbacea* and *Galega orientalis* were used for trapping rhizobia from Mexican soils (from Cuernavaca, Morelos) in pots under natural sunlight, to test if *R. galegae* or other rhizobia could be recovered. Nodules were collected from 2-month-old plants. Isolates were identified by PCR-RFLP of 16S rRNA genes and electrophoretic plasmid profiles using the methods mentioned above.

RESULTS

Isolation of the bacterial symbionts of *Sesbania herbacea*

Only root nodules of *Sesbania herbacea* were collected from the flooded fields of Sierra de Huautla since stem nodules were not observed. The appearance of the nodules was as if they were covered with a cotton-like material. One hundred and four rhizobial isolates were obtained, which we considered fast-growing because single colonies of 2 mm diameter were formed on PY medium after 2 d incubation at 28 °C. These colonies were round, white and shiny and became gummy after incubating them for an additional 3 d. More exopolysaccharides were produced on YMA than on PY medium.

PCR-RFLP analysis of the 16S rRNA genes

The fingerprint patterns of the 16S rRNA gene PCR products of all of the 104 isolates digested with the four restriction enzymes were compared with those obtained with type or reference strains for the defined species (Table 1). The fingerprint patterns among the 104 isolates were identical to each other and with the patterns observed with the type strain for *R. galegae*, HAMBI 540^T. The patterns obtained with the remaining type strains for the various species were distinguishable from those of *R. galegae* and the isolates, and clustered according to their phylogenetic relationships (data not shown) as previously reported (26, 35).

MLEE

Only 66 randomly chosen isolates were included in this analysis because no differences among the 104 isolates by fingerprint analysis of the 16S rRNA genes were detected. However, we included 23 strains of *R. galegae* in addition to the reference strain because the PCR-RFLP pattern of the 16S rRNA genes of HAMBI 540^T and those of the *Sesbania* isolates were identical. Three and 10 ETs were identified among the *Sesbania* isolates and the strains of *R. galegae*, respectively (Tables 1 and 2). The genetic diversity among the strains of *R. galegae* was higher than among the isolates from *Sesbania herbacea* (Table 3). The analysis also included the type strains for *Sinorhizobium terangae* and *Sinorhizobium saheli* because they originated from root nodules of *Sesbania* in Africa. These two species clustered together and were genetically distant (0·94) from clusters formed by our *Sesbania* isolates and the

Table 2. Allele profiles of 12 enzyme loci representing 15 ETs among symbionts of *Sesbania* and *Galega* species

For each enzyme locus, the numbers denote a different allele for that locus. The absence of enzyme activity is scored as a null character state (−). Abbreviations: PGI, phosphoglucose isomerase; MDH, malate dehydrogenase; IDH, isocitrate dehydrogenase; G6P, glucose-6-phosphate dehydrogenase; PGM, phosphoglucomutase; ME, malic enzyme; HEX, hexokinase; XDH, xanthine dehydrogenase; ADK, adenylate kinase; ALD, alanine dehydrogenase; EST, esterase; IPO, indophenol oxidase.

ET	Reference strain	Alleles at indicated enzyme loci											
		PGI	MDH	IDH	G6P	PGM	ME	HEX	XDH	ADK	ALD	EST	IPO
***Rhizobium huautlense* cluster 3**													
1	S02^T	4	4	4	4	4	4	6	4	4	4	3	4
2	S03	4	4	4	4	4	4	4	4	4	4	4	4
3	S103	4	4	4	4	4	4	6	4	4	4	4	4
***Rhizobium galegae* cluster 1**													
4	HAMBI 1461	4	4	3	4	3	3	6	4	4	6	7	2
5	HAMBI 540^T	6	4	3	6	3	3	6	4	4	6	8	2
6	HAMBI 1207	7	4	3	6	2	3	7	4	4	6	8	2
7	HAMBI 1428	4	4	3	6	3	3	6	4	4	6	8	2
***Rhizobium galegae* cluster 2**													
8	HAMBI 1143	4	4	4	6	4	4	7	4	4	6	3	2
9	59A2	4	4	4	4	4	4	8	4	4	6	2	1
10	HAMBI 1146	4	4	4	4	4	4	8	4	4	6	3	2
11	HAMBI 1145	7	4	4	4	4	4	5	4	6	6	7	2
12	HAMBI 1185	6	4	4	6	4	4	4	4	4	6	7	2
13	HAMBI 1186	7	4	4	4	4	4	6	4	4	6	7	2
Sinorhizobium terangae													
14	ORS 1009	8	8	8	9	6	7	6	–	9	9	9	9
Sinorhizobium saheli													
15	ORS 609	8	8	8	6	6	8	6	–	9	9	9	9

Table 3. Genetic diversity (*h*) at 12 enzyme loci among *R. huautlense* and *R. galegae*

See Table 2 for abbreviations and the cluster composition.

Enzyme	Cluster 1 (4 ETs)		Cluster 2 (6 ETs)		Clusters 1+2 (10 ETs)		Cluster 3 (3 ETs)	
	Alleles	*h*	Alleles	*h*	Alleles	*h*	Alleles	*h*
PGI	2	0·833	3	0·689	3	0·733	1	0
MDH	1	0	1	0	1	0	1	0
IDH	1	0	1	0	2	0·533	1	0
G6P	2	0·500	2	0·534	2	0·556	1	0
PGM	2	0·500	1	0	3	0·600	1	0
ME	1	0	1	0	2	0·533	1	0
HEX	2	0·500	5	0·932	5	0·822	2	0·668
XDH	1	0	1	0	1	0	1	0
ADK	1	0	2	0·334	2	0·200	1	0
ALD	1	0	1	0	1	0	1	0
EST	2	0·500	3	0·733	4	0·778	2	0·668
IPO	1	0	2	0·334	2	0·200	1	0
Mean	1·4	0·236	1·9	0·300	2·3	0·409	1·2	0·111

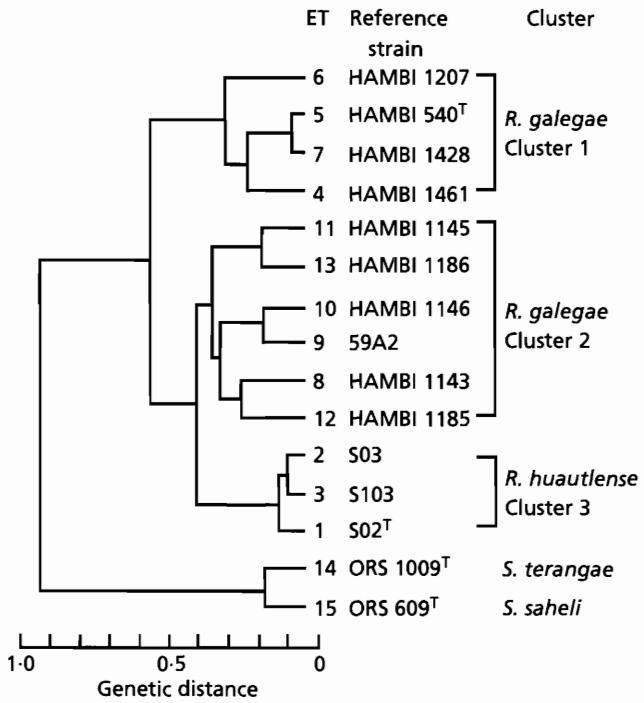

Fig. 1. Genetic relationships among *Rhizobium huautlense*, *R. galegae* and type strains for *Sinorhizobium saheli* and *Sinorhizobium terangae* based on the MLEE data. In the case of *R. huautlense* the three strains indicated represent three ETs among the 66 isolates that were analysed. In the case of *R. galegae*, strains indicated represent 10 ETs among 23 strains that were analysed.

strains from the *Galega* host plant species (Fig. 1). Ten ETs were identified among the strains of *R. galegae* which formed two clusters (clusters 1 and 2) separated at a genetic distance of 0·56. The two clusters identified among *R. galegae* corresponded with hosts nodulated since the strains of cluster 1 originated from *G. orientalis*, except HAMBI 1207, and those of cluster 2 from *G. officinalis* (28). Three ETs forming a single cluster (cluster 3) were identified among the 66 isolates from *Sesbania herbacea*. Cluster 3 and cluster 2 were separated at a genetic distance of 0·39.

The I_A values were $1·49 \pm 0·38$ when all the three clusters, 1, 2 and 3, were considered together, and $1·64 \pm 0·43$ and $1·00 \pm 0·46$, respectively, when the two subsets, clusters 1 and 2 or clusters 2 and 3 were analysed. The fact that all of these I_A values differed significantly from zero might indicate linkage disequilibrium relationships between these three clusters. However, results of linkage disequilibrium analysis might be questionable when sample sizes are small as is the case in each of the three clusters 1, 2 and 3.

DNA G+C content and DNA–DNA hybridization

The DNA G+C contents for the two representative isolates, S02^T and S03, were 57·0 and 58·9 mol%, respectively. Four isolates from *Sesbania herbacea*, which represented the three ETs, shared DNA homology above 70% as determined by the filter method (Table 3). The DNA homology of isolate S02^T and the type strains of the described species, including *R. galegae*, ranged from 9 to 22% determined with the filter method. When determined by the hydroxyapatite method the DNA homology of isolate S02^T with the *R. galegae* type strain HAMBI 540^T and strain 59A2 was 12 and 26%, respectively. The DNA homology between strain 59A2 and HAMBI 540^T of *R. galegae* representing cluster 2 and cluster 1 in the MLEE analysis was 40 and 65% as determined by the filter

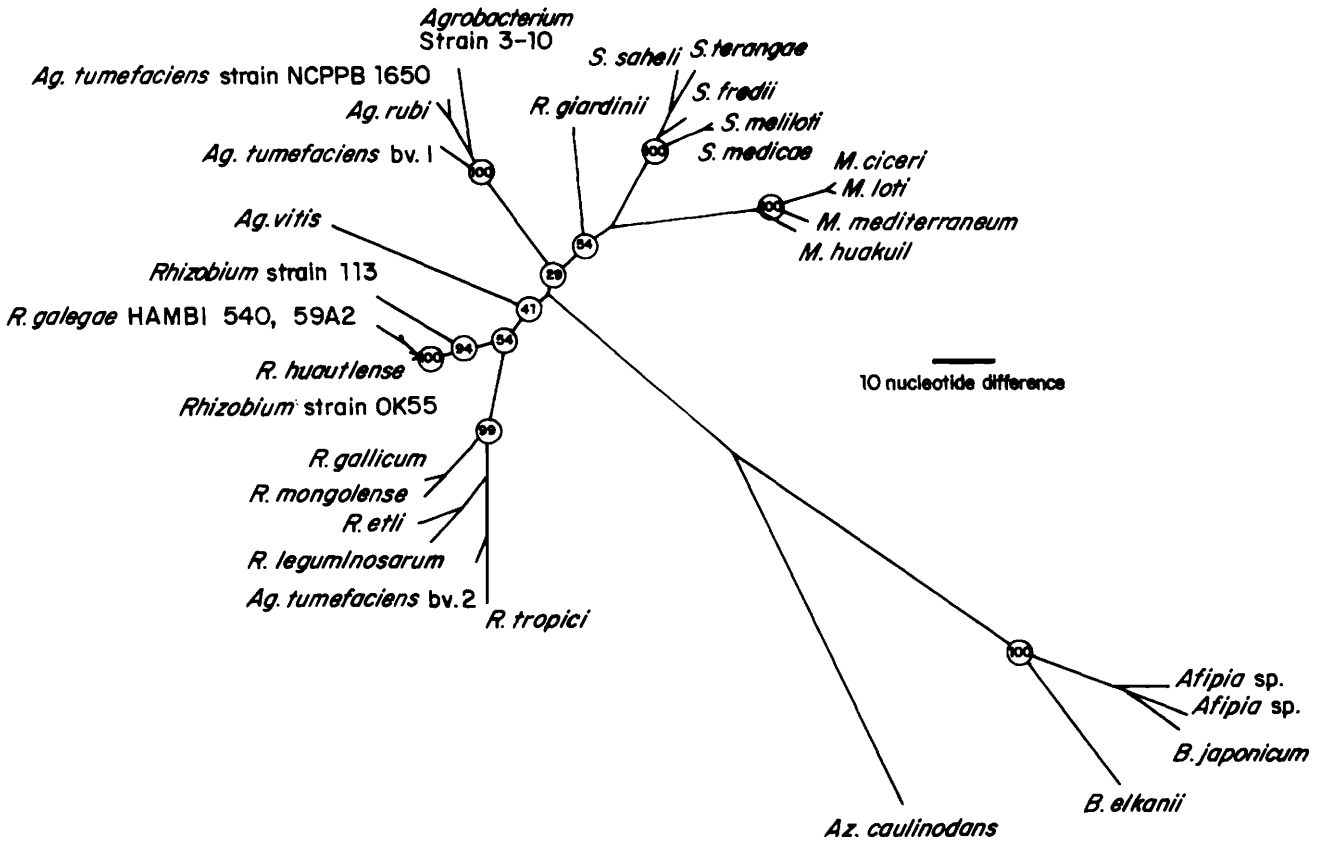

Fig. 2. Phylogenetic relationships of *Rhizobium huautlense* and *R. galegae* within the *α-Proteobacteria* based upon aligned sequences of the small subunit rRNA genes. Jukes–Cantor distances were derived from the aligned sequences to construct an optimal unrooted tree using the neighbour-joining method. The levels of support for the presence of several relevant nodes are indicated. Representative sequences in the tree obtained from GenBank which were used in the phylogenetic analysis were: *Agrobacterium rubi* (D14503), *Agrobacterium vitis* (D14502), *Agrobacterium tumefaciens* bv. 2 strain NCPPB 2991 (D14501), *Agrobacterium tumefaciens* strain NCPPB 1650 (D14506), *Agrobacterium tumefaciens* bv. 1 strain NCPPB 2437 (D14500), *Agrobacterium* sp. strain 3-10 (Z30542), *Afipia clevelandensis* (M69186), *Afipia felis* (M65248), *Azorhizobium caulinodans* (X94200), *Bradyrhizobium elkanii* (U35000), *B. japonicum* (U69638), *Mesorhizobium ciceri* (U07934), *M. huakuii* (D13431), *M. loti* (X67229), *M. mediterraneum* (L38825), *Rhizobium etli* (U28916), *R. galegae* (X67226), *R. gallicum* (U86343), *R. giardinii* (U86344), *R. leguminosarum* bv. viciae (U29386), *R. mongolense* (U89817), *R. tropici* (U89832), *Rhizobium* sp. strain OK55 (D14510), *Rhizobium* sp. strain 113 (D14512), *Sinorhizobium fredii* (X67231), *Sinorhizobium medicae* (L39882), *Sinorhizobium meliloti* (X67222), *Sinorhizobium saheli* (X68390) and *Sinorhizobium terangae* (X68387). The sequences were aligned using the PILEUP program and the aligned sequences were analysed using the DNADIST and NEIGHBOR programs of the software package PHYLIP version 3.5c (17) and the tree was constructed using RETREE and DRAWTREE. The bootstrap analysis was done using the Molecular Evolutionary Genetics Analysis (MEGA) package version 1.01 (24).

and hydroxyapatite methods, respectively. The DNA homology values of 59A2 and several additional strains of *R. galegae* varied between 32 and 94% when determined by the filter method, which in some cases was lower than previously reported (29, 57).

Phylogeny of the isolates based upon 16S rRNA gene sequences

Phylogeny was reconstructed with only the representative isolate S02^T since fingerprint patterns obtained by PCR-RFLP analysis of the 16S rRNA gene products of all 104 isolates were identical. However, the 16S rRNA gene sequence of 59A2 also was determined to reconstruct the phylogeny of the cluster 2 strains of *R. galegae*. The 16S rRNA gene sequence of isolate S02^T was most similar to that of *Sesbania* strain OK55 (99·9%) and was 99·5 and 99·7% similar to those of *R. galegae* HAMBI 540^T and 59A2. The nucleotide sequence of the 16S rRNA gene of isolate S02^T was less similar to that of *Agrobacterium vitis* (96·1%) than to that of *R. leguminosarum* (96·3%). Apart from *R. galegae*, within the genus *Rhizobium* the 16S rRNA gene sequence of isolate S02^T and that of *R. mongolense* (55) was most similar (96·8%). The nucleotide sequence similarities of the 16S rRNA genes of 59A2 with those of *R. leguminosarum*, *R. mongolense* and *Agrobacterium vitis* were 96·2, 96·7 and 96·2%,

respectively. The aligned 16S rRNA gene sequences were used to reconstruct a phylogenetic tree (Fig. 2). The representative isolate from *Sesbania herbacea*, S02^T, clustered with strains HAMBI 540^T and 59A2 of *R. galegae*, and the isolates OK55 and 113 from *Sesbania aculeata* and *Mimosa invisa* (41), respectively. The node of this cluster was shared with that of the other species of *Rhizobium* and *Agrobacterium tumefaciens* bv. 2 strain NCPPB 2991. In our phylogenetic reconstruction the position of *Agrobacterium vitis* became uncertain in comparision to previous reports (46, 60) since *Agrobacterium vitis* and *R. galegae* no longer shared a common node. Instead, the branch with *Agrobacterium vitis* was independent and had a node close to that of the other species of *Agrobacterium*. However, results of the bootstrap analysis using 500 replications would indicate a low level of confidence in the relative positions of the nodes separating *R. galegae* and *R. leguminosarum*, *Rhizobium* and *Agrobacterium vitis*, and *Agrobacterium vitis* and *Agrobacterium tumefaciens* bv. 1. We observed shifts in the relative positions of *R. galegae* and *Agrobacterium vitis* and a change in the confidence values of the positions of these nodes as we reconstructed phylogenies after progressively removing sequences from the alignment. After removing the 16S rRNA gene sequences of S02^T, 113, OK55, 59A2, AF3-10, *R. etli*, *R. gallicum* (2) and *R. mongolense* from the alignment, tree topology was restored to that reported by Willems & Collins (60).

Determinations of plasmid profiles and identification of the symbiotic plasmid

The majority of the 66 isolates originating from *Sesbania herbacea* had two plasmids with approximate molecular sizes of 900 and 400 kb (Table 1, Fig. 3). We concluded that the symbiotic plasmid was the smaller of the two since it hybridized with probes containing the cloned *nod* and *nif* genes. However, only the 900 kb plasmid was observed with isolate S41 and in the case of isolate S172 an additional plasmid of 220 kb was identified. No plasmid bands were observed in isolates S32 and S182, however by hybridization to *nif* genes we observed bands that could respond to very large megaplasmids (data not shown). Strains of *R. galegae* had plasmids which differed in molecular size from those detected in the isolates from *Sesbania herbacea* (Table 1). All the strains of *R. galegae* had a megaplasmid (> 1000 kb) and either one or no additional plasmids. The molecular size of the second plasmid was either 340 or 180 kb. Cluster 1 strains had only the megaplasmid (ETs 4 and 5), or the megaplasmid and a 180 kb plasmid (ETs 6 and 7). The cluster 2 strains either had the megaplasmid alone (ETs 9 and 12), or the megaplasmid and either the 340 kb plasmid (ETs 8 and 11) or the 180 kb plasmid (ETs 10 and 13). The megaplasmid of HAMBI 540^T was identified as the symbiotic plasmid, which agreed with previous reports (23, 40).

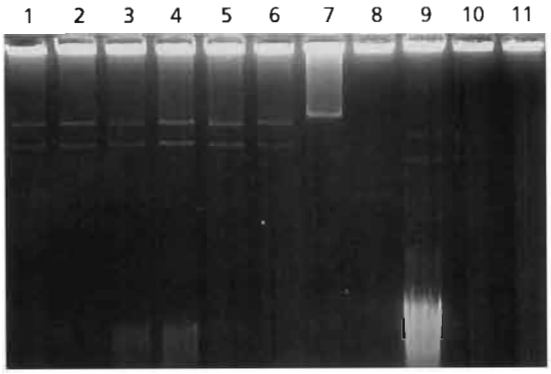

Fig. 3. Plasmids of *Rhizobium huautlense*. Plasmids were visualized by using the modified Eckhardt procedure (20) and their mobilities were determined in 0·7% agarose gels. Lanes: 1–6, *R. huautlense* S02^T, S94, S03, S11, S103, S124, representing three ETs, respectively; 7, *R. galegae* HAMBI 540^T; 8, *Azorhizobium caulinodans* ORS 571; 9, *Agrobacterium tumefaciens* IAM 13129; 10, *R. etli* CFN 42^T cured of the 630 kb plasmid; 11, *R. tropici* CFN 299. The approximate molecular size of the plasmids is shown in Table 1.

Phenotypic characterization

The distinctive phenotypic characters of 15 isolates from *Sesbania herbacea*, nine strains of *R. galegae* and the type strain of *Agrobacterium vitis* are listed in Table 4. The isolates could be distinguished from *R. galegae* by growth on YMA at 40 °C or at pH 9·0. Growth of the isolates on the basal medium was significantly improved by the addition of thiamin but not pyridoxine. Strains of *R. galegae* did not grow on basal medium supplemented with the two vitamins because of the requirement of calcium panthotenate as growth factor. Differences between these two groups also were resistance to ampicillin and the utilization of adenine, methionine and dulcitol. Growth in LB or on YMA in the presence of 1·5% NaCl distinguished *Agrobacterium vitis* from the 15 isolates from *Sesbania herbacea* and the nine strains of *R. galegae*. Further distinctive characters of the *Agrobacterium vitis* type strain were resistance to chloramphenicol, growth with glycine and cystine, the production of acid with adenine and maltose, and the production of alkaline with aspartate and glutamate. Strains of *R. galegae* belonging to the two clusters could not be distinguished by phenotypic characters.

The isolates from *Sesbania herbacea* did not grow on YMA at pH 4·5. All produced acid on YMA and were resistant to 300 μg lincomycin ml^{-1} and 5 μg ml^{-1} each of erythromycin, neomycin and kanamycin. All were sensitive to 5 μg tetracycline ml^{-1}, 50 μg ml^{-1} each of ampicillin, chloromycin, gentamicin and neomycin, and 100 μg kanamycin ml^{-1}. All could grow and produce acid with L-leucine, L-lysine, L-tryptophan, L-arginine, L-isoleucine and L-phenylalanine as sole nitrogen sources, and did not grow with *p*-flouro-DL-phenylalanine and anthranilic acid. All used D-xylose, D-fructose, inositol, D-galactose, D-glucose, melobiose,

Table 4. DNA–DNA hybridization of *R. huautlense* strain S02^T and *R. galegae* strain 59A2 with type strains of related species

DNA–DNA hybridization was overnight at 65 °C and the membranes were washed twice in 2 × SSC at room temperature for 10 min and in 1 × SSC for 15 min at 65 °C. Values are means ± standard error.

Species	Strain	DNA homology (%) with:	
		S02^T	59A2
Rhizobium huautlense	S02^T	100	16 ± 3
	S94	102 ± 8	
	S03	80 ± 10	18 ± 1
	S103	79 ± 3	
Rhizobium galegae			
Cluster 1	HAMBI 540^T	22 ± 2	41 ± 3
	HAMBI 1141	17 ± 3	32 ± 3
Cluster 2	HAMBI 1146		94 ± 6
	HAMBI 1183	21 ± 3	44 ± 5
	HAMBI 1143		40 ± 2
	HAMBI 1185		36
	HAMBI 1191		36 ± 0
	59A2	24 ± 1	100
Rhizobium leguminosarum	USDA 2370^T	20 ± 2	
Bradyrhizobium japonicum	USDA 6^T	9 ± 1	
Azorhizobium caulinodans	ORS 571^T	9 ± 0	
Mesorhizobium loti	NZP 2213^T	14 ± 1	
Sinorhizobium meliloti	USDA 1002^T	13 ± 0	
Sinorhizobium saheli	ORS 609^T	13 ± 0	
Sinorhizobium terangae	ORS 1009^T	13 ± 0	
Agrobacterium vitis	IAM 14140^T	15 ± 1	
Agrobacterium rubi	IAM 13569^T	13 ± 0	
Agrobacterium tumefaciens	IAM 13129^T	16 ± 0	
Agrobacterium rhizogenes	IAM 13570^T	14 ± 1	

D-arabinose, rhamnose, fumarate, succinate and sucrose as sole carbon substrates for growth, but propionate was not utilized.

Nodulation tests

R. galegae HAMBI 540^T did not nodulate *Sesbania herbacea* and isolate S02^T did not nodulate *G. officinalis*. Besides nodulation of the original trap host, S02^T also nodulated *L. leucocephala*, *Sesbania rostrata* and *T. repens*, but nodules were small and ineffective with the latter two legumes. Although the nodules on *L. leucocephala* were small, improved plant growth compared to the uninoculated control indicated that a nitrogen-fixing symbiosis had been established.

Plant trapping tests

Ten plants of *Galega orientalis* did not form nodules in soils from Cuernavaca. *Sesbania herbacea* plants formed root nodules and some nodule-like structure on the stems. The majority of the root nodules were formed by isolates which had identical RFLP patterns of 16S rRNA genes with *Mesorhizobium* cluster U strain ORS 1037. The other isolates were similar to *R. tropici* B strain CIAT 899^T and *R. etli* CFN 42^T according to the 16S rRNA gene RFLP analysis. No isolate was similar to those isolated from Sierra de Huautla. One *R. tropici* type-B-like isolate was checked for the plasmid profiles and it showed three bands which were totally different from those of all isolates from flooded fields at Sierra de Huautla. Different bacteria were isolated from each of the nodule-like stem structures. Some of them had RFLP patterns of 16S rRNA genes similar to *Sinorhizobium* species. Further work is needed to test the nodulation ability and taxonomic positions of these isolates.

DISCUSSION

The ability of *Sesbania* species to nodulate is well-documented (1, 11). Several rhizobial symbionts of *Sesbania* species originating from plants growing in Africa have been classified within the genera *Azorhizobium* (15) and *Sinorhizobium* (11). However, there are some indications that species of *Sesbania* growing

outside the African continent form symbioses with *Rhizobium*, but their detailed characterization has not been reported. Isolates originating from *Sesbania aculeata* growing on the Asian continent (42, 46) would appear to have close phylogenetic affinities with *R. galegae*. The partial description of two Brazilian isolates of *Sesbania marginata* indicate that soils of the Americas also harbour symbionts of *Sesbania* within the genus *Rhizobium* (11). However, their phylogeny was not determined. We chose to work with *Sesbania herbacea* growing in an undisturbed nature reserve in Mexico, since nodulation of this legume has not been determined. The results of a polyphasic approach to characterize the 104 isolates we obtained led to the conclusion that at least this species of *Sesbania* growing in the Northern hemisphere of the American continent forms symbioses with bacteria within the genus *Rhizobium*. It would appear from our data that *Sesbania herbacea* growing in the nature reserve at Huautla formed symbioses only with *Rhizobium*, although it is possible that symbionts of other genera were not isolated.

In current reconstructions of the phylogeny of rhizobia based upon 16S rRNA gene sequences, *R. galegae* and the other species within the genus *Rhizobium* do not appear to share a common ancestry (46, 60, 61). Instead, *R. galegae* would seem to have more affinity with *Agrobacterium vitis*, which has prompted a call for the possible revision of *R. galegae* from a species of *Rhizobium* to one of *Agrobacterium* (11). In our reconstruction of the phylogeny of *R. huautlense*, the revised phylogenetic tree would tend to indicate common ancestry between *R. galegae* and the type species of the genus, *R. leguminosarum*. This would agree with the reported sequence similarities of the common nodulation genes of *R. galegae* and *R. leguminosarum* (52). The reassignment of *R. galegae* (and *Rhizobium huautlense*) to a species of *Agrobacterium* or to a new genus, as was suggested previously (11, 62), would not be consistent with the evidence we present.

The diversity among the isolates was very limited. It is possible that these isolates are the most competent rhizobia in flooded conditions for this plant because *Sesbania herbacea* formed nodules with several other rhizobia, but not *R. huautlense*, when grown in soil. The limited geographic area from which the isolates originated may have contributed to the apparent genetic homogeneity of *R. huautlense*. This is in contrast to the diversity identified within *R. etli* originating from soils in Mexico (50) as well as among the strains of *R. galegae* which were included in our analysis. These isolates and strains originated from a wider geographic region and from either more promiscuous host plants in the case of *R. etli* or from more than one species of host plant in the case of *R. galegae*. This is relevant in the case of *R. huautlense* because it has been suggested that origin and host-plant-specificity may influence bacterial genetic diversity (7, 33, 49).

Although *R. huautlense* and *R. galegae* are closely related, we suggest that they are distinct species. Our suggestion is supported by their genetic differences mainly based upon the low estimates of DNA relatedness between the two species. Further differences were observed in plasmid content and the size of the symbiotic plasmids. *R. huautlense* and *R. galegae* appear to originate from different geographic regions since there are no native *Galega* species in Morelos (O. Dorado, unpublished data) and no *R. galegae* in the tested Mexican soils as shown from the plant trapping tests performed. The geographic separation is a barrier to genetic exchange (32) and we propose that this could have led to their divergence into separate species.

We are unable to make conclusions about the possible genetic relationships between *R. huautlense* and *Sesbania* isolates OK55 and SIN-1 which originate from Asia because their characterization has not been sufficiently detailed. However, we are able to point out some common features as well as some differences. Both OK55 (41) and *R. huautlense* require thiamin as a growth factor. The reported DNA G+C content of OK55 and related strains (62·7–63·9 mol%) is higher than that of *R. huautlense* (57·0–58·9 mol%). Characteristics shared by strain SIN-1 (42) and *R. huautlense* are that both harbour two plasmids with the symbiotic genes located on the smaller one, both grow at 37 °C, both utilize gluconate and lactose but not acetate and citrate as sole carbon substrates, both are sensitive to chloramphenicol, erythromycin, kanamycin, tetracycline and streptomycin. Resistance to ampicillin and growth in YMA in the presence of 2% NaCl distinguished SIN-1 from *R. huautlense*.

Description of *Rhizobium huautlense* sp. nov.

Rhizobium huautlense (huau.tlen'se. N.L. gen. n. *huautlense* of Huautla, the region in Mexico where the organisms were isolated).

Aerobic, Gram-negative, non-spore-forming rods. Grows on YMA and PY at temperatures up to 40 °C. On YMA, the colonies are circular, cream-coloured, semi-translucent and mucilagenous within 2–4 d. Older colonies have a white centre. On PY medium, the colonies produce less polysaccharides than on YMA and acid is produced. The generation time is 2·0–2·2 h. Growth occurs on YMA supplemented with 1·0% NaCl and at pH 9·0. Resistant to 300 μg lincomycin ml^{-1}, 5 μg ml^{-1} each of ampicillin, erythromycin, neomycin and kanamycin. Sensitive to 50 μg ml^{-1} each of ampicillin, chloramphenicol, gentamicin and neomycin, and 5 μg tetracycline ml^{-1}, and 100 μg kanamycin ml^{-1}. Adenine, L-leucine, L-lysine, methionine, L-tryptophan, L-arginine, L-isoleucine, L-phenylalanine, glutamate and aspartic acid are used as sole nitrogen substrates for growth, but *p*-fluoro-DL-phenylalanine and anthranilic acid are not utilized. Dulcitol, inositol, xylose, D-fructose, D-galactose, lactose, melibiose, D-arabinose, rhamnose, fumarate

and succinate are used as sole carbon substrate for growth, but citrate and propionate are not utilized. Thiamin is required for growth. The species nodulates and forms nitrogen-fixing symbioses with *Sesbania herbacea* and *L. leucocephala*. At the molecular level, *Rhizobium huautlense* is differentiated from related species by cellular plasmid content, the molecular size of the symbiotic plasmid, variation in electrophoretic mobilities of 12 enzyme loci, low estimates of DNA relatedness and by the nucleotide sequence of 16S rRNA gene. The DNA G+C content is 57·0–58·9 mol% (T_m). S02^T is the type strain of *R. huautlense*, and it has the characteristics given above for the species. All 66 isolates of *R. huautlense* characterized in our study have been deposited in the USDA ARS National *Rhizobium* Resource Center collection, USA, and in the Culture Collection of Centro de Investigación sobre Fijación de Nitrógeno (CFN) at UNAM, Cuernavaca, Mexico.

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