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Nodulation of *Lupinus albus* by Strains of *Ochrobactrum lupini* sp. nov.

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The nodulation of legumes has for more than a century been considered an exclusive capacity of a group of microorganisms commonly known as rhizobia and belonging to the α-*Proteobacteria*. However, in the last 3 years four nonrhizobial species, belonging to α and β subclasses of the *Proteobacteria*, have been described as legume-nodulating bacteria. In the present study, two fast-growing strains, LUP21 and LUP23, were isolated from nodules of *Lupinus honoratus*. The phylogenetic analysis based on the 16S and 23S rRNA gene sequences showed that the isolates belong to the genus *Ochrobactrum*. The strains were able to reinfect *Lupinus* plants. A plasmid profile analysis showed the presence of three plasmids. The *nodD* and *nifH* genes were located on these plasmids, and their sequences were obtained. These sequences showed a close resemblance to the *nodD* and *nifH* genes of rhizobial species, suggesting that the *nodD* and *nifH* genes carried by strain LUP21^T were acquired by horizontal gene transfer. A polyphasic study including phenotypic, chemotaxonomic, and molecular features of the strains isolated in this study showed that they belong to a new species of the genus *Ochrobactrum* for which we propose the name *Ochrobactrum lupini* sp. nov. Strain LUP21^T (LMG 20667^T) is the type strain.

Plants from the family *Leguminosae* are usually capable of dinitrogen fixation because of their symbiotic interaction with nodulating bacteria belonging to the order *Rhizobiales*. Most bacteria that establish a symbiosis with legume plants, including some nonrhizobial species of *Methylobacterium* (16, 45) and *Devosia* (40, 41), belong to the α subclass of *Proteobacteria*, although some species from genera of the β subclass, such as *Ralstonia* and *Burkholderia*, can also nodulate legumes (6, 32, 53). In the last few years there has been an increasing amount of research focused on bacteria that nodulate stems or roots of tropical legume species. However, the identity of many of the endosymbionts of temperate legumes still remains unknown. The genus *Lupinus* groups up to 200 species of herbs and small shrubs, broadly distributed in the Mediterranean area and in the American continent, where they colonize very different environments. Despite the agronomic and ecological interest of *Lupinus*, this plant has been poorly studied with respect to its symbionts. Plants from the genus *Lupinus* are nodulated by fast- and slow-growing rhizobia; however, slow-growing rhizobia are more frequently isolated from this legume (3, 5, 21, 30). The data obtained from the small-subunit (SSU) rRNA gene indicate a very close relationship between some bradyrhizobia isolated from *Lupinus* and *Bradyrhizobium japonicum* (3, 12, 30). However, bacterial strains nodulating *Lupinus* plants have been poorly characterized thus far, and fast-growing species nodulating this legume have not been not officially described; nevertheless, in the past the species *Rhizobium lupini* was proposed (15) and was later abandoned (14).

During a study of rhizobia nodulating *Lupinus* plants in several geographical regions, we isolated two fast-growing strains from nodules of two *Lupinus honoratus* plants. According to the rRNA gene sequences, they were identified as members of the genus *Ochrobactrum* within the α_2 subclass of *Proteobacteria*. This genus belongs to the family *Brucellaceae* and was first described by Holmes et al. in 1988 (11) with a single species, *Ochrobactrum anthropi*. Later, four more species were described from different origins: *Ochrobactrum intermedium* from clinical sources (54), *Ochrobactrum grignonense* and *Ochrobactrum tritici* from soil and the wheat rhizoplane (23), and *Ochrobactrum gallinifaecis* from chicken feces (17).

Two nonrhizobial species belonging to the α subclass of *Proteobacteria* have been described hitherto as the legume endosymbionts *Devosia neptuniae* (41) and *Methylobacterium nodulans* (16). Recently, a strain isolated from *Acacia mangium* (33) was reported to belong to the genus *Ochrobactrum* in the family *Brucellaceae*, although there are no data about the symbiotic genes carried by this strain, nor its species affiliation. Therefore, we sought here to examine the phylogenetic relationships of strains LUP21 and LUP23 and to detect and characterize the symbiotic genes that encode for nodulation and nitrogen fixation. Finally, a polyphasic study of the isolates was also performed to establish the taxonomic position of the new strains.

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MATERIALS AND METHODS

Isolation from plant nodules. Strains LUP21^T and LUP23 were isolated from root nodules of *L. honoratus* growing in Argentina. Isolations were made according to the method of Vincent (56) with yeast mannitol agar (YMA) (4). The

TABLE 1. Symbiotic characteristics of the strains from this study

Treatment	Nodule location	No. of nodules/plant[a]	Shoot dry matter[b] (g)
Control without N	None	0	0.29
O. lupini LUP21^T	At the intersections of the main and secondary roots	8	0.44
O. lupini LUP23	At the intersections of the main and secondary roots	7	0.42
Bradyrhizobium sp. strain ISLU35	Along secondary roots	14	0.41

[a] The number of nodules per plant was significantly different between the LUP isolates and strain ISLU35 at $P = 0.05$ according to Fisher protected LSD analysis. Both were also significantly different from the control.

[b] The shoot dry matter was not significantly different between the LUP isolates and strain ISLU35 at $P = 0.05$ according to Fisher protected LSD analysis. They were significantly different from the control.

cultures used to inoculate *Lupinus albus* plants were purified from a single colony after 2 days of incubation at 28°C and cultivated on YMA medium.

Nodulation tests. Surface-sterilized seeds of *L. albus* were germinated axenically in petri dishes. Seedlings were transferred to pots with sterile vermiculite and watered with nitrogen-free Rigaud and Puppo (38) nutrient solution. Five plants were inoculated with 1 ml each of a bacterial suspension of LUP21 or LUP23 containing 8×10^8 cells/ml. The inoculated plants were placed for 6 weeks in a plant growth chamber with mixed incandescent and fluorescent lighting (400 microeinsteins m^{-2} s^{-1}; 400 to 700 nm), programmed for a 16-h photoperiod, day-night cycle, with a constant temperature in the range of 25 to 27°C and 50 to 60% relative humidity. The strain *Bradyrhizobium* sp. strain ISLU35, a strain nodulating *Lupinus* in the Canary Islands (12), was used as positive control. As a negative control, uninoculated *L. albus* plants watered with nitrogen-free Rigaud and Puppo solution were used. After 6 weeks, the nodules were counted, and the dry weight of the aerial part of the plants was determined. The data obtained were analyzed by one-way analysis of variance, with the mean values compared by using the Fisher protected least significant difference (LSD) analysis ($P = 0.05$).

Plasmid profile analysis. The isolates were subjected to plasmid profile analysis according to the method of Plazinski et al. (37), except that electrophoresis was done at 2 V cm^{-1} for 90 min, followed by 3 V cm^{-1} for 60 min and finally at 6 V cm^{-1} for 4 h. The 175- and 205-kb plasmids of *Sinorhizobium meliloti* GR4 (48) were used as size markers. Plasmid DNA was capillary transferred to a nylon membrane according to the method of Southern (44) and immobilized by baking at 80°C for 2 h.

***nodD* and *nifH* detection.** Oligonucleotide primers were designed to amplify a conserved fragment of the *nodD* and *nifH* genes among members of the family *Rhizobiaceae* (40). The PCR-amplified fragments of *nifH* and *nodD* genes were sequenced. For use as probes, they were digoxigenin labeled with a DIG DNA Labeling Kit (Roche Diagnostics) according to the manufacturer's instructions. Hybridization was detected with the DIG Nucleic Acid Detection Kit (Boehringer Mannheim) with BCIP (5-bromo-4-chloro-3-indolylphosphate) and nitroblue tetrazolium as substrates for alkaline phosphatase according to the manufacturer's instructions.

DNA extraction and sequence analysis. The 16S rRNA, 23S rRNA, *nodD*, and *nifH* genes were amplified and sequenced as previously described (40, 52). The sequences obtained were compared to those from GenBank by using the BLAST program (1). Sequences were aligned by using the CLUSTAL X software (46). The distances were calculated according to Kimura's two-parameter method (18), and phylogenetic trees were inferred by using the neighbor-joining method (42). Bootstrap analysis was based on 1,000 resamplings. The MEGA2 package (19) was used for all analyses.

Phenotypic characterization. Colony morphology was studied on yeast mannitol (56) and nutrient agars. Cells were Gram stained according to the method of Doetsch (8). For electron microscopy the cells were grown on nutrient agar for 2 days and then stained with 0.2% (vol/vol) uranyl acetate. Thin sections were examined at 80 kV with a Zeiss EM 209 transmission electron microscope. Physiological studies were performed as described in reference 55, and the API 50CH, API 20E, and API 20NE systems were used according to the manufacturer's instructions. Catalase and oxidase activity were tested as described previously (41). Susceptibility to various antibiotics was examined by using penicillin (10 U), ampicillin (2 μg), oxytetracycline (30 μg), neomycin (5 μg), cloxacillin (1 μg), erythromycin (2 μg), cefuroxime (30 μg), ciprofloxacin (5 μg), polymyxin B (300 IU), gentamicin (10 μg), and streptomycin (300 μg) disks (Becton Dickinson) with Antibiotic agar (Oxoid) as the basal medium.

Extraction and analysis of fatty acids. For fatty acid methyl ester (FAME) analysis, strains were grown for 24 h at 28°C on TSA plates containing 30 g of Trypticase soy broth (BBL) supplemented with 15 g of Bacto agar (Difco) liter of distilled water^{-1}. Cells were saponified, and fatty acids were methylated to FAMEs and extracted by using Sherlock Microbial Identification System version 3.0 (MIDI) (31, 29). FAMEs were separated on an Agilent 6890A series gas chromatograph with a 7683 autoinjector and an autosampler tray module (Agilent Technologies). Separation of FAMEs was achieved with a fused-silica capillary column (25 m by 0.2 mm) with cross-linked 5% phenylmethyl silicone (film thickness, 0.33 μm; HP Ultra2). Hydrogen served as carrier gas. Peak integration and identification were performed by using the Hewlett Packard Chemstation and Sherlock software.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins. Strains were grown on nutrient agar (Oxoid CM3) at 28°C for 24 h. Whole-cell protein extracts were prepared and separated by electrophoresis by using small modifications of the procedure of Laemmli (22) as described previously (7).

TP-RAPD patterns. Crude DNA (2 μl) was used as a template for obtaining two-primer–[random(ly) amplified polymorphic DNA] (TP-RAPD) patterns. PCR was performed by using an AmpliTaq Gold reagent kit (Perkin-Elmer Biosystems) according to the manufacturer's instructions. Primers 8F (5′-AGA

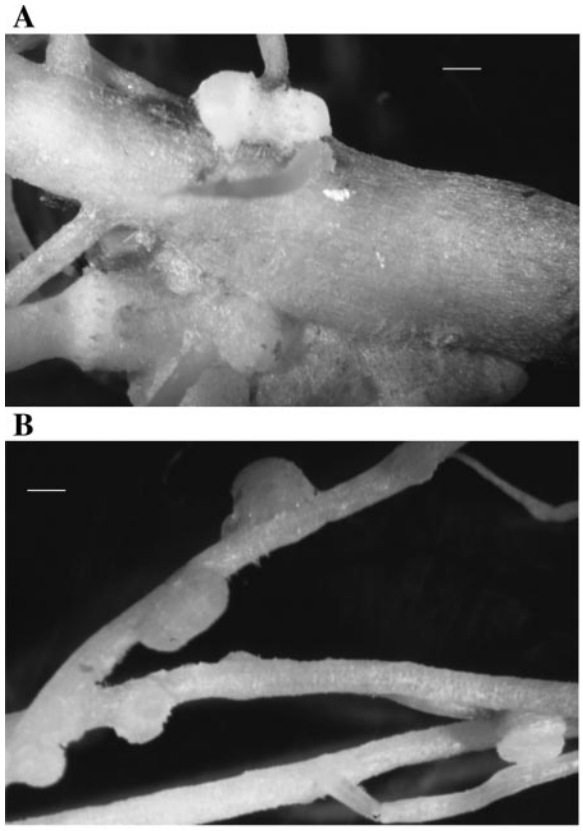

FIG. 1. Nodules induced by strain LUP21^T (A) and *Bradyrhizobium* sp. strain ISLU35 (B) in *L. albus* roots. Bars, 0.2 cm.

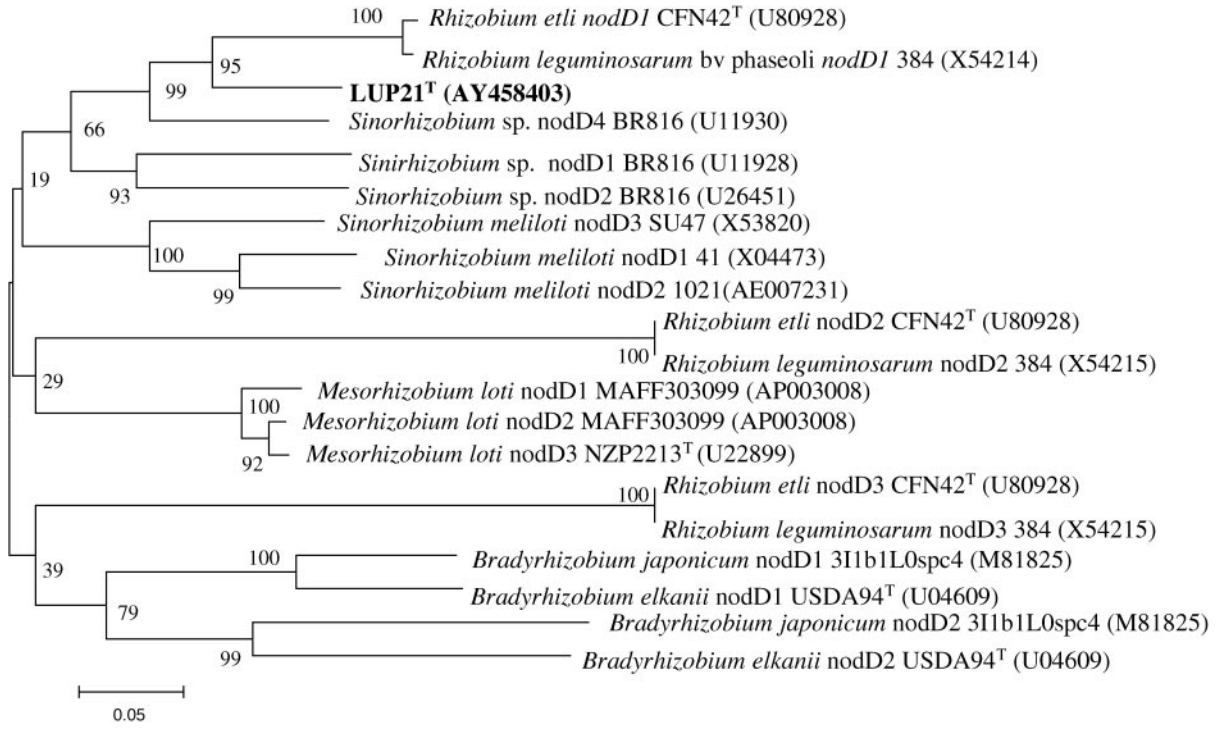

FIG. 2. Comparative sequence analysis of *nodD* gene sequences from strain LUP21^T and representative related strains from GenBank. The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets. The analysis was based on 376 nt. Bar, 5 nt substitutions per 100 nt.

GTTTGATCCTGGCTCAG-3′, *Escherichia coli* positions 8 to 27) and 1522R (5′-GGTTACCTTGTTACGACTT-3′, *E. coli* positions 1509 to 1491) were used at 2 μM final concentration (39). PCR conditions were as follows: preheating at 95°C for 9 min; followed by 35 cycles of denaturing at 95°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 2 min; with a final extension at 72°C for 7 min. The PCR products were separated by electrophoresis on a 1.5% agarose gel in TBE buffer (100 mM Tris, 83 mM boric acid, 1 mM EDTA [pH 8.5]) at 6 V cm^{-1}, stained in a solution containing 0.5 μl of ethidium bromide ml^{-1}, and photographed under UV light. Standard VI (Boehringer-Roche) was used as a size marker. Then, 3 μl of 6× loading solution (40% sucrose and 0.25% bromophenol blue) was added to each sample.

DNA base composition and DNA-DNA hybridization. DNA was extracted by the procedure of Marmur (26). To determine the DNA base composition, DNA was degraded enzymatically into nucleosides (28). The resulting nucleoside mixtures were separated by high-performance liquid chromatography using a Waters Symmetry Shield C8 column at 37°C. The solvent was 0.02 M $NH_4H_2PO_4$ (pH

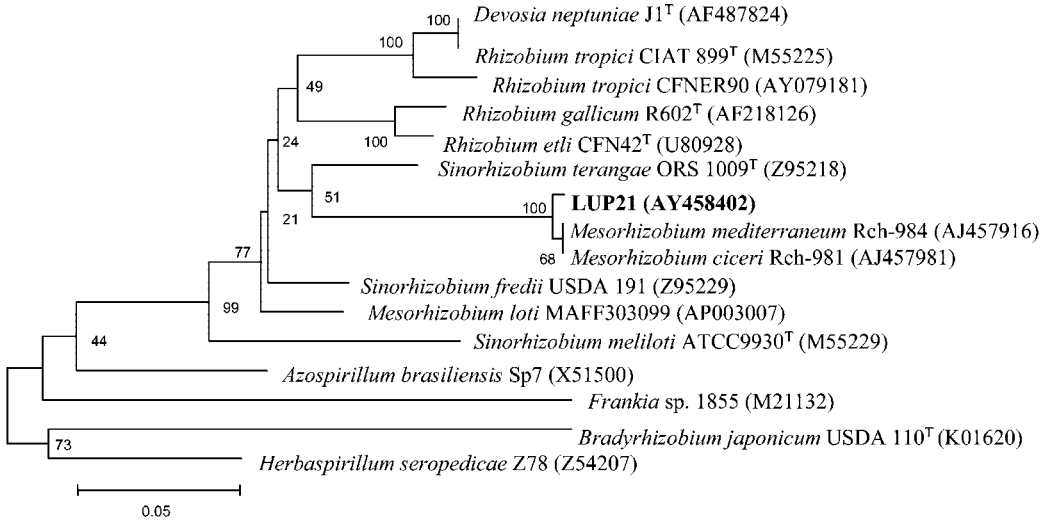

FIG. 3. Comparative sequence analysis of *nifH* gene sequences from strain LUP21^T and representative related strains from GenBank. The significance of each branch is indicated by a bootstrap value for 1,000 subsets. The analysis was based on 438 nt. Bar, 5 nt substitutions per 100 nt.

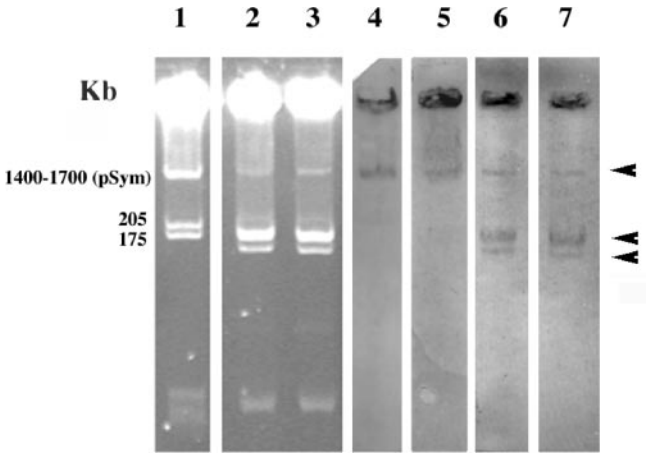

FIG. 4. Plasmid profile in a horizontal 0.7% agarose gel. Lanes: 1, *S. meliloti* GR4; 2, strain LUP21^T; 3, LUP23. The results of hybridization (marked by arrows), using the *nodD* probe, for strain GR4 (lane 4) and strains LUP21^T and LUP23 (lane 6) are also shown. The results of the hybridization using the *nifH* probe for strain GR4 (lane 5) and strains LUP21^T and LUP23 (lane 7) are as indicated.

4.0) with 1.5% acetonitrile. Nonmethylated lambda phage DNA (Sigma) was used as the calibration reference. DNA-DNA hybridizations were performed by using a microplate method modified from Ezaki et al. (9) as described by Willems et al. (60), with hybridizations carried out at 45°C.

RESULTS AND DISCUSSION

Nodulation of *Lupinus*. The fast-growing isolates LUP21^T and LUP23 were able to nodulate *Lupinus albus* plants that had a lower number of nodules than those inoculated with strain ISLU35 used as a positive control. The strains LUP21^T and LUP23 induced a mean of 8 nodules per plant, whereas *Bradyrhizobium* sp. strain ISLU35 used as control formed a mean of 14 nodules per plant (Table 1). This was a significant difference at $P = 0.05$ according to Fisher protected LSD analysis. The morphology of nodules induced by strains isolated in the present study (Fig. 1A) was different from that of nodules induced by strain ISLU35 (Fig. 1B). The nodules induced by the LUP strains were formed at the intersection of the main and secondary roots, whereas those induced by strain ISLU35 were formed along the secondary roots. At 6 weeks postinoculation, no significant differences were observed in the dry matter of plants inoculated with strain ISLU35 and the strains from the present study (Table 1). Significant differences were observed in the dry matter of plants inoculated with the strains from the present study and the negative controls, suggesting that strains LUP21^T and LUP23 are able to fix nitrogen in symbiosis with *L. albus*.

***nodD* and *nifH* amplification and phylogenetic analysis of strain LUP21^T.** The *nodD* and *NifH* genes (accession numbers AY458403 and AY458402, respectively) of strain LUP21^T

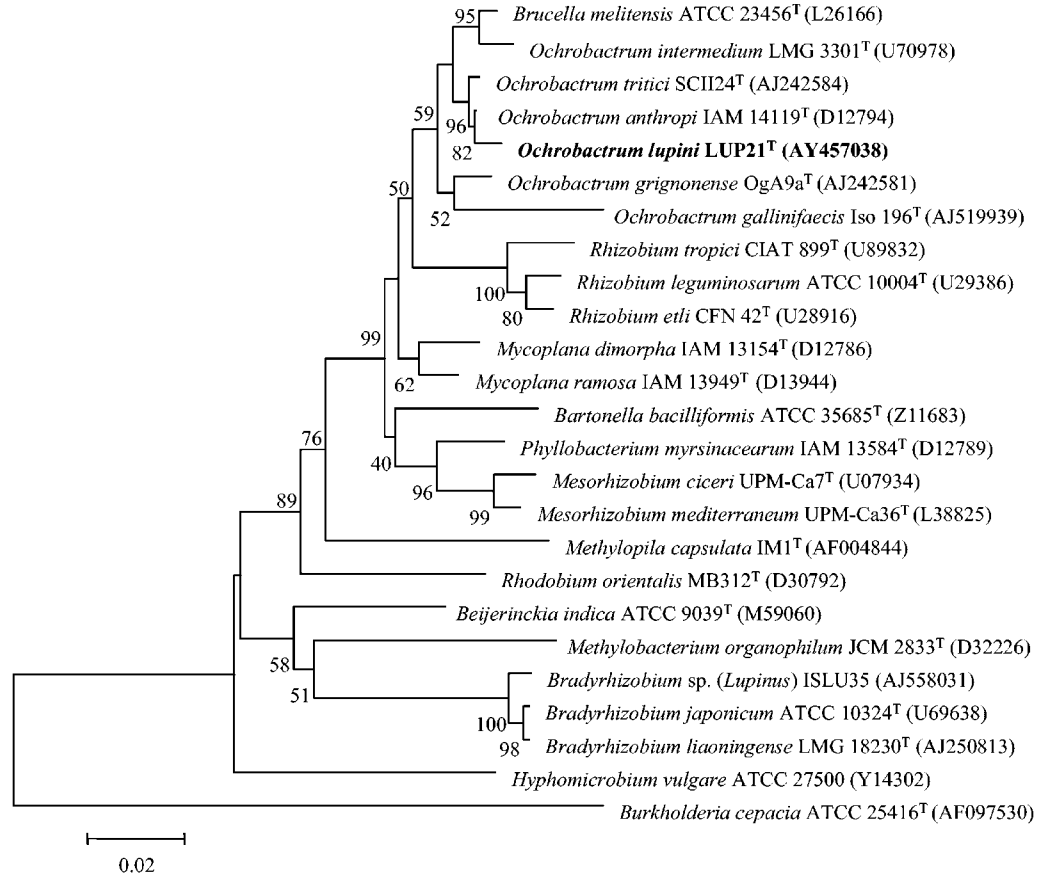

FIG. 5. Comparative sequence analysis of 16S rRNA from strain LUP21^T and representative related strains from GenBank. The significance of each branch is indicated by a bootstrap value for 1,000 subsets. The analysis was based on 1,481 nt. Bar, 2 nt substitutions per 100 nt.

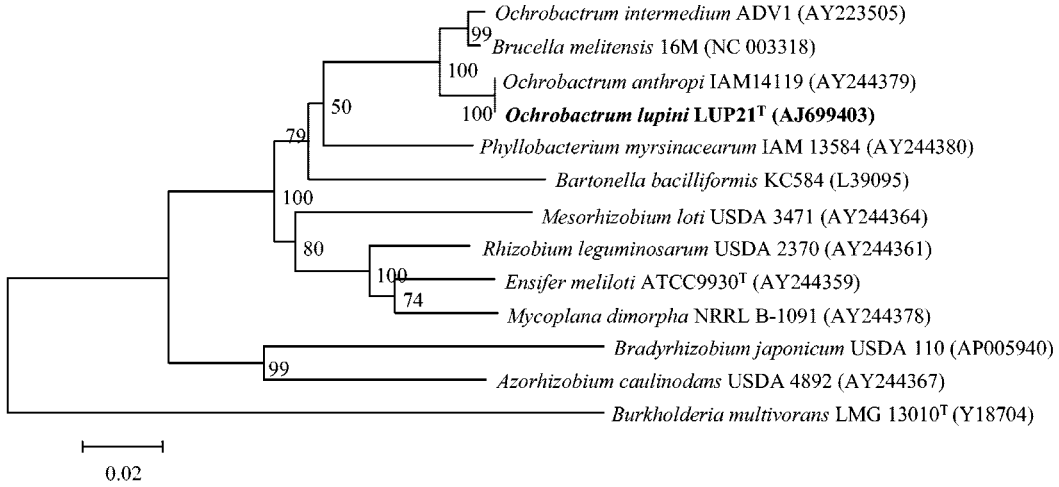

FIG. 6. Comparative sequence analysis of 23S rRNA from strain LUP21^T and representative related strains from GenBank. The significance of each branch is indicated by a bootstrap value for 1,000 subsets. The analysis was based on 1,890 nt. Bar, 2 nt substitutions per 100 nt.

were amplified and sequenced by using specific primers (40). The phylogenetic analysis of the sequences obtained is shown in Fig. 2 and 3. The *nodD* gene sequence of strain LUP21^T showed 86.4% similarity with respect to the *nodD* gene sequence of *Rhizobium etli* CFN42^T. The sequence of the *nifH* gene showed 99.6% similarity with several strains of *Mesorhizobium ciceri* reported to nodulate chickpea in Africa (25). These results differ from those obtained previously based on *nifD* sequences from *Bradyrhizobium* strains that showed a great degree of local divergence (36); nevertheless, additional studies including other rhizobial species from various geographical locations should be performed to establish definitive conclusions about the relation between the phylogeny of *nif* genes and the geographical origin of strains carrying these genes.

Plasmid profile analysis and location of *nifH* and *nodD* genes. In fast-growing species nodulating legumes, the symbiotic genes encoding for nodulation and nitrogen fixation are located on plasmids. These symbiotic plasmids can be transferred in the rhizosphere conferring the ability to nodulate legumes to nonrhizobial species (32, 40, 45). Figure 4 shows the plasmid profile of strains LUP21^T and LUP23 (lanes 2 and 3) carrying a megaplasmid of more than 1,700 kb (plasmid A) and two megaplasmids of ca. 200 kb (plasmid B) and 150 kb (plasmid C), respectively. The probes used allowed the detection of both *nodD* and *nifH* genes in the three plasmids carried by the strains isolated in the present study (Fig. 4, lanes 6 and 7).

rRNA sequencing and analysis. A single colony of each strain was used for all molecular analyses. The complete 16S rRNA sequences of strains LUP21^T (accession number AY457038) and LUP23 were obtained before and after their reisolation from *Lupinus* nodules to check the purity of the strains. They were also compared to sequences from the public databases. The 16S rRNA sequence of strain LUP23 showed 100% identity with that of the strain LUP21^T. Isolates LUP21^T and LUP23 were tentatively identified as members of the genus *Ochrobactrum* after a comparison with the sequences deposited in the GenBank. This genus is located in the order *Rhizobiales* (α_2 subclass of *Proteobacteria*), in the family *Brucellaceae*, and does not contain nitrogen fixing, legume-nodulating species (Fig. 5). The closest relatives found were *O. anthropi* with 99.5% sequence similarity, followed by *O. tritici* with 99.3% sequence similarity.

The results of 16S rRNA sequencing clearly showed that the strains from the present study do not belong to the rhizobial genera described until now. However, it was recently reported that fragments of 16S rRNA may be transferred among bacteria, including rhizobia, by lateral gene transfer, creating mosaic genes in which different segments have affinities to different relatives (51, 57), obscuring classification on the basis of

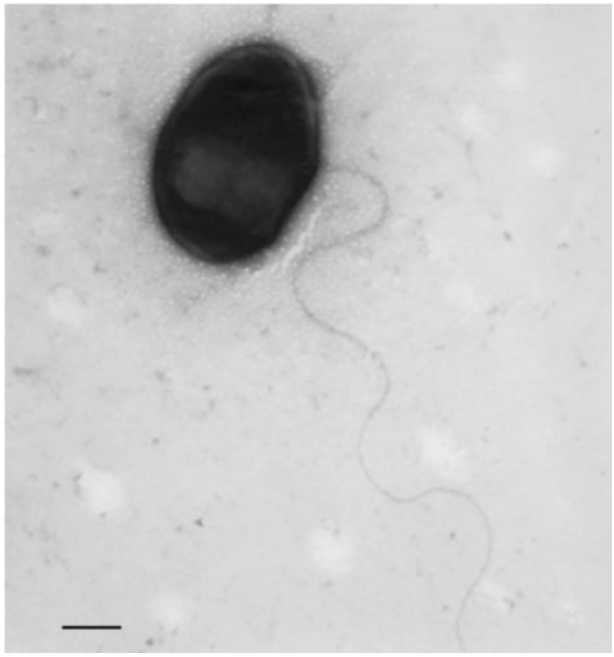

FIG. 7. Transmission electron micrograph of strain LUP21^T grown in nutrient agar for 48 h. Bar, 0.7 μm.

TABLE 2. Differentiating physiological characteristics[a]

Parameter	Physiological characteristics of strain[a]:												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Reference	This study	This study	11	23	23	54	17	20, 61, 62	20, 62	34, 35	55	43, 63	27, 63
Type of flagellation	Polar	Polar	Subpolar or peritrichous	Subpolar or peritrichous	Subpolar or peritrichous	Polar	Nonmotile	Polar or subpolar	ND	ND	ND	ND	Subpolar or peritrichous
Incubation time at 28°C in YMA or TSA	12–24 h	12–24 h	12–24 h	12–24 h	12–24 h	12–24 h	12–24 h	4–7 days	6–10 days	3–5 days	4–5 days	2–3 days	1–2 days
Growth at 40°C	w	w	v	+	+	ND	ND	−	−	+	+	−	+
Growth at pH 10	+	+	ND	−	−	ND	ND	−	−	+	−	−	−
Growth in 2% NaCl	+	+	ND	ND	ND	ND	ND	−	−	+	+	−	+
β-Galactosidase	−	−	−	−	−	−	−	−	−[c]	−	−	+	+
Urease	+	+	+	−	+	+	ND	ND	ND	ND	ND	+	+
Nitrate reduction	−	−	+	+	+	+	ND	+	ND	ND	ND	ND	+
Arginine dehydrolase	−	−	ND	−	−	−	ND	ND	ND	ND	ND	ND	−[f]
Esculin	+	+	−	+[b]	−	v	−	v	−	ND	ND	ND	+[f]
Assimilation of:													
L-Arabinose	+	+	+	+	−	−	+	+	+	+	+	+	+[f]
Adipate	−	+	v	−	−	−	−	ND	ND	ND	ND	ND	+[f]
Citrate (24h)	+	+	−	−	−	+	−	v	v	ND	ND	−[e]	V[h]
Phenyl-acetate	+	−	−	−	−	−	−	ND	ND	ND	ND	ND	−[f]
N-Acetylglucosamine	+	+	+	−[b]	+	+[b]	−	ND	ND	+	+	ND	+[f]
D-Mannose	+	+	+	+	−	v	+	+	+	+	+	+	+[f,g]
Maltose	+	+	+	−	+	−	−	+	−	+	+	+[g]	+
Rhamnose	+	+	+	+	+	−[b]	−	+	v	+	+	ND	ND
Mannitol	+	+	+	−	+[c]	−	ND	+	v	+	+	+	+
D-Turanose	−	−	+	+	+	ND	ND	+	v	+	+	ND	ND
Melibiose	+	+	−	+	−	−	−	v	−	+	+	ND	ND
D-Arabitol	−	−	+	+	+	ND	ND	+	v	+	+	+[i]	v[j]
Antibiotic resistance													
Chloramphenicol	S-I	S-I	R	S-I	I	R	ND	I	I	R	S	S[j]	R[k]
Polymyxin B (300 IU)	R	R	S	R	S	v	ND	R[d]	ND	R	S	S[g]	R[f,g]
G+C content (mol%)	56.8	57	56–59	58	59	57–59	ND	61–65	60–64	63–64	63–64	59–64	59–64

[a] Strains: 1, *O. lupini* LUP21^T (LMG 22726^T); 2, *O. lupini* LUP23 (LMG 22727); 3, *O. anthropi*; 4, *O. grignonense*; 5, *O. tritici*; 6, *O. intermedium*; 7, *O. gallinifaecis*; 8, *B. japonicum*; 9, *B. liaoningense*; 10, *M. ciceri*; 11, *M. mediterraneum*; 12, *Rhizobium etli*; 13, *R. tropici*. +, Positive; −, negative; v, variable; w, weak; ND, no data; S, sensitive; R, resistant; I, intermediate.

[b] Kämpfer et al. (17).

[c] This study.

[d] Yao et al. (62).

[e] Amarger et al. (2) and Wei et al. (59).

[f] Zurdo-Piñeiro et al. (64).

[g] van Berkum et al. (49).

[h] Amarger et al. (2).

[i] Wei et al. (59).

[j] Segovia et al. (43) and Wei et al. (54).

[k] Martinez-Romero et al. (27) and Wei et al. (59).

[l] Vincent (56).

TABLE 3. Fatty acid profile of strains LUP21^T and LUP23 compared to those of the remaining species from the genus *Ochrobactrum* and to those of the representative rhizobial species

Fatty acid	Fatty acid profile compared to strain[a]:												
	1[b]	2[b]	3[b]	4[b]	5[b]	6[b]	7[c]	8[d]	9[d]	10[d]	11[d]	12[d]	13[d]
10:0 3OH		0.3											
11:0 ISO 3OH			1.5										
12:0 2OH	0.5	0.2											
12:0 3OH	0.8	0.5						<0.1		0.3	0.1		
13:0 iso 3OH										0.2			
14:0		0.2					0.7	<0.1			0.4		
15:0 2OH												0.7	
15:0 iso 3OH													3.4
16:0	4.2	5.5	7.2	2.7	3.9	2.5	8.9	12.0	14.8	13.4	10.3	2.5	7.7
16:0 3OH	0.7	0.4										1.2	3.9
16:1 ω5c								1.1	0.9				
17:0	1.6	1.1	1.6	1.6	0.9	2.3		0.2	0.8	1.3	1.7	0.3	<0.1
17:0 iso										4.2	4.2		0.9
17:0 iso 3OH													0.3
17:0 cyclo										0.8	0.9		0.7
17:1ωw6c	1.4	1.2		0.4		1.1		0.2	0.7				
17:1ω8c				0.2				0.5	1	0.2	0.5	<0.1	
18:0	3.0	2.6	5.2	5.4	3.5	3.2	3.7	0.6	0.4	3.3	4.2	8.7	2.9
18:0 3OH	1.0	0.8		0.5	1.9								
18:1 2OH	6.2	13.4	2.4	2.6	12.9	3.9	1.5						0.8
18:1ω7c	70.8	59.5	62.8	68.3	68.6	55.8	28.8						
11 methyl 18:1ω7c								1.9		11.9	5.6	1.5	0.6
19:0 10 methyl	0.7	0.8								0.2	0.1	<0.1	
19:0 cyclo ω8c	4.3	8.3	14.5	16.4	6.3	30.5	47.2	1.2	5.0	37.3	33.4	10.2	49.3
20:1ω9t										0.1	0.5	1	
20:1ω7c				0.4									
20:2ω6,9c									0.1	1	0.8		0.4
20:3ω6,9,12c												2	1.8
Summed feature 1[f]	0.5	0.2											
Summed feature 2[g]	1.9	1.4	1.6		0.6							6.0	2.3
Summed feature 3[h]	2.3	3.2	2.1	1.5	1.4	0.8	3.7	1.1	0.4	0.3		0.2	0.2
Summed feature 7[i]								81.2	75.5	24.7	35.8	66.2	24.6
Unknown 11.799		0.4											
Unknown 18.814			1.2										

[a] Strains: 1, *O. lupini* LUP21^T (LMG 22726^T); 2, *O. lupini* LUP23 (LMG 22727); 3, *O. anthropi* LMG 3331^T; 4, *O. grignonense* LMG 18954^T; 5, *O. tritici* LMG 18957^T; 6; *O. intermedium* LMG 3301^T; 7, *O. gallinifaecis* DSM 15295^T; 8, *B. japonicum*; 9, *B. liaoningense*; 10, *M. ciceri*; 11; *M. mediterraneum*; 12, *R. etli*; 13, *R. tropici.*
[b] Data from this study.
[c] Kämpfer et al. (17).
[d] Tighe et al. (47).
[e] Fatty acid with values lower than 0.1% are not shown.
[f] Summed feature 1: 13:0 OH/15:1 iso I/15:1 iso H.
[g] Summed feature 2: 12:0 unknown aldehyde with ECL 10.928/16:1 iso I/14:0 3OH.
[h] Summed feature 3: 16:1 ω7c/15:0 iso 2OH.
[i] Summed feature 7: 18:1 ω7cis/ω9trans/ω12trans, 18:1ω7cis/ω9cis/ω12trans.

this molecule. The 16S rRNA sequence of the strains isolated in the present study notably differs from all rhizobial species along the complete gene. For example, with respect to *R. leguminosarum* ATCC 10004^T and *B. japonicum* LMG 6138^T, variations are located along the complete 16S rRNA gene showing differences in 80 and 151 nucleotides (nt), respectively. These observations exclude the possibility of horizontal transfer of a 16S rRNA fragment from *Ochrobactrum* to the strains from the present study. Nevertheless, a partial sequence of the 23S rRNA gene (1,890 nt, corresponding to *E. coli* positions 208 to 2233 [10]) was obtained to confirm the results obtained by 16S rRNA sequencing. The sequences of strains LUP21^T (accession number AJ699403) and LUP23 were identical. A comparison with the sequences held in the GenBank showed that the sequence obtained was 99.8% similarity to that of *O. anthropi* LMG 3331^T. Figure 6 shows the phylogenetic location of strain LUP21^T within the family *Brucellaceae*. This result confirms that the isolates from the present study belong to the genus *Ochrobactrum* and are distinct from rhizobial genera currently reported.

Very recently a strain classified in the genus *Ochrobactrum* (*Ochrobactrum* sp. strain DASA 35030) was reported as an endosymbiont of *Acacia mangium*; however, its taxonomic status within the genus was not fully established (33). When the deposited 16S rRNA sequence of strain DASA 35030 with 1,151 nt was compared to the sequence of LUP21^T, the similarity found was to be 93.1%.

Phenotypic characterization. Strains LUP21^T and LUP23 were gram-negative short-rods and motile by a polar flagellum (1.4 to 1.5 μm by 0.2 to 0.4 μm). Figure 7 shows the cell morphology of isolate LUP21^T as observed by transmission electronic microscope. Cells form white mucoid colonies on YMA medium. The generation times were 3 to 4 h in YMB, and both strains were catalase and oxidase positive.

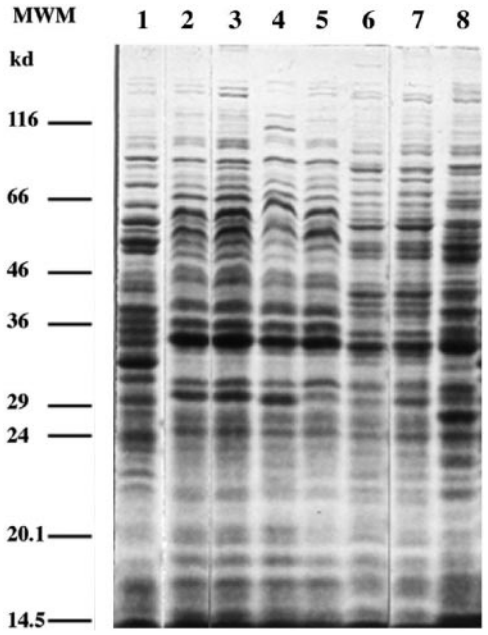

FIG. 8. SDS-PAGE protein profiles of *O. grignonense* LMG 18954^T (lane 1), *O. lupini* LUP21^T (lane 2), LUP23 (lane 3), *O. anthropi* 3331^T (lane 4), *O. anthropi* LMG 371 (lane 5), *O. tritici* LMG 18957^T (lane 6), *O. tritici* MG 18958 (lane 7), and *O. intermedium* LMG 3301^T (lane 8). Molecular weight markers (MWM): lysozyme, 14.5 kDa; trypsin inhibitor, 20.1 kDa; trypsinogen, 24 kDa; carbonic anhydrase, 29 kDa; GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 36 kDa; egg albumin, 45 kDa; bovine albumin, 66 kDa; β-galactosidase, 116 kDa.

The phenotypic characteristics of strains LUP21^T and LUP23 were compared to those of other *Ochrobactrum* species and various representatives from rhizobial genera. Differentiating physiological characteristics are presented in Table 2. According to the data, the strains from the present study clearly differ from those representing the genera *Rhizobium*, *Mesorhizobium*, and *Bradyrhizobium*. Isolates LUP21^T and LUP23 showed a growth rate similar to that of other *Ochrobactrum* species, which is faster than rhizobial strains. LUP21^T and LUP23 differed from their phylogenetic closest neighbors, *O. anthropi* and *O. tritici*, in nitrate reduction and D-turanose and D-arabitol assimilation (which were negative for strains LUP21^T and LUP23) and in esculin hydrolysis and melibiose and citrate assimilation (which were positive for these strains). In addition to these differences, the strains from the present study also differed from *O. anthropi* in resistance to polymyxin B, which was positive for strains LUP21^T and LUP23, and from *O. tritici* in their ability to assimilate mannose and L-arabinose. The differences with respect to the remaining *Ochrobactrum* species and representative strains of *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium* are recorded in Table 2.

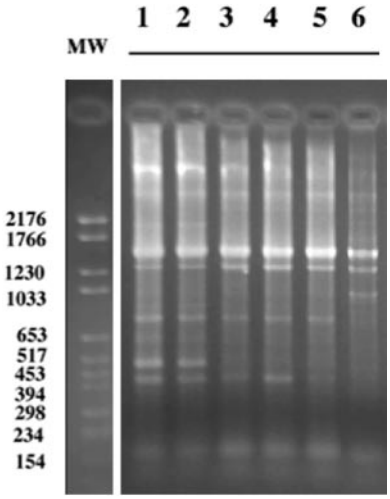

FIG. 9. TP-RAPD profiles of *O. lupini* LUP21^T (lane 1), *O. lupini* LUP23 (lane 2), *O. anthropi* LMG 3331^T (lane 3), *O. anthropi* LMG 371^T (lane 4), *O. anthropi* LMG 33 (lane 5), *O. tritici* LMG 18957^T (lane 6). MW, Standard VI of Boehringer-Roche.

Fatty acid analysis. The cellular fatty acid patterns of strains LUP21^T and LUP23 and other *Ochrobactrum* and rhizobial strains are recorded in Table 3. The profile is dominated for all *Ochrobactrum* strains by $C_{18:1\omega7c}$ which makes up 55 to 70% of the fatty acid methyl esters. Also present in all *Ochrobactrum* strains tested, in quantities of more than 2% are the straight chain fatty acids $C_{16:0}$ and $C_{18:0}$, the branched chain $C_{18:1\ 2OH}$ and the cyclic fatty acid $C_{19:0\ cyclo\ \omega8c}$. The hydroxy fatty acid $C_{18:1\ 2OH}$, present (at least 2%) in all *Ochrobactrum* strains tested, is not present in significant amounts in the genera *Bradyrhizobium*, *Mesorhizobium*, *Sinorhziobium*, or *Rhizobium* (13, 47). The overall fatty acid profiles of the new isolates support their inclusion in the genus *Ochrobactrum*.

SDS-PAGE of whole-cell proteins. The protein profiles (Fig. 8) for strains LUP21^T and LUP23 were nearly identical, indicating that both strains most probably belong to the same species. Their protein profile was similar to that of *O. anthropi* LMG 3331^T but had some differences in the higher-molecular-weight part of the profile. It had some more differences with the protein pattern of *O. tritici* LMG 18957^T and was clearly different from that of *O. intermedium* LMG 3301^T and *O. grignonense* LMG 18954^T. These results are in line with the fact that, also according to 16S rRNA sequence similarities, *O. anthropi* (and *O. tritici*) are the nearest neighbors of the *Lupinus* isolates.

TP-RAPD patterns. In a previous study we showed that TP-RAPD patterns allow the differentiation among rhizobial species (39) and that, within a species, the TP-RAPD pattern is identical for different strains. Figure 9 shows the TP-RAPD patterns of the strains isolated in the present study compared to those of *O. anthropi* and *O. tritici*. As expected, strains LUP21^T and LUP23 showed the same pattern (Fig. 9, lanes 1 and 2), which differed from the patterns of *O. anthropi* (lanes 3, 4, and 5) and *O. tritici* (lane 6). Nevertheless, the profiles of LUP21^T and LUP23 were closer to that of *O. anthropi* since only one difference between these profiles was found.

DNA-DNA hybridization. The results of DNA-DNA hybridization were 95% between strain LUP21^T and LUP23. Hybridization of these strains with labeled DNA of *O. anthropi* LMG 3331^T resulted in 68 and 65% reassociation, respectively. The second strain, *O. anthropi* LMG 371 confirmed this observation, with 65% hybridization with LUP21^T and LUP23. Both *O. anthropi* strains showed 86% hybridization. In light of the recommendation of a threshold value of 70% DNA-DNA similarity for definition of a species (58), these results indicate that strains LUP21^T and LUP23 can be regarded as a separate

genospecies, distinct from *O. anthropi*, although closely related.

In conclusion, the 16S rRNA and 23S rRNA sequences demonstrate that the isolates from *Lupinus* described here are phylogenetically unrelated to the rhizobia and represent a new species of the family *Brucellaceae*. To our knowledge, this is the first report of a member of this family with the ability to nodulate the legume *Lupinus*. The high similarity of the *nodD* and *nifH* sequences of strain LUP21^T to the corresponding genes of rhizobial species suggests that this strain may have acquired these symbiotic genes by horizontal transfer from rhizobia. This new finding of a nonrhizobial species nodulating legumes is in line with recent reports of other new species outside the rhizobia, in the genera *Devosia* (40, 41) *Methylobacterium* (16, 45), *Ralstonia* (6), *Blastobacter* (50), *Ochrobactrum* (33), and *Burkholderia* (32, 53). It demonstrates the need for more studies on the diversity of bacteria nodulating legumes from both tropical and temperate soils.

On the other hand, the genotypic, phenotypic, and chemotaxonomic data presented here support the classification of strains LUP21^T and LUP23 as new species of the genus *Ochrobactrum* for which the name *O. lupini* sp. nov. is proposed with isolate LUP21^T representing the type strain. The strains were deposited in the LMG culture collection as LMG 22726^T and LMG 22727, respectively.

Description of *Ochrobactrum lupini* sp. nov. *Ochrobactrum lupini* (lu.pi′ni. N.L. masc. *lupinus* legume, N.L. gen. nov. *lupini* of the legume, referring to the isolation source of this microorganism, nodules of *L. albus*).

Cells are nonmotile, non-spore-forming, gram-negative rods. Good growth occurs on yeast-mannitol agar and nutrient agar at 25 to 30°C. Colonies on these media are white to beige mucoid with entire edges and have a diameter of between 2 and 3 mm. The two strains are oxidase and catalase positive. The fatty acid profiles were mainly composed of $C_{18:1\omega7c}$ (50 to 70%), $C_{16:0}$ (4 to 5%), and $C_{18:0}$ (2 to 3%). Carbon source utilization includes L-arabinose, citrate, erythritol, D-fucose, L-fucose, gluconate, 2-ketogluconate, 5-ketogluconate, glucose, *N*-acetylglucosamine, lactose, D-mannose, maltose, mannitol, rhamnose, melibiose, and L-xylose. The following carbon sources are not assimilated: amygdalin, D-arabinose, cellobiose, ducitol, D-fructose, galactose, glycerol, glycogen, inositol, inulin, melezitose, α-methyl-D-glucoside, α-methyl-D-mannoside, D-raffinose, salicin, sorbitol, L-sorbose, trehalose, and turanose. Both strains hydrolyze esculin and urease. Various physiological differences between the two strains and other *Ochrobactrum* species are given in Table 2. The G+C contents of strains LUP21^T and LUP23 were 56.8 and 57 mol%, respectively. The strains nodulate *L. honoratus* and *L. albus*. The type strain is LUP21^T (LMG 22726^T).

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