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Novel Alphaproteobacterial Root Nodule Symbiont Associated with *Lupinus texensis*▽

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Phylogenetic analysis of rRNA gene, *recA*, *nodA*, *nifD*, and *nifH* sequences suggested that nitrogen-fixing symbionts from two populations of *Lupinus texensis* acquired the capacity for nodule symbiosis separately from other rhizobia in the alphaproteobacteria. Their closest 16S rRNA relatives were the nonsymbiotic taxa *Chelatococcus*, *Bosea*, and *Balneomonas*.

Legume nodule symbionts are found in several lineages in the alphaproteobacteria and at least two lineages in the betaproteobacteria (5, 17). Four genera of alphaproteobacteria (*Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Bradyrhizobium*) constitute the predominant symbionts for most legume species in habitats throughout the world. However, additional nodule-forming lineages of alphaproteobacteria that may have a more restricted geographic distribution or limited host range have been discovered in recent years (8, 14, 16, 22, 28, 31, 32). This suggests that further sampling of other host taxa and locations is likely to uncover additional lineages of nodule bacteria.

The legume genus *Lupinus* includes approximately 275 species of herbs and shrubs (11). Studies of most *Lupinus* species within their native range (4, 15, 21, 24), as well as outside their native distribution (6, 26, 30), indicate that strains of *Bradyrhizobium* are the predominant nodule symbionts. We sampled nodule bacteria in the native range of *Lupinus texensis* in Texas (13) and found that none of the isolates resembled *Bradyrhizobium* symbionts associated with other *Lupinus* species. Twenty-eight root nodules were collected from multiple plants at two sites 20 km apart (Williamson County and Travis County), and one bacterial isolate per nodule was obtained as previously described (25). DNA purification and PCR experiments used standard protocols (5). Amplification of the 5′ intervening sequence region of the 23S rRNA gene (27) indicated that all 28 isolates had a unique size variant (473 bp) that differed from length variants found in North American *Bradyrhizobium* strains (510 or 537 bp) (18, 20, 21). A 431-bp 5′ portion of the 23S rRNA gene was sequenced in five Texas isolates (GenBank accession numbers EF191402 to EF191406), and each sequence was the same. Blast searches indicated that this sequence was distantly related to all currently known lineages of legume nodule symbionts. The closest match among rhizobia was *Bradyrhizobium japonicum* USDA 110 (80% similarity). Other genera of alphaproteobacterial and betaproteobacterial symbionts had similarities ranging from 62 to 77%.

To screen the remaining *L. texensis* nodule isolates, PCR assays were performed with a primer pair (LUTf1 and 23LUTr2; Table 1) matching unique portions of the *L. texensis* symbiont 5′ 23S rRNA sequence. All isolates yielded the same 167-bp amplification product. Other genera of nodule bacteria (*Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Rhizobium*) failed to yield an amplification product with these primers.

A nearly full-length portion of the 16S rRNA gene (1,408 bp) was sequenced for two isolates (Lut5, Lut6). Both isolates had the same sequence (EF191407, EF191408). The maximum-likelihood (ML) tree showed that the *L. texensis* symbionts formed a lineage well separated from traditionally recognized genera of rhizobia (Fig. 1). The *L. texensis* symbionts were grouped along with a set of ecologically diverse taxa (*Chelatococcus*, *Balneomonas*, and *Bosea thiooxidans*) with high bootstrap support (96 to 100%). These taxa are not known to form symbiotic relationships with legumes. *Chelatococcus asaccharovorans* has been isolated from soil and water in various locations (1, 10). *Balneomonas flocculans* was collected from a hot spring in Japan (29). *Bosea thiooxidans* was isolated from agricultural soils in India (7). A second *Bosea* strain (ORS1414), found as a commensal inhabitant of legume nodules in Tunisia (although not capable of inducing nodule formation [36]), was also grouped in this lineage.

Two nodule symbionts considered to be members of the genus *Methylobacterium* were also clustered in the group with the *L. texensis* symbionts and *Chelatococcus*, *Balneomonas*, and *Bosea thiooxidans* (Fig. 1). Strain WSM 3686 was isolated from the legume *Lotononis* in Zambia (DQ838529), and strain AC72a was sampled from *Phaseolus vulgaris* in Ethiopia (35). However, the identity of these two strains on the genus level is uncertain. Five species in the genus *Methylobacterium* that were included in the phylogenetic analysis formed a well-supported group (100% bootstrap value) that was clearly distinct from the lineage encompassing strains WSM 3686 and AC72a and the *L. texensis* symbionts (Fig. 1).

These results leave unresolved the proper genus name of the *L. texensis* nodule symbionts. The *L. texensis* symbionts had a 16S rRNA sequence closely affiliated with those of nonsymbiotic species classified into three separate genera (*Balneomonas flocculans*, 95.5% similarity; *Bosea thiooxidans*, 96.5% similarity; *Chelatococcus asaccharovorans*, 97.8% similarity). Because

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TABLE 1. Oligonucleotide primers used in this study

Primer	5′→3′ nucleotide sequence	Product size (bp)	Target gene
LUTf1	CGGAGGGTCAGATGAAGGGATTA	167	23S rRNA
23LUTr2	GGTTCCGATGACGATCAGGTTGG		
recAf	GGCAGTTCGGCAAGGGCTCGAT	529	*recA*
recAr	ATCTGGTTGATGAAGATCACCAT		
nifD.f1	TCGGACTTCCAGGAAAAGGACAT	521	*nifD*
nifD.r2	CCGWACTTYTCTTCCATGTGGC		
nifH.f2	TAYGGNAARGGGGGGATYGGYAAGTC	437	*nifH*
nifH.r3	TCGCCGGACATGACGATGTAGAT		
LUT5af	AGAAAATAGCAAGAAAGAGGAGGT	619	*nodA*
LUTar1	AAHATGGATKRGGACCGTCGTC		

there was high bootstrap support for the placement of these taxa in a lineage distinct from *Methylobacterium* (Fig. 1), it appears to be inappropriate to classify the *L. texensis* symbionts in that genus.

Lateral transfer of portions of the 16S rRNA gene can be a potential source of phylogenetic distortions, by creating mosaic genes where different segments have different ancestries (33). However, several analyses failed to detect evidence for this as a factor affecting inferences about *L. texensis* symbiont 16S rRNA relationships. First, analysis of 16S rRNA sequences with GENECONV (23) did not identify any mosaic structure of the *L. texensis* symbiont 16S rRNA gene. Second, a split decomposition analysis (3) with SplitsTree (12) yielded a strictly dichotomously branching tree. Thus, there was no evidence for multiple alternative pathways of relationship indicative of mosaic 16S rRNA gene structure in the *L. texensis* symbionts. Finally, when 16S rRNA data were divided into two equal portions, both the 5′ and 3′ halves resulted in trees that grouped the *L. texensis* symbionts with *Chelatococcus*, *Balneomonas*, and *Bosea thioxidans* and placed this group apart from all other rhizobial genera, as in Fig. 1. Thus, substitutions throughout different portions of the 16S rRNA gene supported the same conclusions about relationships of the *L. texensis* symbionts.

A 484-bp portion of the *recA* gene was sequenced in three isolates of the novel *L. texensis* symbionts. Two isolates (Lut5, Ltg2) had identical sequences, which differed by 96 bp from the sequence of isolate Lut6 (EF191415 to EF191417). Blast searches did not identify close matches to either sequence among known genera of nodule bacteria (or other bacteria). Comparison of the Ltg2/Lut5 *recA* sequence with those of 16 taxa of other rhizobia (from five genera, i.e., *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*) showed similarity percentages that ranged from 71% (*B. japonicum* USDA 110) to 76% (*Rhizobium gallicum*). Percent *recA* similarity for strain Lut6 ranged from 71% (*B. japonicum* USDA 110) to 76% (*R. leguminosarum*). *recA* sequences are not currently available for key taxa identified as relatives of the *L. texensis* symbionts based on the 16S rRNA data (*Balneomonas*, *Chelatococcus*, *Bosea thiooxidans*), so the specific topology of Fig. 1 cannot be confirmed with these data. Nevertheless, the results provide further evidence that the *L. texensis* symbionts are not closely related to known nodule bacteria.

A complete sequence for the *nodA* gene (591 bp; Table 1 contains the primers used for amplification) was obtained for two of the novel *L. texensis* isolates. Both isolates had the same sequence (EF191413, EF191414). Because the origin of *nod* genes is not well resolved, an unrooted tree was used to depict relationships (Fig. 2). The tree topology indicated that the novel *L. texensis* symbiont formed a well-supported branch with *Mesorhizobium plurifarium* strains that were isolated from *Acacia tortilis* in Africa (2). These results imply that the *L. texensis* strains evolved to become legume nodule symbionts independently of the most closely related rhizobial taxon in the 16S rRNA tree (*Methylobacterium nodulans*). This conclusion is based on the observation that all of these taxa have a closer 16S rRNA relationship to nonsymbiotic taxa than they do to one another (Fig. 1) and the fact that *M. nodulans* and the *L. texensis* symbionts do not cluster as each other's closest relatives in the *nodA* tree (Fig. 2).

Partial sequences of the *nifD* (476 bp) and *nifH* (388 bp) genes were obtained for two isolates of the novel *L. texensis* symbionts, and for both genes, the two isolates proved to be identical (EF191409 to EF191412). The ML tree topologies for both the *nifD* and *nifH* genes were broadly similar but differed considerably from the ribosomal and *nodA* trees. Phylogenetic analysis of the partial *nifD* sequences (Fig. 3) showed that the *L. texensis* symbionts formed a group with *Rhizobium*, *Mesorhizobium*, and *Sinorhizobium* (92 to 97% bootstrap values). The *nifH* tree topology (not shown) also placed the novel *L. texensis* nodule bacteria together with *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium* with high bootstrap support (86 to 92%). For both trees, *Bradyrhizobium* formed a well-separated lineage. A partition homogeneity test (9) indicated that both *nif* tree topologies were highly incongruent with the 16S rRNA tree ($P < 0.001$). To eliminate the possibility that these *nif* sequences came from another bacterial strain that somehow became mixed with the *L. texensis* strain cultures, a spontaneous streptomycin-resistant mutant was obtained for strain Lut5 and its partial *nifD* sequence was determined. The mutant had a *nifD* sequence identical to that of the Lut5 parent strain, and its 5′ 23S rRNA PCR product (obtained with lineage-specific primers LUTf1 and 23LUTr2) was also identical. Thus, there was no evidence that the parent strain was a mixed culture.

These results suggest that the novel *L. texensis* symbionts may have acquired their *nif* genes from a phylogenetically distant source through lateral transfer. Partition homogeneity tests also indicated that there was significant incongruence of both *nifD* and *nifH* phylogenetic trees from the *nodA* tree ($P < 0.027$ and $P < 0.003$, respectively).

Symbiotic behavior. Inoculation experiments with *L. texensis* symbionts used standard protocols (34), and no nodules formed on uninoculated control plants in any experiment. When *L. texensis* plants were inoculated with three isolates (Lut3, Lut5, Lut6), all plants formed nodules (mean = 8 to 11 nodules per plant), and acetylene reduction assays indicate

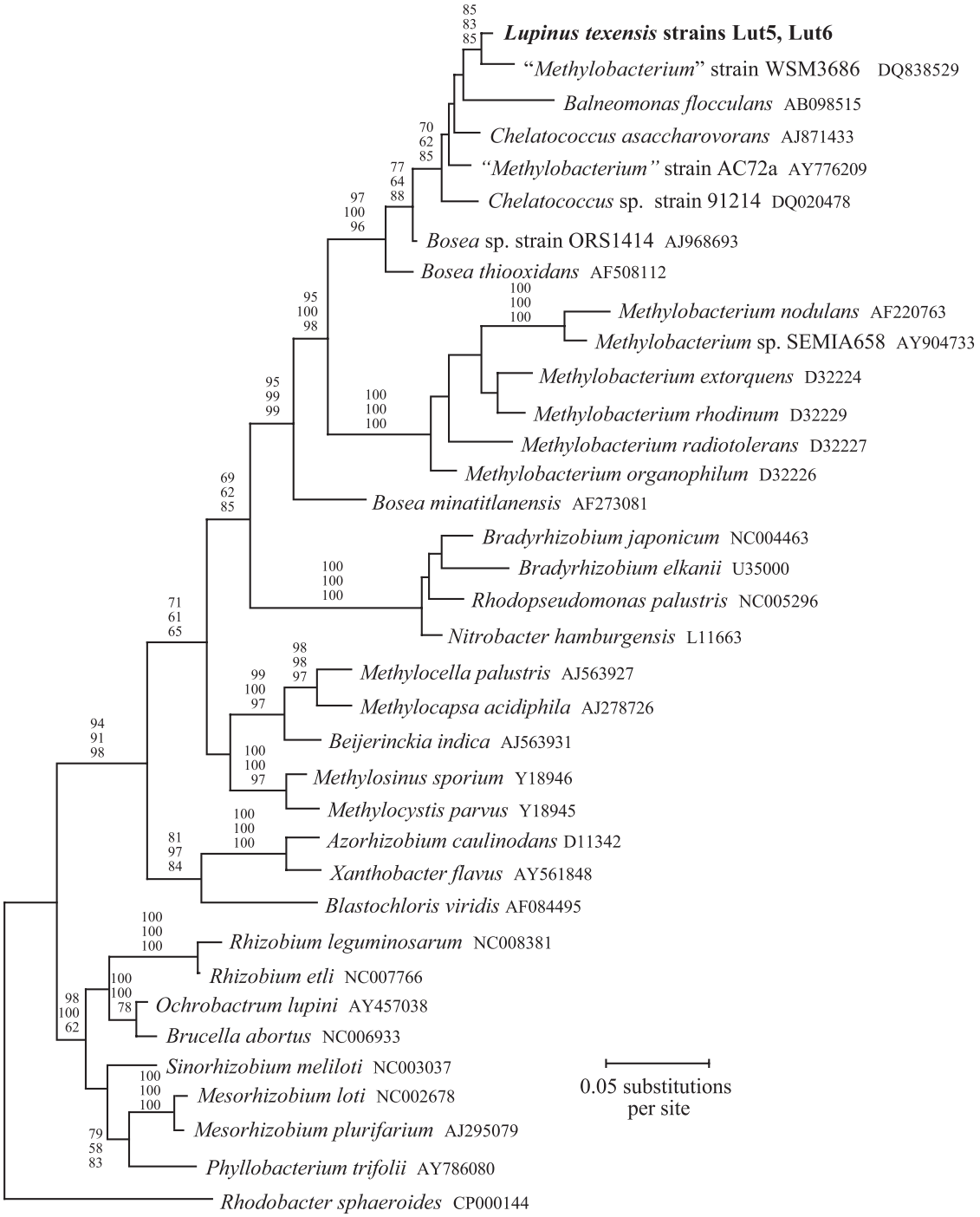

FIG. 1. ML phylogenetic tree for 16S rRNA genes (1,408 bp) from *L. texensis* symbionts and other alphaproteobacteria. Values near branches are bootstrap percentages for maximum-parsimony (top), neighbor-joining (center), and ML (bottom) analyses.

that there was substantial nitrogenase activity (0.6 to 1.1 μmol ethylene/plant/min). These values are within the range commonly found for *Bradyrhizobium* strains with various legume hosts (19, 20) and indicate that nodules were functional for nitrogen fixation. The identity of the nodule occupants was verified by reisolating one bacterial culture from a surface-sterilized nodule for each inoculation isolate and then sequencing the 5′ 23S rRNA region. The nucleotide sequences from the nodule isolates were identical to those from the inoculants. This confirms that the novel *L. texensis* strains were responsible for inducing nodule formation in this experiment.

Plants of *Phaseolus vulgaris*, *Mimosa pudica*, and *Desmodium canadense* all failed to form nodules when inoculated with these three isolates, while *Cytisus scoparius* and *Macroptilium atropurpureum* formed tiny nodules (mean = 0.1 and 7 nodules/plant, respectively). However, acetylene reduction assays

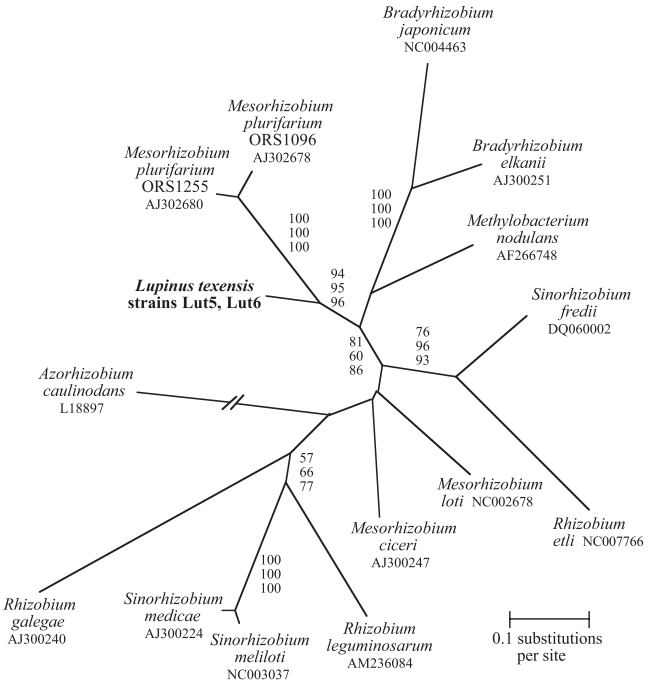

FIG. 2. Unrooted phylogenetic tree derived from ML analysis of *nodA* sequences (591 bp) from *L. texensis* symbionts and other rhizobia. Values near branches are bootstrap percentages for maximum-parsimony (top), neighbor-joining (center), and ML (bottom) analyses.

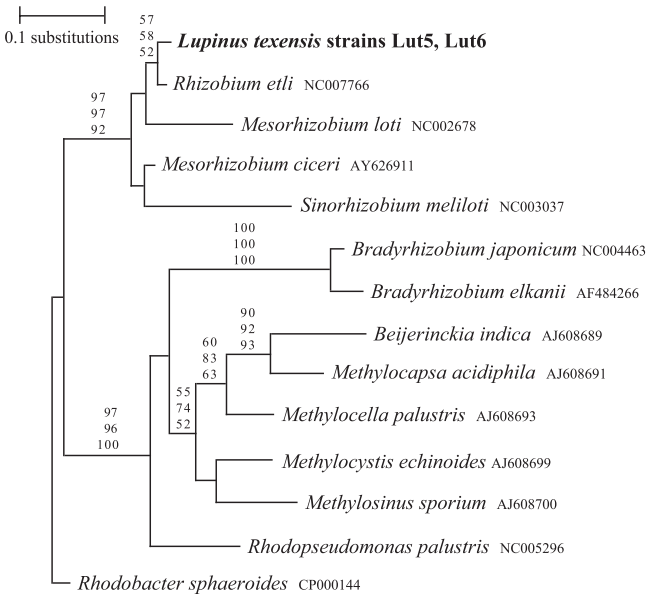

FIG. 3. ML phylogenetic tree for partial *nifD* sequences (476 bp) from *L. texensis* symbionts and other alphaproteobacteria. Values near branches are bootstrap percentages for maximum-parsimony (top), neighbor-joining (center), and ML (bottom) analyses.

failed to detect any nitrogenase activity in these plants. A bacterial culture was reisolated from a surface-sterilized nodule from both *Cytisus* and *Macroptilium* plants. PCR assays with the 23S rRNA primers specific to the novel *L. texensis* symbionts (LUTf1, 23LUTr2) indicated that the *Cytisus* and *Macroptilium* nodule isolates exhibited the same 167-bp product as the inoculant *L. texensis* strains.

L. texensis plants were grown for 44 days after inoculation with the same three isolates and with two *Bradyrhizobium* strains isolated from other *Lupinus* species (nine per treatment). The two *Bradyrhizobium* strains failed to form nodules on *L. texensis*, and the growth of plants inoculated with these strains was not different from that of uninoculated controls. Two isolates (Lut5 and Lut6) resulted in mean plant biomass values (0.43 ± 0.08 [standard error] and 0.46 ± 0.08 g) that were significantly higher than those of uninoculated controls (0.20 ± 0.03 g; $P < 0.05$ [analysis of variance]). A third isolate (Lut3) showed a smaller growth increase (0.28 ± 0.03 g), which was not significantly greater than that of the controls. Thus, the novel *L. texensis* bacteria can benefit their host legume under nitrogen-limiting conditions, but isolates vary in the capacity to improve plant growth.

When plants of three other species of *Lupinus* (*L. perennis*, *L. succulentus*, *L. microcarpus*) were inoculated with four *L. texensis* isolates (Lut3, Lut5, Lut6, and Ltg2), no nodules developed on any of the plants. All three of these *Lupinus* species did form nodules when inoculated with various strains of *Bradyrhizobium* isolated from North American *Lupinus* species. These results suggest that the capacity for nodule symbiosis with the novel *L. texensis* symbionts may not be widespread in the genus *Lupinus* and that *L. texensis* symbionts may be more or less specialized on their original host.

Conclusions. We have shown that natural populations of *L. texensis* harbor a novel lineage of nitrogen-fixing nodule symbionts that are distantly related to the *Bradyrhizobium* strains utilized by other species of *Lupinus*. The fact that the closest known relatives of these strains in the 16S rRNA tree are nonsymbiotic taxa (*Chelatococcus*, *Balneomonas*, *Bosea thioxidans*; Fig. 1) implies that the *L. texensis* symbionts acquired the capacity for nodule symbiosis separately from other rhizobial groups of alphaproteobacteria. Further studies of sequence variation in relatives of this bacterial lineage and studies of their biogeographic distribution and host range are needed to better understand the origin of this symbiotic interaction.

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