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Rhizobia from Lanzarote, the Canary Islands, That Nodulate *Phaseolus vulgaris* Have Characteristics in Common with *Sinorhizobium meliloti* Isolates from Mainland Spain▽

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The stable, low-molecular-weight (LMW) RNA fractions of several rhizobial isolates of *Phaseolus vulgaris* grown in the soil of Lanzarote, an island of the Canary Islands, were identical to a less-common pattern found within *Sinorhizobium meliloti* (assigned to group II) obtained from nodules of alfalfa and alfalfa-related legumes grown in northern Spain. The *P. vulgaris* isolates and the group II LMW RNA *S. meliloti* isolates also were distinguishable in that both had two conserved inserts of 20 and 46 bp in the 16S-23S internal transcribed spacer region that were not present in other strains of *S. meliloti*. The isolates from *P. vulgaris* nodulated bean but not *Medicago sativa*, while those recovered from *Medicago*, *Melilotus*, and *Trigonella* spp. nodulated both host legumes. The bean isolates also were distinguished from those of *Medicago*, *Melilotus*, and *Trigonella* spp. by *nodC* sequence analysis. The *nodC* sequences of the bean isolates were most similar to those reported for *S. meliloti* bv. mediterranense and *Sinorhizobium fredii* bv. mediterranense (GenBank accession numbers DQ333891 and AF217267, respectively). None of the evidence placed the bean isolates from Lanzarote in the genus *Rhizobium*, which perhaps is inconsistent with seed-borne transmission of *Rhizobium etli* from the Americas to the Canaries as an explanation for the presence of bean-nodulating rhizobia in soils of Lanzarote.

A remarkable attribute of *Phaseolus vulgaris* (common bean) is its ability to nodulate with rhizobia from at least 20 different legume genera (summarized in reference 1). Of particular relevance is the report by Ishizawa (16), who described *P. vulgaris* nodulation ranging from doubtful to good by 14 strains recovered from *Medicago sativa*, *Medicago denticulata*, and *Melilotus alba*, while nodulation of the latter three legumes by four bean strains was negative.

At the time of the host range experiments, such as those described by Ishizawa (16), rhizobial nomenclature depended on the legume host of origin; the taxonomy of the strains was based on cross-inoculation groups. Consequently, no information was available about the genetic relationships among the rhizobial strains that originated from the different host legume genera and formed nodules on *P. vulgaris*. Eventually, rhizobial nomenclature based on the cross-inoculation groups was abandoned because of the many unexplainable and incongruous nodulation data (44). The cross-inoculation groups consisted of different rhizobial species within the single genus *Rhizobium*. Eventually, rhizobial taxonomy was expanded to several different genera based on estimates of their phylogeny (38). Phylogenies of bean-nodulating rhizobia were estimated from variations in the 16S rRNA gene sequence (39), even though subsequently it became clear that this method is significantly limited by histories of genetic exchange and recombination (6, 40). Most reported phylogenies of rhizobia nodulating *P. vulgaris* have placed them in the genus *Rhizobium* (3, 39), but several surveys with isolates from North Africa and Spain have demonstrated that rhizobia in the genus *Sinorhizobium* also nodulate this legume species (12, 23, 24, 25, 41), supporting the nodulation data originally published by Ishizawa (16). The number of isolates described as originating from nodules of *P. vulgaris* in the genus *Sinorhizobium* is small, and for the most part, from the published evidence, it has been suggested that they are affiliated with *Sinorhizobium fredii*. However, nodules of *P. vulgaris* growing in a single Tunisian soil where beans are cultivated yielded four isolates that, according to the data, appeared to support an affiliation with *Sinorhizobium meliloti* rather than *S. fredii* (25). Whether these four cultures were of the same rhizobial genotype constituting a single example of *S. meliloti* isolated from *P. vulgaris* is unknown.

P. vulgaris was introduced into Europe as a crop plant as early as the 16th century (31) but never became a very important part of agriculture in Lanzarote, one of the Canary Islands that lie in the Atlantic Ocean to the west of the North African coast. Since there is no record of any nodulation studies with *P. vulgaris* cultivated on Lanzarote Island, the first objective of this study was to examine bean plants that had grown in Lanzarote soil for nodulation. Considering that the diversity of rhizobia able to nodulate bean plants is extremely wide, the

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TABLE 1. Characteristics of the strains analyzed in this study

Strain(s)	Geographical origin	Host of isolation	RAPD pattern	LMW RNA group	Plasmid profile	Nodulation of[c]:	
						Alfalfa	Common bean
GVPV01, GVPV02, GVPV04, GVPV05, GVPV06, GVPV16	Guatiza Vega[a]	*P. vulgaris*	a	II	A	−	+
GVPV08, GVPV09,	Guatiza Vega	*P. vulgaris*	b	II	A	−	+
GVPV11	Guatiza Vega	*P. vulgaris*	b	II	B	−	+
GVPV12, GVPV13	Guatiza Vega	*P. vulgaris*	c	II	B	−	+
RMA20, RMA30, RMA31, RMA32, RMA33	Riego de la Vega[b]	*Melilotus alba*	ND[d]	II	C	+	+
RMOF01	Riego de la Vega	*Melilotus officinalis*	ND	II	C	+	+
RTM02, RTM06, RTM08, RTM11, RTM15, RTM17	Riego de la Vega	*T. monspelliaca*	ND	II	C	+	+
RTF15	Riego de la Vega	*T. foenum-graecum*	ND	II	C	+	+
RMSA04, RMSA36, RMSA42	Riego de la Vega	*M. sativa*	ND	II	C	+	+
S. meliloti ATCC 9930^T	Virginia	*M. sativa*	d	I	D	+	+

[a] Guatiza Vega, Lanzarote, Canary Islands, Spain.
[b] Riego de la Vega, Spain.
[c] +, positive; −, negative.
[d] ND, not determined.

second objective was to characterize the isolates originating from the nodules of plants grown in Lanzarote soil.

(Part of this work was presented as a poster at the First International Meeting on Microbial Phosphate Solubilization, Salamanca, Spain, July 2002.)

MATERIALS AND METHODS

Isolation and nodulation tests. Isolates were made from effective root nodules of *P. vulgaris* (Table 1), according to the method of Vincent (42), by using yeast mannitol agar as the bacterial growth medium. Seeds of *P. vulgaris* var. "pinta" were surface sterilized with sodium hypochlorite for 20 min, washed 10 times with sterile distilled water, and sown in pots with a soil from Guatiza Vega (Lanzarote, Canary Islands) that had been collected in a location where this legume has not recently been cultivated. Plants were grown in a greenhouse in the Canaries without supplemental lighting for 30 days before being harvested; nodules were selected from five randomly chosen plants. Isolation and characterization of rhizobia from *Medicago*, *Melilotus*, and *Trigonella* spp. originating from León, Spain, have been described by del Villar et al. (5).

The isolates were examined for their effectiveness for nitrogen fixation with *P. vulgaris* var. "pinta" and *M. sativa* var. "Aragon" cultivated in a growth chamber using modified Leonard jars (21) filled with vermiculite moistened with N-free Rigaud and Puppo nutrient solutions (29). Noninoculated nitrogen-free and nitrogen-supplemented plants were used as controls. Five replicates for each treatment were used, and plants were harvested 6 weeks after planting to determine the shoot dry weight and the number of nodules. *Rhizobium etli* CFN42^T and *S. meliloti* ATCC 9930^T were included for reference.

RAPD analysis. Randomly amplified polymorphic DNA (RAPD) PCR was done on the isolates and controls using the primer M13 (5′-GAGGGTGGCG GTTCT-3′) according to the method described by Rivas et al. (30). The PCR products were separated according to molecular size by horizontal agarose gel electrophoresis (30) using Standard VI (Boehringer-Roche, Indianapolis, IN) as a size marker.

LMW RNA analysis. Low-molecular-weight (LMW) RNA extractions were done as described by Höfle (13). The LMW RNA mixtures in each sample were separated according to molecular size by staircase electrophoresis (4) using 400- by 360- by 0.4-mm gels in a vertical slab unit (Poker Face SE 1500 Sequencer; Hoeffer Scientific Instruments, San Francisco, CA) as described by Velázquez et al. (41). Molecular size markers used were obtained from Boehringer Mannheim (Mannheim, Germany) and Sigma (St. Louis, MO) and included 5S rRNA from *Escherichia coli* MRE 600, tRNA specific for tyrosine from *E. coli*, and tRNA specific for valine from *E. coli*. After electrophoresis was complete, the gels were silver stained according to the method described by Haas et al. (11).

DNA-DNA hybridization analysis. The DNA-DNA hybridization analysis was done according to the method described by Ezaki et al. (7) by following the recommendations of Willems et al. (43). The analysis was done with representative strains from each of the RAPD groups and the type strain of *S. meliloti*, LMG 6133^T.

Sequence analyses of 16S rRNA genes, *nodC*, and the 16S-23S ITS regions. PCR amplification and sequence analysis of 16S rRNA genes, *nodC*, and the 16S-23S internal transcribed spacer (ITS) regions were carried out as previously described (19, 20). Selected sequences in GenBank, obtained by searches using the BLASTN program (2), were aligned with the DNA sequences obtained for the isolates by using Clustal W (35). Distances calculated according to Kimura's two-parameter model (17) were used to infer phylogenetic trees by using the neighbor-joining method (34) with MEGA 2.1 software (18). Confidence values for nodes in the trees were generated by bootstrap analysis using 500 permutations of the data sets.

Plasmid profile analysis. Rhizobial cells were incubated in tryptone-yeast extract medium at 25°C at 180 rpm on a rotary shaker until cultures reached a concentration of 1×10^6 cells/ml. Cells were collected from 1.5 ml of broth in a centrifuge set at $9{,}000 \times g$ for 5 min. Separation of plasmids by electrophoresis was done according to the method described by Plazinski et al. (28), with the exception that the conditions used were 2 V cm^{-1} for 90 min followed by 3 V cm^{-1} for 60 min and finally 6 V cm^{-1} for 240 min. The pRmeGR4b (205 kb) and pRmeGR4a (175 kb) plasmids of *S. meliloti* GR4 (36) were used as molecular markers.

RESULTS AND DISCUSSION

Isolation of rhizobia from bean nodules. The bean plants that had grown in soil from Lanzarote for 30 days were well nodulated, indicating the presence of bean-nodulating rhizobia in the soil of Lanzarote. Nodules for isolation of the rhizobia were randomly selected from five plants and yielded cultures that were numbered with the prefix GVPV (Table 1). The observation of bean-nodulating rhizobia in the soil of Lanzarote is similar to reports of bean-nodulating rhizobia in different soils of Andalucía (12, 32), because in each case, there had been no recent history of bean cultivation. The results of studies from Spain are different from those from Tunisia, where normal-sized nodules were formed only when beans were grown in soils that had a recent history of bean cultivation (22, 23, 24). Even though there is no recent history of bean cultivation or of rhizobial inoculation in the Spanish locations, both Herrera-Cervera et al. (12) and Rodríguez-Navarro et al. (32) postulated that bean rhizobia may well have been introduced into Spanish soils from the Americas during the last five cen-

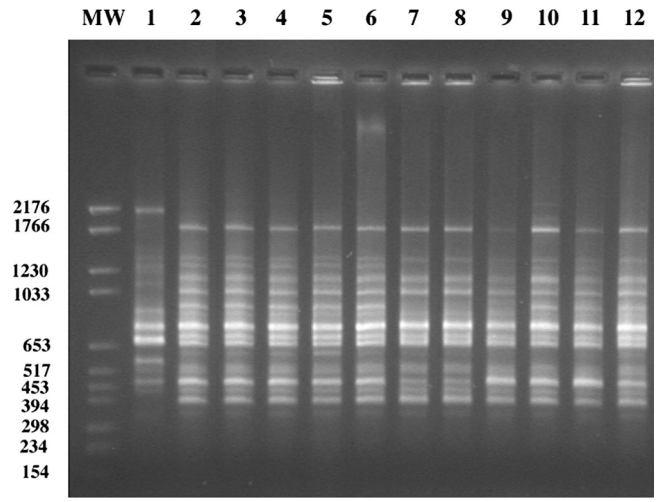

FIG. 1. Horizontal agarose gel electrophoresis of RAPD using DNA of bean isolates from Guatiza Vega (Lanzarote) as the template with results for *S. meliloti* LMG 6133, GVPV01, GVPV02, GVPV04, GVPV05, GVPV06, GVPV08, GVPV09, GVPV11, GVPV12, GVPV13, and GVPV16 shown in lanes 1 through 12, respectively.

turies, because Pérez-Ramírez et al. (27) reported that bean seeds naturally carry rhizobia on their testa. In support of their hypothesis were the high numbers of isolates (54% and 71%, respectively) they categorized as being members of *R. etli*.

RAPD, DNA-DNA hybridization, and 16S rRNA gene sequence analyses. RAPD analysis, as used in other rhizobial studies to determine diversity (15, 26, 37), was applied to the bean isolates from Lanzarote. From the fingerprint patterns obtained with this approach, evidence was obtained that all isolates were almost identical and could be placed into only three highly similar groups (Fig. 1 and Table 1).

The 16S rRNA gene sequences obtained with a representative isolate from each of the three almost-indistinguishable RAPD groups were identical to each other. Also, the 16S rRNA gene sequences of the bean isolates were 99.8% similar to the gene sequence of the *S. meliloti* type strain USDA 1002^T (ATCC 9930, LMG 6133). Since genus affiliation commonly is decided from the 16S rRNA gene sequence (9), and because histories of recombination (40) exclude inferences of species boundaries, perhaps a suggestion that the bean isolates obtained from Lanzarote should be grouped in the genus *Sinorhizobium* would be justified (Fig. 2). Further supporting evidence was obtained by a DNA-DNA hybridization analysis, because a mean homology value of 75% was obtained between the bean isolate GVPV12 and the type strain of *S. meliloti*, LMG 6133^T. Similarly, isolates from nodules of *P. vulgaris* that had grown in Southern Spain as well as Tunisia have been placed in the genus *Sinorhizobium* (12, 22, 23, 24, 32). None of the representatives of the isolates of bean from Lanzarote were placed in the genus *Rhizobium*, a finding which is significantly different from the reported prevalence of *Rhizobium* in the nodules of *P. vulgaris* in the other studies. Consequently, seed-borne transmission of bean rhizobia in the genus *Rhizobium* from the Americas to the Canaries and subsequent extensive interspecific symbiotic gene exchange, as inferred by Herrera-Cervera et al. (12) to explain the diversity of bean-nodulating rhizobia in European soils, appears to have less support in the case of the isolates from Lanzarote.

LMW RNA analysis. Gel electrophoresis of the stable, LMW RNA fraction of single bacterial strains is a high-resolution method for rapid genotypic identification and classification of bacteria (13). This approach was applied to obtain additional evidence for the placement of the bean isolates from Lanzarote in the genus *Sinorhizobium* and to provide further support for their close affiliation with *S. meliloti*. A representative LMW RNA group II strain of *S. meliloti* (5) originating from *Trigonella monspelliaca* (RTM17) was included in the analysis in addition to the type strains for *S. fredii*, *Sinorhizobium medicae*, and *S. meliloti* (Fig. 3). The 5S rRNA zones of the bean-nodulating isolate GVPV12 from Lanzarote, *S. fredii* USDA 205^T, *S. medicae* USDA 1037^T, and *S. meliloti* LMG

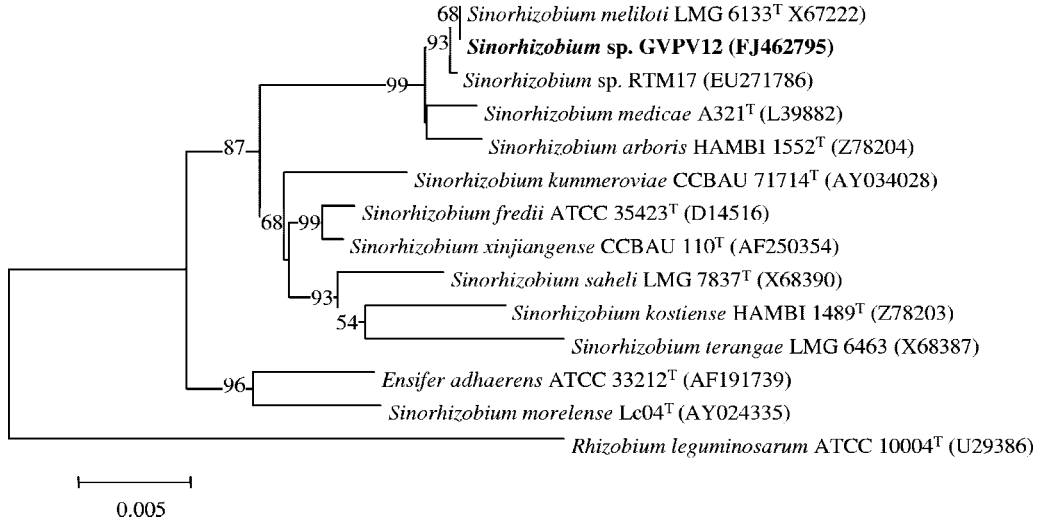

FIG. 2. Placement of the bean isolates on a neighbor-joining tree constructed from variations in the 16S rRNA gene sequences. Permutations of the data set (1,000) were used in a bootstrap analysis to derive a majority-rule consensus tree, and the levels of support for the presence of nodes are indicated. The alignment length of the sequences was 1,479 nucleotides.

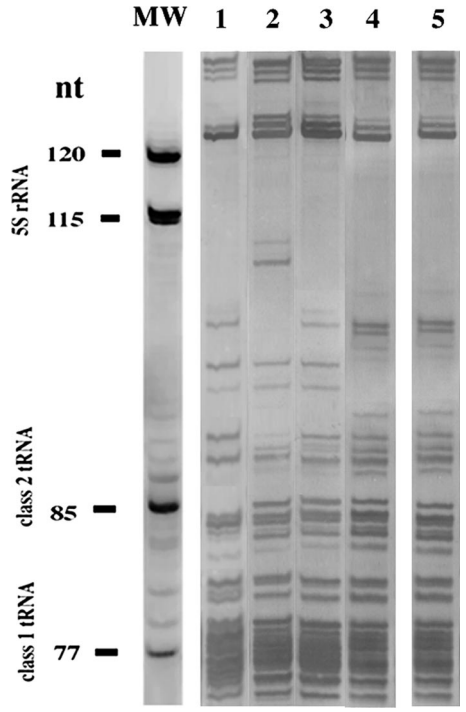

FIG. 3. LMW RNA patterns of *S. fredii* USDA 205^T (lane 1), *S. medicae* USDA 1037^T (lane 2), *S. meliloti* LMG 6133^T (lane 3), RTM17 (lane 4), and GVPV12 (lane 5).

6133^T were identical and supported the placement of the bean isolates in the genus *Sinorhizobium*. The class 1 and class 2 tRNA patterns of RTM17 and the bean isolate were identical, while the two tRNA patterns produced in the lanes with the type strains for *S. fredii*, *S. medicae*, and *S. meliloti* were dissimilar. Therefore, the bean isolates from Lanzarote were placed in LMW RNA group II of *S. meliloti* as described recently by del Villar et al. (5).

Analysis of ITS sequences. Further evidence for the placement of the bean isolates was gathered by sequence analysis of the ITS (Fig. 4) in representatives of both LMW RNA I and II groups of *S. meliloti*, since the 16S-23S ITS region has sections that are hypervariable and useful for distinguishing intraspecific groups (19, 33, 37). Two inserts, of 20 and 46 bp, located between positions 565 to 585 and 644 to 690 relative to the sequence of the type strain of *S. meliloti*, LMG 6133^T (GenBank accession number AF345286), were present in the ITS of all LMW RNA group II rhizobia, irrespective of their host of origin. The ITS in the LMW RNA group II isolates RTM17 and GVPV12 were 94.8% and 95% similar to the region in *S. meliloti* LMG 6133^T (LMW RNA type I), respectively. Within each LMW RNA group, the ITS regions were more than 99.5% similar. Therefore, an analysis of ITS regions provided further evidence for the placement of the bean isolates in the genus *Sinorhizobium*, perhaps supporting an inferred description of them as *S. meliloti*, but recombination as an explanation for the presence of similar ITS sequences should not be disregarded.

TABLE 2. Results of inoculation experiments[a]

Treatment	*P. vulgaris*			*M. sativa*		
	NN	SDW (g)	PN (mg)	NN	SDW (mg)	PN (mg)
GVPV12	97 (c)	1.3 (a)	325 (a)	0	NA	NA
GVPV04	89 (c)	1.0 (a)	250 (a)	0	NA	NA
RTM17	37 (b)	1.2 (a)	300 (a)	7 (a)	57.2 (b)	2.0 (b)
RMA31	12 (a)	1.3 (a)	325 (a)	8 (a)	65.3 (b)	2.3 (b)
S. meliloti ATCC 9930^T	37 (b)	1.4 (a)	350 (a)	9 (a)	38.0 (a)	1.0 (a)
R. etli CFN42^T	97 (c)	1.1 (a)	275 (a)	ND	ND	ND

[a] Letters in parentheses indicate groups of values that are not significantly different from each other ($P = 0.05$, Fisher's protected least significant differences). NN, number of nodules per plant; SDW, shoot dry weight per plant; PN, total nitrogen per plant; ND, not determined; NA, not applicable.

Host range for nodulation, plasmid content, and *nodC* sequence analysis. LMW RNA group II isolates originating from bean or alfalfa-related legume hosts were used in reciprocal cross-inoculation studies to determine their host range. Nodulation levels of bean by GVPV04 and GVPV12, representing the bean isolates from Lanzarote with different plasmid profiles, and the type strain for *R. etli* (CFN42^T), were not significantly different (Table 2). Also, there were no significant differences in plant dry weights or in total plant nitrogen levels

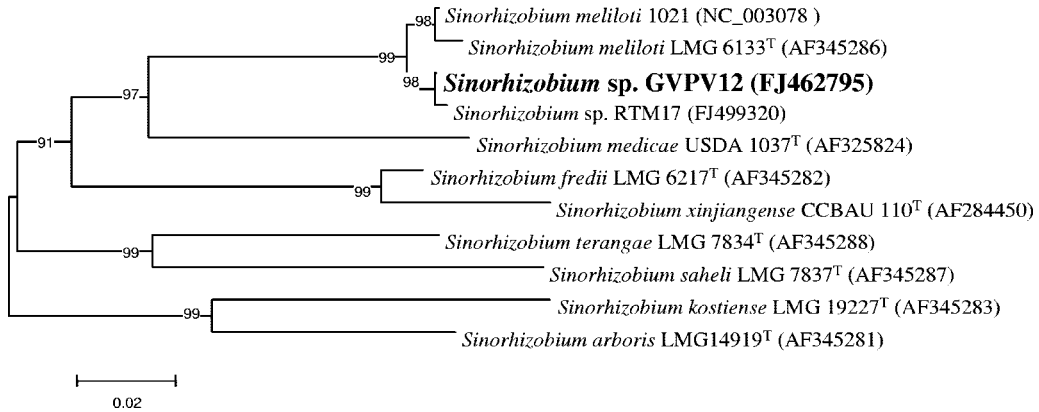

FIG. 4. Placement of LMW RNA group II isolates on a neighbor-joining tree constructed from sequence variation of 16S-23S ITS fragments. Permutations of the data set (1,000) were used in a bootstrap analysis to derive a majority-rule consensus tree, and the levels of support for the presence of nodes are indicated. The alignment length was 726 nucleotides.

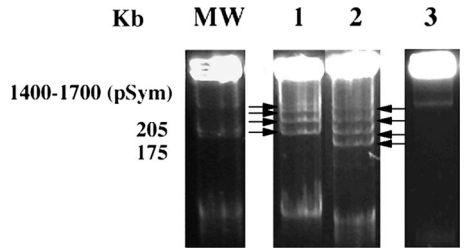

FIG. 5. Plasmids of GVPV04 (lane 1), GVPV12 (lane 2), and RTM17 (lane 3). MW, *S. meliloti* GR4. Plasmids in lanes 1 and 2 are indicated with arrows.

among the treatments. Significantly fewer nodules on bean resulted from inoculation with RMA31 and RTM17, which are LMW RNA group II isolates from *Melilotus alba* and *T. monspelliaca*, respectively. However, there were no significant differences in plant dry weights or nitrogen contents among any of these treatments. Nodulation levels for *M. sativa* by RMA31, RTM17, and the type strain for *S. meliloti* (ATCC 9930^T) were similar. Plant dry weights and total nitrogen levels produced by RMA31 and RTM17 were significantly higher than those for ATCC9930^T. The bean isolates from Lanzarote failed to nodulate *M. sativa* (Table 2), indicating that they have a more-restricted range for nodulation than LMW RNA group II rhizobia that originated from alfalfa-related legumes grown in soil from Riego de la Vega, Northern Spain.

Cellular plasmid contents of LMW RNA group II isolates of *S. meliloti* were compared because of the difference in the nodulation responses with *M. sativa*. RTM17, chosen to represent LMW group II isolates from *Medicago*, *Melilotus*, and *Trigonella*, harbored a single plasmid (Fig. 5, lane 3). GVPV04 and GVPV12, chosen to represent LMW RNA group II isolates from bean, harbored four plasmids each (Fig. 5, lanes 1 and 2). Based upon relative mobilities, two different plasmid contents were observed among the bean isolates, ranging from approximately 200 to 1,500 kb or 90 to 1,500 kb, respectively. Three plasmids, ranging from approximately 60 to 1,700 kb, were observed with the reference strain of *S. meliloti*, GR4. Therefore, isolates GVPV04 and GVPV12 originating from bean appeared to harbor three additional plasmids compared to RTM17 despite the implied similarity of their chromosomes.

In addition to differentiation based on nodulation of *M. sativa* and plasmid content, distinct *nodC* gene sequences were observed among the LMW RNA group II isolates (Fig. 6). The *nodC* gene sequences of isolates RTM17 and RMA31 (data for RMSA36, RMOF01, and RTF15 not shown) from *T. monspelliaca*, *M. sativa*, *Melilotus alba*, *Melilotus officinalis*, *and Trigonella foenum-graecum*, respectively, were almost identical to each other and to the sequence of the type strain for *S. meliloti*, USDA 1002^T (GenBank accession number EF428922). The *nodC* gene sequence of bean isolates GVPV04 and GVPV12 from Lanzarote and GR-06 from Andalucía (12) (GenBank accession number AF217269) were identical to one another but different from RTM17, RMSA36, RMA31, RMOF01, and RTF15, which were isolated from different alfalfa-related legumes in northern Spain (Fig. 6). Placement of bean isolate GR-06 in *S. fredii* has been proposed (20), and the biovar mediterranense has been suggested to distinguish it from the soybean-nodulating strains of *S. fredii* (25). However, the *nodC* sequences of the bean isolate 16b1 from Tunisia (GenBank accession number AF481764), also with a proposed placement in *S. fredii* (23), and GVPV04 and GVPV12 were dissimilar (Fig. 6). Evidence for placement of both isolate GR-06 and isolate 16b1 in *S. fredii* was provided by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes and by a 536-bp sequence of 16S rRNA gene in the case of strain GR-06 (20, 23). The *nodC* gene sequence of GVPV04 and GVPV12 also was similar to that of the bean isolate LILM4H41 (GenBank accession number DQ333891) placed in *S. meliloti* with evidence provided from 16S rRNA gene sequence analysis (25). Based on *nodC* gene sequence analysis, Mnasri et al. (25) proposed to distinguish the bean-nodulating isolate LILM4H41 from *Medicago*-nodulating *S. meliloti* by assigning it the biovar mediterranense, as was done for *S. fredii* bean-nodulating isolates from Andalucía and Tunisia. Additional evidence for the placement of GR-06, 16b1, and LILMH4H41 in the species *S. fredii* and *S. meliloti* was not provided. This limitation is significant, because Gevers et al. (8) have indicated that classification of prokaryotic species by rRNA gene sequence alone is unsatisfactory since sequence similarity is subject both to simple stochastic variation and to the influence of recombination or horizontal gene transfer. Certainly, evidence has been provided that sections within the 16S rRNA genes of rhizobia have undergone recombination, influencing the placement of species on a phylogenetic tree (40). Consequently, it may have been premature to propose biovars of *S. fredii* and *S. meliloti*, because perhaps insufficient evidence was provided to suggest that GR-06, 16b1, and LILMH4H41 are rhizobia belonging to these two prokaryotic species. Also, consideration should perhaps be given to whether it is sensible to assign biovar status to different rhizo-

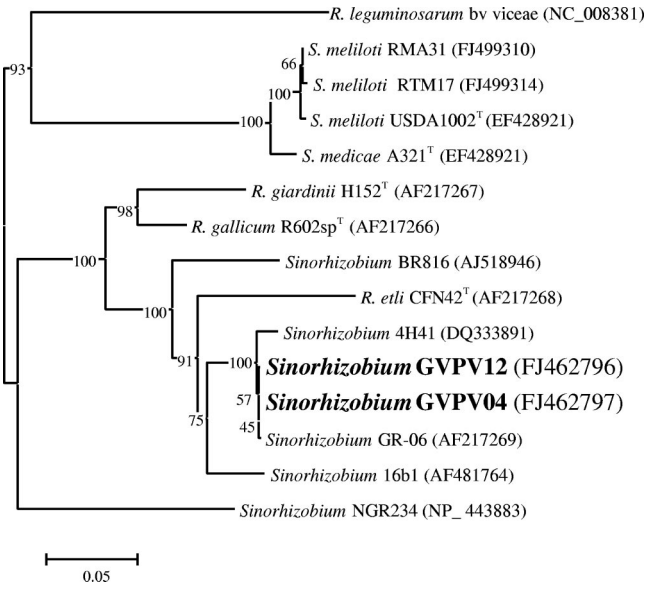

FIG. 6. Comparative sequence analysis of *nodC* gene sequences from strains isolated in this study and related representative strains from GenBank. The significance of each branch is indicated by a bootstrap value calculated for 500 subsets. The alignment length was 834 nucleotides.

bia that nodulate *P. vulgaris*, since this host legume is so promiscuous that it nodulates with rhizobia of legumes that range from the temperate shrub *Caragana arborescens*, native to Siberia and Manchuria (10), to the tropical shrub *Leucaena leucocephala* (14).

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