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Characterization of Rhizobial Isolates of *Phaseolus vulgaris* by Staircase Electrophoresis of Low-Molecular-Weight RNA

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Low-molecular-weight (LMW) RNA molecules were analyzed to characterize rhizobial isolates that nodulate the common bean growing in Spain. Since LMW RNA profiles, determined by staircase electrophoresis, varied across the rhizobial species nodulating beans, we demonstrated that bean isolates recovered from Spanish soils presumptively could be characterized as *Rhizobium etli*, *Rhizobium gallicum*, *Rhizobium giardinii*, *Rhizobium leguminosarum* bv. viciae and bv. trifolii, and *Sinorhizobium fredii*.

The common bean (*Phaseolus vulgaris*), an important leguminous crop, is promiscuous because it forms nitrogen-fixing symbioses with a wide diversity of rhizobia, including six proposed species of *Rhizobium* and one of *Sinorhizobium* (1, 2, 6, 8, 10, 12, 14, 17). Because of this diversity, it would be useful to have a rapid method for the presumptive identification of newly isolated cultures that would also provide additional criteria to evaluate the taxonomic position of bean-nodulating rhizobia. While 16S rRNA gene sequencing might be considered the method of choice, this method is not always readily available to all laboratories and the expense involved would make the analysis of many isolates prohibitive. Analysis of LMW RNA cell content by staircase electrophoresis (20) is an alternative approach that may permit the characterization of numerous bean isolates by a method that is both more readily available and cheaper than sequencing analysis.

To explore the potential of this method, we first obtained the LMW RNA profiles of the type strain and other strains belonging to the different proposed rhizobial species that nodulate beans (Table 1). LMW RNA, extracted as described by Höfle (9), was separated according to molecular size using staircase electrophoresis (5) and the gels were silver-stained (7). Based on results with the reference strains, we concluded that the analysis of LMW RNA was useful for the presumptive identification of unknown bean isolates because the banding patterns within species were consistent, although they varied across species (data not shown). The banding patterns across the different biovars of *Rhizobium etli* (22) and *Rhizobium gallicum* (2) were identical with those of the type strains. However, the results obtained with ATCC 14482 (presumed to be *Rhizobium leguminosarum* bv. phaseoli) were different from those obtained with the type strain for *R. leguminosarum* and were identical with *R. etli* (data not shown). The reason for this may well be the misclassification of ATCC 14482 as *R. leguminosarum* bv. phaseoli since this is a bean isolate originally obtained by the U.S. Department of Agriculture in 1959 (3I6c15, USDA 2668), and a systematic analysis of this strain by other methods has not been done. We also were able to distinguish *R. leguminosarum* bv. viciae and bv. trifolii, which were included in our analysis to complement the data obtained with ATCC 14482. Analyses of other presumptive strains of *R. leguminosarum* bv. phaseoli were not included and, therefore, it is not known whether the banding pattern would have been different from one or both of the other two biovars of this rhizobial species.

The staircase electrophoresis method was used to characterize bean isolates obtained from two regions of Spain, León and Andalucía. We used the bean isolates originating from Andalucía that were characterized by Rodríguez-Navarro et al. (14). Nodules from bean plants growing in a field in León were used to isolate rhizobia according to the method of Vincent (21). Purified isolates were confirmed to nodulate beans by standard plant tests replicated five times using $NaClO_3$-surface-sterilized seeds and with plants grown in pots containing sterilized vermiculite moistened with N-free Rigaud and Puppo nutrient solution (13) and inoculated with approximately 10^9 cells. Plants were grown for 30 days in a growth chamber with a 16-h photoperiod at 26°C and 50 to 60% rH. The isolates from León were characterized by the production of 10 exocellular glucosidases using 1:1 mixtures of bacterial suspensions (6×10^9 CFU/ml) grown for 4 days in Bergensen minimal medium (3) at 28°C and *para*-nitrophenyl substrates at 0.4% concentrations (wt/vol) in 50 mM phosphate buffer, pH 7.0. Chromophores were developed by adding equal volumes of 4% $NaCO_3$. From these results we placed the isolates RPVR07, RPVR32, and RPVN03 within *R. leguminosarum* bv. viciae and the isolates RPVR06, RPVR09, RPVN02, and RPVN03 within *R. leguminosarum* bv. trifolii (Table 2). Our presumptive placement of these isolates by using exocellular enzyme profiles was supported by the results of LMW RNA profiles (Fig. 1A). The profiles of RPVR07, RPVR32, RPVN03, and *R. leguminosa-*

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TABLE 1. Strains used in this study

Strain	Host	Geographical origin	Source or reference
RPVR06, RPVR07, RPVR09, RPVR32	*Phaseolus vulgaris* var. "redonda"	León, Spain	This study
RPVN02, RPVN03, RPVN05	*Phaseolus vulgaris* var. "negra"	León, Spain	This study
14-C1, 14-C3, 8-C3, 4C-4, 15C-4	*Phaseolus vulgaris* var. "canellini"	Andalucía, Spain	14
5NJ-1, 5NJ-2, 16NJ-2	*Phaseolus vulgaris* var. "negra"	Andalucía, Spain	14
4PR-2, 21PR-1, 18PR-2	*Phaseolus vulgaris* var. "presenta"	Andalucía, Spain	14
R. etli bv. mimosae			
Mim 1-4, Mim 1, Mim 1-2, Mim 4-2, Mim 7-5, Mim 2, Mim 11	*Mimosa affinis*	Huautla, Mexico	22
R. etli			
F6, F8	*Phaseolus vulgaris*	Jalisco, Mexico	11
Viking 1	*Phaseolus vulgaris*	Belize	11
Bra 1	*Leucaena leucocephala*	Brazil	11
Rhizobium tropici IIA			
Br 832, Br 833, Br 835, Br 836	*Leucaena leucocephala*	Brazil	11
Br 10043	*Phaseolus vulgaris*		11
R. tropici IIB			
Br 850, Br 864, Br 859, Br 852, Br 857	*Leucaena leucocephala*	Brazil	11
R. gallicum bv. phaseoli			
PhI21, PhD12	*Phaseolus vulgaris*	Gers, France	2
R. gallicum bv. gallicum			
PhP222, PhF29	*Phaseolus vulgaris*	France	2
R. leguminosarum bv. phaseoli			
8002	*Phaseolus vulgaris*	Norwich	
Rhizobium mongolense			
USDA 1884	*Medicago ruthenica*	Hutan Lao, China	18
USDA 1929	*Medicago ruthenica*	Xilinhot, China	18
R. giardinii H152^T	*Phaseolus vulgaris*	Côte d'Or, France	2
R. gallicum R602^T	*Phaseolus vulgaris*	Maine et Loire, France	2
R. tropici IIA CFN 299^T	*Phaseolus vulgaris*	Brazil	16
R. tropici IIB CIAT899^T	*Phaseolus vulgaris*	Colombia	16
R. etli CFN42^T	*Phaseolus vulgaris*	Guanajuato, Mexico	16
R. leguminosarum bv. viceae ATCC 10004^T	*Pisum sativum*	Illinois, United States	
R. leguminosarum bv. trifolii ATCC 14480	*Trifolium repens*		
R. leguminosarum bv. phaseoli ATCC14482	*Phaseolus vulgaris*		
R. mongolense USDA 1844^T	*Medicago ruthenica*	Tongliao, China	18

TABLE 2. Physiological and biochemical characteristics of rhizobia used in this study[a]

Substrate[b]	*R. leguminosarum* bv. viceae	*R. leguminosarum* bv. trifolii	*R. etli*	*R. tropici* IIB	*R. tropici* IIA	*R. gallicum*	*R. mongolense*	*R. giardinii*	*S. fredii*	RPVR07	RPVR32	RPVN03	RPVR06	RPVR09	RPVN02	RPVN05
PNP-N-acglc	+	−	−	+	−	+	+	+	+	+	+	+	−	−	−	−
PNP-α-D-ara	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+
PNP-β-D-ara	−	+	+	+	−	−	−	+	+	−	+	−	−	−	−	−
PNP-α-L-fuco	+	−	+	+	−	−	−	+	+	+	+	+	+	+	+	+
PNP-β-D-fuco	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PNP-α-D-gal	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+
PNP-β-D-gal	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PNP-N-lact	+	−	−	−	+	−	−	−	+	+	+	+	−	−	−	−
PNP-α-D-mal	+	−	+	+	−	+	+	+	+	+	+	+	+	+	+	+
PNP-α-D-xyl	+	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−
PNP-β-D-xyl	+	+	−	−	−	−	−	−	+	+	+	+	−	−	−	−

[a] Type strain was used except for strains isolated in this study.

[b] Abbreviations: PNP, *para*-nitrophenyl; PNP-N-acglc, PNP-N-acetylglucosaminide; PNP-α-D-ara, PNP-α-D-arabinopyranoside; PNP-β-D-ara, PNP-β-D-arabinopyranoside; PNP-α-L-fuco, PNP-α-L-fucopyranoside; PNP-β-L-fuco, PNP-β-L-fucopyranoside; PNP-α-D-gal, PNP-α-D-galactopyranoside; PNP-β-D-gal, PNP-β-D-galactopyranoside; PNP-α-D-mal, PNP-α-D-maltopyranoside; PNP-lact, PNP-lactopyranoside; PNP-α-D-xyl, PNP-α-D-xylopyranoside; PNP-β-D-xyl, PNP-β-D-xylopyranoside.

[c] Plus indicates that compound is hydrolyzed; minus indicates that compound is not hydrolyzed.

rum bv. viciae (Fig. 1A, lanes 2, 6, 7, and 8, respectively) were identical. Similarly, the profiles of RPVR06, RPVR09, RPVN02, RPVN03, and *R. leguminosarum* bv. trifolii (Fig. 1A, lanes 1, 3, 4, 5, and 9, respectively) were identical. These results indicate that in some cases *R. leguminosarum* bv. viciae and bv. trifolii may cross-nodulate onto beans or that within these two biovars variants exist that are able to nodulate beans. Although nodulation of clover and peas by these isolates was not determined, this unexpected result would have been missed using sequencing analysis of the 16S rRNA gene since this locus is conserved among the three biovars belonging to this species.

The isolates from Andalucía were more heterogeneous than those from León, since we distinguished five different LMW RNA profiles (Fig. 1B). Isolates 4C-4, 8C-3, 16NJ-2, 4PR-2, 21PR-1, and 14C-1 (Fig. 1B, lanes 2, 5, 7, 11, and 12) were identified with *R. etli* (lane 14), 5NJ-2 and 14C-3 (lanes 3 and 10) with *R. gallicum* (lane 16), 5NJ-1 and 18PR-2 (lanes 8 and 9) with *Rhizobium giardinii* (lane 15), and 8002 (lane 6) with *R. leguminosarum* bv. trifolii (lane 13). The profile of isolate 15C-4 (lane 1) was identical with that of *Sinorhizobium fredii* (lane 17). The placement of isolate 15C-4 within *Sinorhizobium*

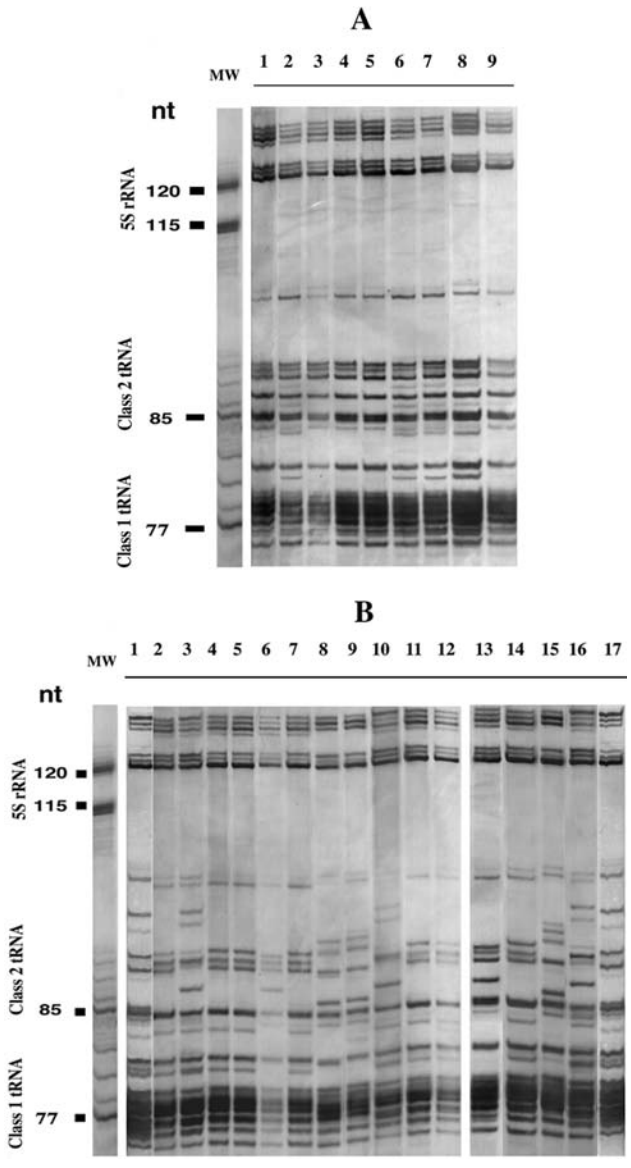

FIG. 1. LMW RNA profiles of rhizobial isolates from Spanish soils. Analysis of isolates (A) from León: lane 1, RPVR06; lane 2, RPVR07; lane 3, RPVR09; lane 4, RPVN02; lane 5, RPVN05; lane 6, RPVR32; lane 7, RPVN03; lane 8, *R. leguminosarum* bv. viciae ATCC 10004^T; lane 9, *R. leguminosarum* bv. trifolii ATCC 14480; and (B) from Andalucía: lane 1, 15C-4; lane 2, 8C-3; lane 3, 14C-3; lane 4, 4C-4; lane 5, 16NJ-2; lane 6, 8002; lane 7, 4PR-2; lane 8, 18PR-2; lane 9, 5NJ-1; lane 10, 5NJ-2; lane 11, 21PR-1; lane 12, 14C-1; lane 13, *R. leguminosarum* bv. trifolii ATCC 14480; lane 14, *R. etli* CFN42^T; lane 15, *R. giardinii* H152^T; lane 16, *R. gallicum* R602^T; and lane 17, *S. fredii* ATCC 35423^T.

was confirmed by full-length sequencing of the 16S rRNA gene according to the method of van Berkum et al. (19) since the sequence was identical with that of *S. fredii* (data not shown). There is a precedence for the nodulation of bean by *S. fredii* (15), and Herrera-Cervera et al. (8) reported the isolation of three bean-nodulating rhizobia from a soil in Granada that had 16S rRNA genes similar to those of *S. fredii*, based on PCR restriction fragment length polymorphism and partial sequencing analysis. Therefore, we conclude that staircase electrophoresis of LMW RNA is a rapid and cost effective method for the presumptive characterization of bean isolates and that in the case of the biovars of *R. leguminosarum* the approach has more resolution than sequencing analysis of the 16S rRNA gene.

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REFERENCES

1. **Amarger, N., M. Bours, F. Revoy, M. R. Allard, and G. Laguerre.** 1994. *Rhizobium tropici* nodulates field-grown *Phaseolus vulgaris* in France. Plant Soil **161:**147–156.
2. **Amarger, N., V. Macheret, and G. Laguerre.** 1997. *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov. from *Phaesolus vulgaris* nodules. Int. J. Syst. Bacteriol. **47:**996–1006.
3. **Bergersen, F. J.** 1961. The growth of *Rhizobium* in synthetic media. Australian J. Biol. **14:**349–360.
4. **Bidle, K. D., and M. Fletcher.** 1995. Comparison of free-living and particle-associated bacterial communities in the Chesapake Bay by stable low-molecular-weight RNA analysis. Appl. Environ. Microbiol. **61:**944–952.
5. **Cruz-Sánchez, J. M., E. Velázquez, P. F. Mateos, and E. Martínez-Molina.** 1997. Enhancement of resolution of low molecular weight RNA profiles by staircase electrophoresis. Electrophoresis **18:**1909–1911.
6. **Diouf, A., P. de Lajudie, M. Neyra, K. Kersters, M. Gillis, E. Martínez-Romero, and M. Gueye.** 2000. Polyphasic characterization of rhizobia that nodulate *Phaseolus vulgaris* in West Africa (Senegal and Gambia). Int. J. Syst. Bacteriol. **50:**159–170.
7. **Haas, H. B., B. Budowle, and G. Weiler.** 1994. Horizontal polyacrylamide gel electrophoresis for the separation of DNA fragments. Electrophoresis **15:** 153–158.
8. **Herrera-Cervera, J. A., J. Caballero-Mellado, G. Laguerre, H. V. Tichy, N. Requena, N. Amarger, E. Martínez-Romero, J. Olivares, and J. Sanjuán.** 1999. At least five rhizobial species nodulate *Phaseolus vulgaris* in a Spanish soil. FEMS Microbiol. Ecol. **30:**87–97.
9. **Höfle, M. G.** 1988. Identification of bacteria by low molecular weight RNA profiles: a new chemotaxonomic approach. J. Microbiol. Methods **8:**235–248.
10. **Jordan, D. C.** 1984. Family III, *Rhizobiaceae* Conn 1938 321^{AL}, p. 234–244. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, Vol. 1, The Williams & Wilkins Co., Baltimore, Md.
11. **Martínez-Romero, E., L. Segovia, F. M. Mercante, A. A. Franco, P. Graham, and M. A. Pardo.** 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int. J. Syst. Bacteriol. **41:**417–426.
12. **Pérez-Ramírez, N. O., M. A. Rogel, E. T. Wang, J. Z. Castellanos, and E. Martínez-Romero.** 1998. Seeds of *Phaseolus vulgaris* bean carry *Rhizobium etli*. FEMS Microbiol. Ecol. **26:**289–296.
13. **Rigaud, J., and A. Puppo.** 1975. Indole-3-acetic catabolism by soybean bacteroids. J. Gen. Microbiol. **88:**223–228.
14. **Rodríguez-Navarro, D. N., A. M. Buendía, M. Camacho, M. M. Lucas, and C. Santamaría.** 2000. Characterization of *Rhizobium* spp. bean isolates from southwest of Spain. Soil Biol. Biochem. **32:**1601–1613.
15. **Sadowsky, M. J., P. Cregan, and H. H. Keyser.** 1988. Nodulation and nitrogen fixation efficacy of *Rhizobium fredii* with *Phaseolus vulgaris* genotypes. Appl. Environ. Microbiol. **54:**1907–1910.
16. **Segovia, L., J. P. W. Young, and E. Martinez-Romero.** 1993. Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type I strains as *Rhizobium etli* sp. nov. Int. J. Syst. Bacteriol. **43:**374–377.
17. **Sessitsch, A., H. Ramírez-Saad, G. Hardarson, A. D. L. Akkermans, and W. M. de Vos.** 1997. Classification of Austrian rhizobia and the Mexican isolate FL27 obtained from *Phaseolus vulgaris* as *Rhizobium gallicum*. Int. J. Syst. Bacteriol. **47:**1097–1101.
18. **van Berkum, P., D. Beyene, G. Bao, T. A. Campbell, and B. D. Eardly.** 1998. *Rhizobium mongolense* sp. nov. is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica* [(L.) Ledebour]. Int. J. Syst. Bacteriol. **48:**13–22.
19. **van Berkum, P., D. Beyene, and B. D. Eardly.** 1996. Phylogenetic relationships among *Rhizobium* species nodulating the common bean (*Phaseolus vulgaris* L.). Int. J. Syst. Bacteriol. **46:**240–244.
20. **Velázquez, E., J. M. Cruz-Sánchez, P. F. Mateos, and E. Martínez-Molina.** 1998. Analysis of stable low-molecular-weight RNA profiles of members of the family *Rhizobiaceae*. Appl. Environ. Microbiol. **64:**1555–1559.
21. **Vincent, J. M.** 1970. A manual for the practical study of root-nodule. Blackwell Scientific Publications, Oxford, United Kingdom.
22. **Wang, E. T., M. A. Rogel, A. García-de los Santos, J. Martínez-Romero, M. A. Cevallos, and E. Martínez-Romero.** 1999. *Rhizobium etli* bv. mimosae, a novel biovar isolated from *Mimosa affinis*. Int. J. Syst. Evol. Microbiol. **49:**1479–1491.