Variability among *Rhizobium* Strains Originating from Nodules of *Vicia faba*

PETER VAN BERKUM,^{1*} DESTA BEYENE,² FRANCISCO TEMPRANO VERA,³
AND HAROLD H. KEYSER⁴

Soybean and Alfalfa Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705¹; Holetta Agricultural Research Center, Institute of Agricultural Research, Addis Ababa, Ethiopia²; Servicio de Investigación Agraria, DGIEA, Junta de Andalucía, Estación Experimental La Rinconada, San José de la Rinconada, Seville, Spain³; and NifTAL Project, University of Hawaii, Paia, Hawaii 96779⁴

Received 27 February 1995/Accepted 21 April 1995

***Rhizobium* strains from nodules of *Vicia faba* were diverse in plasmid content and serology. Results of multilocus gel electrophoresis and restriction fragment length polymorphism indicated several deep chromosomal lineages among the strains. Linkage disequilibrium among the chromosomal types was detected and may have reflected variation of *Rhizobium* strains in the different geographical locations from which the strains originated. An investigation of pea strains with antibodies prepared against fava bean strains and restriction fragment length polymorphism analyses, targeting DNA regions coding for rRNA and nodulation, indicated that *Rhizobium* strains from *V. faba* nodules were distinguishable from those from *Pisum sativum*, *V. villosa*, and *Trifolium* spp.**

Vicia faba was an important legume food crop in ancient civilizations until the introduction of *Phaseolus* species from the Americas. Today, fava bean is still an important crop; its main uses are animal feed, dry seeds and fresh beans for human consumption, and industrial processing of the dry seed to extract protein or to produce flour (1). Some varieties of fava bean are rich in L-DOPA (3,4-dihydroxy-phenylalanine), which is used in the treatment of Parkinson's disease (1).

V. faba is a legume and forms symbioses with soil bacteria of the genus *Rhizobium*. The rhizobia infect the plant roots, stimulate root nodule development, and colonize nodule cortical cells. The symbiosis benefits the host because the microbial symbionts provide the plant with nitrogen derived from biological nitrogen fixation. In the case of *V. faba*, the potential for nitrogen fixation is sufficient to sustain large yields (1).

The microbial symbionts of clover, pea, and in some cases bean are classified as *Rhizobium leguminosarum*. This species is further subdivided into three biovars largely on the basis of host plant specificity for infection and nodulation (10). Strains of fava bean generally are assumed to be classified as *R. leguminosarum* bv. *viciae* (1, 4), probably because of cross-infection of pea.

V. faba is considered to be an isolated plant species because of its failure to cross-pollinate with other *Vicia* spp. (1). However, it would appear that *V. faba* is less isolated symbiotically since the plant nodulates with *R. leguminosarum* bv. *viciae* originating from other plant species. Some evidence would indicate that *V. faba* influences competition for nodulation and is infected by selected groups of rhizobia from within mixed soil populations (8). Consequently, rhizobial strains of fava bean may well be distinguishable from other strains of *R. leguminosarum* bv. *viciae*.

The common bean (*Phaseolus vulgaris*) is nodulated by sev-

eral species of *Rhizobium* (11, 13, 16, 20). Also, reports of linkage disequilibrium among *Rhizobium* species isolated from nodules of *Vicia* species indicate phyletic diversity (21). Similar information about *Rhizobium* strains from *V. faba* nodules is lacking, but since these strains are variable for nitrogen fixation (1, 4) an investigation of their genetic diversity is justified.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The rhizobial strains used in this study (Table 1) are accession strains in the National *Rhizobium* Culture Collection, Agricultural Research Service, U.S. Department of Agriculture. Bacteria were grown in yeast-mannitol broth (24) at 150 rpm and 30°C until late-log phase for the production of antigens for immunization and for the determination of serology with fluorescent antibodies (FAs). Growth was in 200 ml of modified arabinose gluconate (22) until mid-log phase (A_{600} of approximately 0.8) at 150 rpm and 30°C for the preparation of cell extracts for protein gel electrophoresis. Late-log-phase broth cultures in 5 ml of modified arabinose gluconate were used as plant inocula. Cultures were grown at 30°C and analyzed for plasmids according to the methods of Hashem and Kuykendall (7). Cultures were grown in modified arabinose gluconate according to the method of van Berkum et al. (23) for preparation of DNA. Stock cultures were maintained at 4°C on modified arabinose gluconate plates after initially being recovered from glycerol storage at –70°C.

Plant culture. Several replicated plant tests with *V. faba* cultivars Prothabon 101, Petite Windsor, Aladin, Long Pod, Olga, and Trios-Dans-Une and *Pisum sativum* cv. Alaska were grown in Leonard jars as previously described (23) to determine whether the fava bean and pea strains used in our study nodulate both host plants. Each jar contained two plants grown in a growth chamber set at light/dark cycles of 14 h/10 h and temperatures of 24°C/20°C for 28 to 30 and 21 to 28 days for the fava bean and pea plants, respectively. Plants were removed from the Leonard jars, and the vermiculite was shaken off the roots for the inspection of nodulation.

Preparation of sera and FAs and determination of serology. Late-log-phase cultures of USDA 2497, USDA 2498, USDA 2499, USDA 2500, USDA 2501, USDA 2502, and USDA 2503 were used to generate antisera according to the methods described by Somasegaran and Hoben (19). Cell suspensions were shipped to Hazleton Research Products Inc. (Denver, Pa.) to produce antibodies in rabbits, using standard procedures (19). Titers of the test and production bleeds were determined by tube agglutination (24). Each antiserum was cross-absorbed with a mixture of the boiled heterologous antigens to remove background reactions, and FAs were produced with gamma globulins obtained by $(\text{NH}_4)_2\text{SO}_4$ precipitation and subsequent dialysis (19). FAs against *V. faba* TAL 1397, TAL 1399, and TAL 1400 were kindly provided by Padma Somasegaran, NifTAL Project. The FAs were used to investigate cross-reactions with the other 15 *Rhizobium* strains from fava bean and the 35 *Rhizobium* strains from pea.

* Corresponding author. Mailing address: Soybean and Alfalfa Research Laboratory, HH-19, Bldg. 011, BARC-West, Beltsville, MD 20705. Phone: (301) 504-7280. Fax: (301) 504-5728. Electronic mail address: PBERKUM@ASRR.ARSUSDA.GOV.

TABLE 1. Rhizobial strains used in this study

Host plant	Strain	Synonym	Geographical origin	Host plant	Strain	Synonym	Geographical origin
Pea	USDA 2364	SEMIA 0332	Virginia	Fava bean	USDA 2356		Unknown
	USDA 2365		Utah		USDA 2357		Unknown
	USDA 2368		Idaho		USDA 2435	TAL 167	Netherlands
	USDA 2370	ATCC 10004	Illinois		USDA 2475	175F3	Netherlands
	USDA 2372		Illinois		USDA 2476	175F4	Netherlands
	USDA 2374		Montana		USDA 2477	175F7	England
	USDA 2375		Finland		USDA 2478	175F11	Morocco
	USDA 2376		Unknown		USDA 2479	TAL 1399	Canada
	USDA 2377	126C33	Georgia		USDA 2488	175F10	Morocco
	USDA 2379		Washington		USDA 2489	TAL 1400	Canada
	USDA 2380		Iowa		USDA 2490	175F19	Egypt
	USDA 2383		Iowa		USDA 2497	ISL7	Spain
	USDA 2384	SEMIA 0330	Iowa		USDA 2498	ISL13	Spain
	USDA 2385		Iowa		USDA 2499	ISL18	Spain
	USDA 2386	SEMIA 0318	Yugoslavia		USDA 2500	ISL20	Spain
	USDA 2387		Unknown		USDA 2501	ISL23	Spain
	USDA 2388		Unknown		USDA 2502	ISL26	Spain
	USDA 2391		Maryland		USDA 2503	ISL37	Spain
	USDA 2393		Alabama		USDA 2504	ISL43	Spain
	USDA 2395		Alabama		USDA 2505	ISL48	Spain
	USDA 2396		Virginia		USDA 2506	ISL55	Spain
	USDA 2397		Florida		USDA 2507	ISL56	Spain
	USDA 2398		Virginia		USDA 2508	ISL58	Spain
	USDA 2399		Louisiana		USDA 2509	175F9	Morocco
	USDA 2400		Georgia	Clover	USDA 2046	ATCC 14479	Virginia
	USDA 2401		Georgia		USDA 2063	162S5	Florida
	USDA 2402		Yugoslavia		USDA 2116		South Carolina
	USDA 2404		Unknown		USDA 2156	162X47	California
	USDA 2405		Unknown		USDA 2220	162P36	Wisconsin
	USDA 2434	C 1204	Washington		USDA 2227	162S4	Wisconsin
	USDA 2443	TOM	Turkey	Hairy vetch	USDA 2335	ATCC 10314	Virginia
	USDA 2447	128A4	Georgia		USDA 2340		Georgia
	USDA 2448	128A11	Wisconsin				
	USDA 2449	128A13	Brazil				
	USDA 2450	128C4	Idaho				

Preparation of the cell suspensions and subsequent procedures to determine their serology with the FAs were performed according to the protocols outlined by Somasegaran and Hoben (19). Controls included sera produced against *Bradyrhizobium japonicum* USDA 123 and cell suspensions of USDA 123.

Preparation of cell extracts and determination of electrophoretic polymorphism of enzymes. Cultures were centrifuged at $10,000 \times g$ for 10 min at 4°C, and the pelleted cells were washed in 0.85% (wt/vol) NaCl solution. The washed cells were suspended in 3 ml of ice-cold 50 mM phosphate buffer (pH 7.0) containing 15% (vol/vol) glycerol and 1 mM phenylmethylsulfonyl fluoride. The bacterial cells were ruptured by two passages through an ice-cold French pressure cell at 2,700 kg/cm². The supernatant fractions, after centrifugation at $10,000 \times g$ and 4°C, were stored at -20°C until use for protein gel electrophoresis. Proteins in 20 µl of each of the bacterial extracts were separated in continuous nondenaturing vertical polyacrylamide (5.5% [wt/vol] acrylamide and 2.4% [wt/vol] *N,N'*-methylene-bis-acrylamide [cross-linker]) gels at 100 V by using buffers described by Selander et al. (17). Staining solutions to detect alcohol dehydrogenase, adenylate kinase, β-galactosidase, glucose 6-phosphate dehydrogenase, glyceraldehyde-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, 3-hydroxybutyrate dehydrogenase, hexokinase, isocitrate dehydrogenase, indophenol oxidase, malic enzyme, mannitol 1-phosphate dehydrogenase, phosphoglucose mutase, and threonine dehydrogenase were as described by Selander et al. (17). The mobilities for each enzyme were recorded as a measurement of the distance of the well to the stained band divided by the distance of the well to the dye line. Variation in the mobility of each enzyme across strains was equated with alleles at corresponding structural gene loci, and the genetic diversity of each enzyme locus was determined according to the method of Selander et al. (17). Each strain was characterized by its combination of allelic variation over the 14 enzymes assayed to identify electrophoretic types. Distances between pairs of electrophoretic types, measured as the proportion of mismatched loci, were used to derive a phenogram based on the average linkage algorithm, using the ETCLUS computer program kindly supplied by Thomas S. Whittam (Pennsylvania State University). The computer program ETLINK (also supplied by Thomas S. Whittam) was used to derive the index of association (18) among the electrophoretic types.

Preparation of DNA, Southern hybridization, and RFLP analysis. Preparation of DNA, restriction digestion, horizontal gel electrophoresis, Southern transfer, and Southern hybridization analyses were as described before (23). Restriction fragment length polymorphism (RFLP) was determined with *EcoRI*, *BamHI*, and *HindIII* DNA digests by using pBJ142 (15) and pRMJ1 (9) as probes for DNA coding for rRNA and nodulation, respectively. The presence or absence of hybridization bands was scored to produce a rectangular data matrix of simple matching coefficients for each of the two probes. Each of the matrices was analyzed with NTSYS-pc (14), producing two phenograms to reveal diversity among the strains in the region of DNA coding for rRNA or nodulation.

RESULTS

Plasmid profiles of strains from *V. faba*. *Rhizobium* strains originating from *V. faba* contained between two and five large plasmids, as determined by the method described by Hashem and Kuykendall (7). Thirteen different plasmid profiles were observed among 22 strains (Fig. 1). Plasmids were not resolved with USDA 2479 and USDA 2488. The plasmid profiles obtained with USDA 2435, USDA 2476, and USDA 2489 were identical to the profile of USDA 2475 (lane 4); the profiles of USDA 2505 and USDA 2506 were identical to the profile of USDA 2501 (lane 11); and the profiles of USDA 2498, USDA 2499, USDA 2504, and USDA 2507 were identical to the profile of USDA 2509 (lane 9).

Multilocus gel electrophoresis for enzyme polymorphism. The number of electromorphs of each of the 14 enzymes ranged from 4 to 7, while the genetic diversity (*h*) for each locus ranged from 0.37 to 0.81 (Table 2), with a mean diversity

FIG. 1. Plasmid profiles of rhizobial strains originating from the nodules of *V. faba*. Lane 1, USDA 2356; lane 2, USDA 2357; lane 3, USDA 2477; lane 4, USDA 2475; lane 5, USDA 2490; lane 6, USDA 2497; lane 7, USDA 2500; lane 8, USDA 2508; lane 9, USDA 2509; lane 10, USDA 2478; lane 11, USDA 2501; lane 12, USDA 2502; lane 13, USDA 2503. The molecular sizes (in megadaltons) of the three smaller plasmids of USDA 205 (*R. fredii*) used as standard molecular size markers are indicated in the margins.

(*H*) of 0.66. Each of the strains was distinct (Fig. 2). The total number of comparisons in a linkage disequilibrium analysis was 2,334, with a mean number of differences of 8.93. The observed mismatch distribution variance of 7.66 was significantly higher than expected. The expected variance was 2.94, with lower and upper 95% confidence limits of 1.28 and 4.60, respectively. There was no correlation between chromosomal genotype and plasmid profile, with the exception of the cluster containing USDA 2489, USDA 2476, USDA 2435, USDA 2497, USDA 2508, USDA 2475, and USDA 2485, which were the only strains with the plasmid profiles of lanes 4, 6, and 8 (Fig. 1).

Serological relationship among the rhizobial strains of *V. faba* and *P. sativum*. One or more of the FAs produced with strains USDA 2497, USDA 2498, USDA 2499, USDA 2500, USDA 2501, USDA 2502, and USDA 2503 cross-reacted with 20 of the 21 rhizobial strains from fava bean. The one exception was USDA 2477. Cross-reactivity of USDA 2479 (TAL 1399), USDA 2489 (TAL 1400), and USDA 2509 (TAL 1397) with the seven antisera was not determined because antisera prepared against these three strains did not cross-react with the other 21 fava bean strains. Strains USDA 2357, USDA 2435, USDA 2478, USDA 2488, and USDA 2504 gave positive reactions with four, four, four, three, and two of the FAs, respectively. All other strains cross-reacted with a single FA.

TABLE 2. Genetic diversity for 14 enzyme loci among rhizobial strains isolated from nodules of *V. faba*

Enzyme locus ^a	No. of forms	Genetic diversity (<i>h</i>)
ADH	4	0.47
ADK	4	0.48
BGA	5	0.73
6GP	5	0.43
G6P	6	0.78
GPD	4	0.37
HBD	5	0.72
HEX	5	0.77
IDH	4	0.66
IPO	6	0.80
ME	6	0.75
M1P	4	0.71
PGM	7	0.78
THD	6	0.81
Mean	5.1	<i>H</i> = 0.66

^a ADH, alcohol dehydrogenase; ADK, adenylate kinase; BGA, β -galactosidase; 6GP, glucose 6-phosphate dehydrogenase; G6P, 6-phosphogluconate dehydrogenase; GPD, glyceraldehyde-3-phosphate dehydrogenase; HBD, 3-hydroxybutyrate dehydrogenase; HEX, hexokinase; IDH, isocitrate dehydrogenase; IPO, indophenol oxidase; ME, malic enzyme; M1P, mannitol 1-phosphate dehydrogenase; PGM, phosphoglucomutase; THD, threonine dehydrogenase.

FIG. 2. Phenogram of the proportional distance among rhizobial strains originating from the nodules of *V. faba*. The phenogram was based on a matrix of enzyme electrophoretic mobilities used in the derivation of a proportional distance matrix, which was subsequently used in clustering analysis based on the average linkage algorithm.

No obvious correlation between serology and multilocus genotype was evident except for the cluster formed by USDA 2506, USDA 2507, and USDA 2501, which cross-reacted with sera prepared against USDA 2501.

Determinations of the serology of 35 strains originating from the nodules of *P. sativum* with FAs prepared against nine strains originating from nodules of *V. faba* (including TAL 1397 and TAL 1399) were mostly negative, with only five strains cross-reacting. Positive results were obtained between FA 2497 and USDA 2377 (3+), between FA 2499 and USDA 2443 (4+), between FA 2501 and USDA 2443 (3+), and between FA 2502 and USDA 2377 (3+), USDA 2380 (3+), and USDA 2434 (3+), and FA 2503 and USDA 2372 (3+). FA TAL 1397 gave positive results with USDA 2450 (3+). None of the *Rhizobium* strains cross-reacted with FAs prepared against *B. japonicum* USDA 123, and USDA 123 cells did not cross-react with FA prepared against the fava bean strains.

Nodulation of *P. sativum* and *V. faba* with pea and fava bean strains. In single-strain inoculation tests, all pea strains nodulated both pea and fava bean. Similarly, all fava bean strains nodulated both host plants.

RFLP analysis. The DNA of six clover, two pea, two hairy vetch, and four fava bean strains digested with *Eco*RI, *Bam*HI, and *Hind*III generated 60 different bands when hybridizations were done with the rDNA probe pBJ142. The resulting matrix of the 14 strains contained 840 datum points to generate simple matching coefficients. Clustering analysis of the simple matching coefficients resulted in a phenogram which indicated that the clover strains and the pea and hairy vetch strains

FIG. 3. Phenogram indicating similarity in the region of DNA coding for rRNA among strains of *Rhizobium* isolated from nodules of clover, pea, hairy vetch, and fava bean. The tree was derived from an RFLP analysis, using pBJ142 as a probe, which resulted in 840 datum points with which simple matching coefficients were generated for clustering analysis.

formed distinct clusters. The fava bean strains USDA 2498, USDA 2508, and USDA 2497 were diverse in the region of DNA coding for rRNA and branched before clustering of the clover strains and the pea and hairy vetch strains (Fig. 3). Strain USDA 2501 had more similarity to the pea and hairy vetch strains than to the clover strains.

The DNA of four clover, four pea and hairy vetch, and four fava bean strains digested with the same three enzymes generated 39 different bands when hybridizations were done with pRMJ1, the 8.7-kb clone containing *nodDABC* of *R. meliloti*. The resulting matrix of the 12 strains contained 468 datum points to generate simple matching coefficients. Clustering analysis of simple matching coefficients indicated that the 12 strains were grouped into three distinct clusters (Fig. 4). Dissimilarity of the nodulation region in the fava bean strains as a group was greater than that of the clover strains compared with the pea and hairy vetch strains.

DISCUSSION

Characterization of the serology and measurements of genetic diversity indicated that rhizobial strains originating from *V. faba* are diverse. The information presented extends the report of variability in symbiotic potential of fava bean strains (4, 12).

It would appear that fava bean strains are more variable than pea strains since Brockman and Bezdicek (3) identified 18 plasmid profiles and three serogroups in the majority of 192 isolates of *R. leguminosarum* bv. *viciae* originating from nodules of *P. sativum*. However, the interpretation that fava bean strains are more variable than pea strains could be due to differences between the two studies. In our study, analyses were performed with few strains originating from geographically separate locations, while Brockman and Bezdicek (3)

FIG. 4. Phenogram indicating similarity in the region of DNA coding for nodulation among strains of *Rhizobium* isolated from nodules of clover, pea, hairy vetch, and fava bean. The tree was derived from an RFLP analysis, using pRMJ1 as a probe, which resulted in 468 datum points with which simple matching coefficients were generated for clustering analysis.

used isolates from plants growing in several sites within Washington State.

The estimated mean genetic diversity across 14 enzyme loci of the fava bean strains is similar to estimates previously reported for *Rhizobium* species. Linkage disequilibrium was observed among the strains of *V. faba*, which is similar to reports for populations of *R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii* originating from two proximate sites in Oregon (21) and for collections of strains nodulating bean (13, 20) and alfalfa (6, 18). The presence of linkage disequilibrium indicates a low incidence of genetic recombination (5, 20), and in the study of Piñero et al. (13) it was associated with an analysis of more than one species of *Rhizobium* nodulating *P. vulgaris* (11, 16). In our case there is no supporting evidence that the analysis included more than one species of *Rhizobium*. Since Smith et al. (18) concluded that the analysis of samples which are spatially isolated may result in the detection of linkage disequilibrium, our data are more appropriately explained by the diversity in origin of the strains used.

Among the variables influencing the outcome of competitive nodulation of legumes listed by Bottomley (2), plant influence may be of significance in the case of pea and fava bean. Hynes and O'Connell (8) suggested that fava bean and pea regulate nodulation through nutritional factors in the root exudates, with the result that each host plant is nodulated with different members of a mixed rhizobial population. Host influence on the outcome of nodulation could lead to the detection of the differences among *R. leguminosarum* bv. *viciae* strains of pea and fava bean that we report. However, the differences between pea and fava bean strains that we observed may not necessarily be related to plant influence on nodulation but may reflect the diversity in their origin.

ACKNOWLEDGMENTS

We thank Marian Pezzano and K. Lee Nash for technical assistance. We also appreciate the efforts of Bertrand D. Eardly and Padma Somasegaran in commenting on the manuscript.

This work was partially supported by grant CCA-8510/087 from the U.S.-Spain Joint Committee for Scientific and Technological Cooperation.

REFERENCES

- Bond, D. A., D. A. Lawes, G. C. Hawtin, M. C. Saxena, and J. H. Stephens. 1985. Faba bean (*Vicia faba* L.), p. 199–265. In R. J. Summerfield and E. H. Roberts (ed.), Grain legume crops. William Collins Sons & Co. Ltd., London.
- Bottomley, P. J. 1992. Ecology of *Bradyrhizobium* and *Rhizobium*, p. 293–347. In G. Stacey, H. J. Evans, and R. H. Burris (ed.), Biological nitrogen fixation. Chapman and Hall, New York.
- Brockman, F. J., and D. F. Bezdicsek. 1989. Diversity within serogroups of *Rhizobium leguminosarum* biovar viciae in the Palouse region of Eastern Washington as indicated by plasmid profile, intrinsic antibiotic resistance, and topography. Appl. Environ. Microbiol. **55**:109–115.
- Dean, J. R., B. Toomsan, and K. W. Clark. 1980. Rhizobium strain selection for faba beans. Can. J. Plant Sci. **60**:385–397.
- Dykhuizen, D. E., and L. Green. 1991. Recombination in *Escherichia coli* and definition of biological species. J. Bacteriol. **173**:7257–7268.
- Eardly, B. D., L. A. Materon, N. H. Smith, D. A. Johnson, M. D. Rumbaugh, and R. K. Selander. 1990. Genetic structure of natural populations of the nitrogen-fixing bacterium *Rhizobium meliloti*. Appl. Environ. Microbiol. **56**:187–194.
- Hashem, F. M., and L. D. Kuykendall. 1994. Plasmid DNA content of some agronomically important *Rhizobium* strains that nodulate alfalfa, berseem clover, and *Leucaena*, p. 181–188. In P. H. Graham, M. J. Sadowsky, and C. P. Vance (ed.), Symbiotic nitrogen fixation. Kluwer Academic Publishers, Boston.
- Hynes, M. F., and M. P. O'Connell. 1990. Host plant effect on competition among strains of *Rhizobium leguminosarum*. Can. J. Microbiol. **36**:864–869.
- Jacobs, T. W., T. E. Egelhoff, and S. R. Long. 1985. Physical and genetic map of a *Rhizobium meliloti* nodulation gene region and nucleotide sequence of *nodC*. J. Bacteriol. **162**:469–476.
- Jordan, D. C. 1984. Genus 1. *Rhizobium* Frank 1889, 338^{al}, p. 235–242. In N. Krieg (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams and Wilkins, Baltimore.
- Martinez-Romero, E., L. Segovia, F. M. Mercanti, 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.
- Mytton, L. R., M. H. El-Sherbeeney, and D. A. Lawes. 1977. Symbiotic variability in *Vicia faba*. 3. Genetic effects of host plant, *Rhizobium* strain and of host $\times$ strain interaction. Euphytica **26**:785–791.
- Piñero, D., E. Martinez, and R. K. Selander. 1988. Genetic diversity and relationships among isolates of *Rhizobium leguminosarum* biovar phaseoli. Appl. Environ. Microbiol. **54**:2825–2832.
- Rohlf, F. J. 1988. NTSYS-pc numerical taxonomy and multivariate analysis system. Exeter Publishing, Ltd., Setauket, N.Y.
- Scott-Craig, J. S., M. L. Guerinot, and B. K. Chelm. 1991. Isolation of *Bradyrhizobium japonicum* DNA sequences that are transcribed at high levels in bacteroids. Mol. Gen. Genet. **228**:356–360.
- 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.
- Selander, R. K., D. A. Cagant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whittam. 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. **51**:873–884.
- Smith, J. M., N. H. Smith, M. O'Rourke, and B. G. Spratt. 1993. How clonal are bacteria? Proc. Natl. Acad. Sci. USA **90**:4384–4388.
- Somasegaran, P., and H. J. Hoben. 1985. Methods in legume-Rhizobium technology. NifTAL, University of Hawaii, Paia.
- Souza, V., T. T. Nguyen, R. R. Hudson, D. Piñero, and R. E. Lenski. 1992. Hierarchical analysis of linkage disequilibrium in *Rhizobium* populations: evidence for sex? Proc. Natl. Acad. Sci. USA **89**:8389–8393.
- Strain, S. R., K. Leung, T. S. Whittam, F. J. de Bruijn, and P. J. Bottomley. 1994. Genetic structure of *Rhizobium leguminosarum* biovar trifolii and viciae populations found in two Oregon soils under different plant communities. Appl. Environ. Microbiol. **60**:2772–2778.
- van Berkum, P. 1990. Evidence for a third uptake hydrogenase phenotype among the soybean bradyrhizobia. Appl. Environ. Microbiol. **56**:3835–3841.
- van Berkum, P., R. B. Navarro, and A. A. T. Vargas. 1994. Classification of the uptake hydrogenase-positive (Hup⁺) bean rhizobia as *Rhizobium tropici*. Appl. Environ. Microbiol. **60**:554–561.
- Vincent, J. M. 1970. A manual for the practical study of root-nodule bacteria. International Biological Programme handbook no. 15. Blackwell Scientific Publications, Oxford.