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Genetic Diversity among *Rhizobium leguminosarum* bv. Trifolii Strains Revealed by Allozyme and Restriction Fragment Length Polymorphism Analyses

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Allozyme electrophoresis and restriction fragment length polymorphism (RFLP) analyses were used to examine the genetic diversity of a collection of 18 *Rhizobium leguminosarum* bv. trifolii, 1 *R. leguminosarum* bv. viciae, and 2 *R. meliloti* strains. Allozyme analysis at 28 loci revealed 16 electrophoretic types. The mean genetic distance between electrophoretic types of *R. leguminosarum* and *R. meliloti* was 0.83. Within *R. leguminosarum*, the single strain of bv. viciae differed at an average of 0.65 from strains of bv. trifolii, while electrophoretic types of bv. trifolii differed at a range of 0.23 to 0.62. Analysis of RFLPs around two chromosomal DNA probes also delineated 16 unique RFLP patterns and yielded genetic diversity similar to that revealed by the allozyme data. Analysis of RFLPs around three Sym (symbiotic) plasmid-derived probes demonstrated that the Sym plasmids reflect genetic divergence similar to that of their bacterial hosts. The large genetic distances between many strains precluded reliable estimates of their genetic relationships.

Phenotypic characters, such as intrinsic antibiotic resistance (1, 22), bacteriophage sensitivity (5, 24), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10, 29) patterns, are used frequently to characterize strains within naturally occurring populations of *Rhizobium* spp. However, these methods have significant limitations. The ability to discriminate unequivocally between isolates of *Rhizobium* spp. by using intrinsic antibiotic resistance and phage sensitivity is limited by the number of antibiotics or phage types utilized. The subjective comparison of complex patterns of proteins makes definitive identification of closely related strains by the SDS-PAGE technique difficult (29). The problem is compounded by the fact that variation detected by these techniques may be due to genes encoded on endogenous plasmids (e.g., antibiotic resistance determined by IncP-1 plasmids such as RP4 [7]). These plasmids may not be host specific (i.e., broad-host-range plasmids such as IncP-1 plasmids [42]), and therefore the variations may bear no relationships to those found among bacterial genomes. Furthermore, in the absence of analyses, the genetic relationships and diversity of strains of naturally occurring populations remain unresolved.

Allozyme electrophoresis has proven to be a powerful tool in genetic studies of a wide range of eukaryotic organisms (32), and although the technique has been used less widely in prokaryotes, a number of reports have verified its applicability to a range of studies with bacteria. In particular, allozyme data have proven valuable for characterizing strains in epidemiological studies (12), ecological studies (6), population structure (39), and systematics (37). With the exceptions of the recent studies of Pinero et al. (31) and Eardly et al. (11), most studies on *Rhizobium* strains (13, 14, 18, 27, 28, 45, 46) have been based on the examination of a limited number of loci, which has precluded a reliable estimate of genetic relationships among the strains examined.

Restriction fragment length polymorphism (RFLP) analysis detects mutations or rearrangements in the genome which alter the distribution of specific restriction endonuclease recognition sites within defined regions of the genome. Increasingly, RFLPs are being used to study the genetic structure of higher organisms. Analysis of RFLPs has been applied to human (3) and plant (19) genetics, but has only recently been used in genetic studies of phytopathogenic bacteria (9, 15, 23). Recently, RFLP analysis has demonstrated the diversity of Sym (symbiotic) plasmid types within naturally occurring populations of *Rhizobium leguminosarum* (8, 35, 47).

In this study of 18 *R. leguminosarum* bv. trifolii strains of diverse geographic origin, 1 strain of *R. leguminosarum* bv. viciae, and 2 strains of *R. meliloti*, we used the techniques of allozyme electrophoresis and RFLP analysis to (i) find genetic markers useful to characterize strains; (ii) examine the level of genetic diversity of both bacterial strains and their Sym plasmids, and (iii) estimate the genetic relationships among strains.

MATERIALS AND METHODS

Bacterial strains. The strains of *Rhizobium* spp. used in this study are listed in Table 1. *R. leguminosarum* bv. trifolii SU489 (a less effective variant of strain TA1 [16]) and SU410, a colony type variant, are reisolates of TA1 and TA2, respectively.

Allozyme electrophoresis. Each strain was grown in 50 ml of GM broth (10). The bacterial culture was harvested by centrifugation at 5,000 × *g* for 10 min, resuspended in 1 ml of 0.15 M phosphate buffer (pH 7.0), and transferred to sterile microcentrifuge tubes. The cell suspension was centrifuged for 2 min, and the supernatant was discarded. The cell pellet was resuspended in 0.5 ml of lysis buffer (100 mg of NADP and 500 μl of 2-mercaptoethanol liter^{-1}) and sonicated in

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TABLE 1. Strains of *Rhizobium* spp. and plasmids

Strain or plasmid	Host plant or relevant characteristics	Origin or source
R. leguminosarum bv. trifolii		
CC2480a	*Trifolium subterraneum* L.	Greece
CC2480c	*Trifolium subterraneum* L.	Greece
CC2238b	*Trifolium subterraneum* L.	Israel
CC2247d	*Trifolium subterraneum* L.	Israel
WU95	*Trifolium subterraneum* L.	W.A., Australia
WA67	*Trifolium subterraneum* L.	W.A., Australia
NA30	*Trifolium subterraneum* L.	New South Wales, Australia
NN10	*Trifolium subterraneum* L.	New South Wales, Austraila
CC10	*Trifolium subterraneum* L.	Victoria, Australia
TA1	*Trifolium subterraneum* L.	Tasmania, Australia
TA2	*Trifolium subterraneum* L.	Tasmania, Australia
SU410	Laboratory reisolate of TA1	
SU489	Laboratory reisolate of TA2	
CC275e	*T. repens*	Tasmania, Australia
US2007	*T. repens*	Sweden
T1	Unknown	United States
SU298	Unknown	Australia
CC235	Unknown	Unknown
R. leguminosarum bv. viciae		
TA101	*Pisum sativum*	Tasmania, Australia
R. meliloti		
CC169	*Medicago rugosa*	S.A., Australia
NA39	Unknown	United States
Plasmid		
pRt1013	1-kb *PstI-XhoI* fragment carrying an *R. leguminosarum* bv. trifolii reiterated chromosomal DNA sequence from pRt654 (35) subcloned into pUC18	This study
pIM178	pLAFR1 cosmid containing ca. 25 kb of chromosomal DNA from *R. leguminosarum* bv. viciae	J. A. Downie (9a)
pRt722	Two tandemly repeated *Bam*HI-linked, ca. 250-bp *ClaI-AvaI nifHDK* promoter (RtRS1) fragments from *R. leguminosarum* bv. trifolii ANU843 (43) cloned into pUC18	This study
pRt585	6.5-kb *Hin*dIII restriction fragment carrying the *nifHDK* operon of *R. leguminosarum* bv. trifolii ANU843 cloned into pBR328	34
pRt587	14-kb *Hin*dIII restriction fragment carrying the clover-specific nodulation genes of *R. leguminosarum* bv. trifolii ANU843 cloned into pBR328	36

three 5-s bursts while being kept chilled in an ice-water bath. The extracts were then centrifuged for 5 min at 2,000 × *g* to remove cellular debris, and small (ca. 5 μl) aliquots were transferred into 20-μl disposable glass pipettes. The end of each tube was sealed, and the tubes were stored at −20°C.

Soluble enzyme extracts of each strain were separated by electrophoresis on cellulose-acetate gels (Cellogel; Chemetron) and stained for specific enzyme activities as described in detail by Richardson et al. (32) and Selander et al. (37). Of the 44 enzymes initially examined, 28 loci stained with sufficient intensity and resolution for reliable and reproducible interpretation. The 28 enzymes are listed in Table 2.

RFLP analysis. Small-scale preparations of total bacterial DNA were obtained by growing each strain in 5 ml of TY medium (2) for 3 days at 28°C on an orbital shaker. The cells from 1.5 ml of each culture were pelleted in a microcentrifuge tube for 2 min, the supernatant was decanted, and the cell pellet was resuspended in 300 μl of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA). Sixteen microliters of 25% (wt/vol) SDS and 65 μl of pronase in TE (10 mg/ml) were added to each cell suspension; the tubes were thoroughly mixed by inverting and incubated at 37°C for 30 min. Thirty-five microliters of 5 M NaCl was added to each suspension, and the tubes were mixed as above and incubated at 65°C for a further 30 min. Each sample was then extracted three times with phenol-chloroform-isoamyl alcohol (50:48:2), equilibrated with 100 mM Tris–500 mM NaCl (pH 8.0), and extracted once with chloroform-isoamyl alcohol (49:1). The DNA was precipitated by adding 1 volume of cold isopropanol (−20°C) and collected by centrifugation for 15 min in a microcentrifuge. The resulting DNA pellet was washed twice with 70% ethanol and once with 95% ethanol and then allowed to air dry. The dried DNA was dissolved in 100 μl of TE by heating at 65°C for 2 h. This procedure routinely yielded 100 to 300 ng of DNA μl^{-1} that was sufficiently pure to be digested with restriction enzymes.

Samples (5 to 8 μg) of DNA were digested completely (12 to 16 h) with *Hin*dIII restriction endonuclease (10 to 15 U), electrophoresed on a 1% (wt/vol) agarose (SeaKem; FMC Corp.) gel in TAE buffer (40 mM Tris [pH 7.8], 20 mM sodium acetate, 2 mM EDTA), and transferred to nylon membranes (Hybond-N; Amersham Corp.) as described previously (34). ^{32}P-labelled DNA probes were prepared by random priming (44). Five DNA probes were used in this study: two cloned chromosomal DNA fragments, pRt1013 and pIM178; and three cloned DNA fragments from the Sym

TABLE 2. Allelic profiles at 28 enzyme loci for 16 ETs represented by 13 strains of *R. leguminosarum* bv. trifolii, 1 strain of *R. leguminosarum* bv. viciae, and 2 strains of *R. meliloti*

Strain	ET	Alleles at indicated enzyme loci[a]																											
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
R. leguminosarum bv. trifolii																													
T1	1	c	c	c	a	a	d	c	a	e	d	f	b	a	b	d	d	d	d	b	a	e	b	d	a	d	b	b	b
CC2480a[b]	2	d	c	c	a	d	d	d	a	e	d	f	b	a	c	d	d	d	c	e	a	b	c	i	a	c	b	b	b
TA1[c]	3	f	c	c	a	c	d	c	a	f	b	b	b	a	c	e	e	d	c	c	a	b	d	i	a	g	a	b	b
SU298	4	e	a	c	b	b	d	b	a	e	e	c	b	a	a	d	d	d	c	a	a	e	d	d	a	f	b	b	—[d]
WU95	5	a	b	c	b	d	d	c	a	d	d	e	b	c	b	c	e	b	e	h	a	f	i	e	a	c	b	b	b
NA30	6	a	b	b	b	d	d	c	c	d	d	e	b	b	b	b	d	b	e	h	a	f	h	c	a	e	b	b	b
CC10	7	e	b	b	b	d	d	d	a	d	d	e	c	b	b	c	d	a	e	h	a	d	g	f	a	c	b	a	b
CC235	8	b	c	c	b	d	d	c	a	d	d	i	a	b	b	c	d	d	a	h	a	a	g	j	a	c	a	b	b
CC275e	9	c	c	c	b	d	d	c	a	c	d	i	a	b	b	c	c	b	b	d	a	b	g	h	a	c	c	b	b
US2007	10	c	d	c	b	b	d	c	a	e	d	g	a	b	b	c	d	d	b	i	a	a	g	i	a	c	c	b	b
CC2238b[e]	11	f	c	c	c	b	d	c	a	d	d	i	a	b	b	b	e	b	e	d	a	d	e	h	a	d	b	b	b
NN10	12	g	c	c	c	a	d	c	a	d	d	fi[f]	b	b	b	b	e	d	e	cj	b	—	f	h	a	—	—	—	b
WA67	13	g	c	c	d	a	d	c	a	d	d	h	b	b	b	b	e	d	c	e	b	c	f	g	b	e	b	b	b
R. leguminosarum bv. viciae																													
TA101	14	d	d	c	a	a	a	c	a	f	c	f	b	b	d	d	d	b	e	g	c	d	c	j	a	b	b	b	b
R. meliloti																													
CC169	15	h[g]	b	a	b	b	b	a	b	a	a	a	b	d	d	a	a	c	e	f	d	—	a	b	c	a	—	b	b
NA39	16	$\underline{i}$	$\underline{e}$	$\underline{a}$	a	a	$\underline{c}$	b	a	$\underline{b}$	b	$\underline{d}$	b	c	b	c	$\underline{b}$	b	c	c	$\underline{d}$	—	$\underline{a}$	$\underline{a}$	$\underline{c}$	$\underline{a}$	$\underline{d}$	b	$\underline{a}$
No. of alleles per locus		9	5	3	4	4	4	4	3	6	5	9	3	4	4	5	5	4	5	10	4	6	9	10	3	7	4	2	2

[a] The following 28 enzymes were assayed for: 1, triose-phosphate isomerase; 2, glucose phosphate isomerase; 3, pyruvate kinase; 4, isocitrate dehydrogenase; 5, phosphoglycerate kinase; 6, malate dehydrogenase; 7, phosphoglucomutase; 8, glyceraldehyde 3-phosphate dehydrogenase; 9, adenylate kinase; 10, 6-phosphogluconate dehydrogenase; 11, fumarate hydratase; 12, superoxide dismutase; 13, phosphoglyceromutase; 14, peptidase C; 15, xanthine dehydrogenase; 16, alkaline phosphatase; 17, glutathione reductase; 18, peptidase B; 19, hexokinase; 20, enolase; 21, aspartate aminotransferase; 22, leucine-amino peptidase; 23, guanine deaminase; 24, UDP-glucose pyrophosphorylase; 25, adenosine deaminase; 26, leucine dehydrogenase; 27, alanine dehydrogenase; 28, uridine monophosphate kinase.

[b] The same allelic profile was observed for CC2480c.

[c] The same allelic profile was observed for TA2, SU410, and SU489.

[d] —, no enzyme activity was detected.

[e] The same allelic profile was observed for CC2247d.

[f] Two alleles were recorded for this locus.

[g] The underlined alleles designate alleles unique to *R. meliloti*; i.e., they were not observed for any *R. leguminosarum* strains.

plasmid of *R. leguminosarum* bv. trifolii ANU843, namely, pRt722, pRt585, and pRt587 (Table 1). The Southern blots were prehybridized in Southern buffer (41) for >1 h at 65°C and then hybridized for at least 16 h at 65°C in the same buffer in the presence of the ^{32}P-labelled DNA probe. The Southern blots were washed briefly with 2× SSC (1× SSC is 0.03 M sodium citrate plus 0.3 M NaCl) at room temperature and twice with 2× SSC at 65°C and then exposed to Kodak X-ray film at −70°C, using an intensifying screen. DNA probes were removed from the Southern blots by washing them three times (20 min each time) in an initially hot (100°C) solution of 0.1% (wt/vol) SDS.

Data analyses. The allelic profile of each strain, for the 28 enzyme loci, was compared with that of each other strain. A matrix of the genetic distance between strains was calculated as the percentage of mismatched alleles at all loci, and cluster analysis was performed by the Unweighted Pair Group Method with Averages (UPGMA) (40). Genetic diversity per locus was calculated as $h = 1 - \Sigma x_i^2$, where x_i is equal to the frequency of the ith allele at the locus. The mean genetic diversity is equal to the arithmetic mean of the genetic diversities of all loci (37).

A similar approach was used to analyze the RFLP results. The pRt1013 and pIM178 RFLP pattern of each strain was compared with that of each other strain. The number of strongly hybridizing corresponding fragments was recorded. A matrix of distances was computed on the basis of the equation $D = 1 - [2n_{xy}/(n_x + n_y)]$, where D equals the percentage of mismatched fragments between strains x and y and n_{xy} equals the total number of strongly hybridizing fragments of strains x and y, respectively (9). Cluster analysis was done by UPGMA.

RESULTS

Allozyme analysis. All 28 enzyme loci were polymorphic for the strains examined; the number of alleles per locus ranged from 2 (alanine dehydrogenase and uridine monophosphate kinase) to 10 (hexokinase and guanine deaminase) (Table 2). The average number of alleles per locus was 5.5. The mean genetic diversity per enzyme locus estimated from the total sample of *Rhizobium* strains was 0.58. Unexpectedly, additional alleles were observed for *R. leguminosarum* bv. trifolii NN10 at two loci, fumarate hydratase and hexokinase. Among the 21 strains of *Rhizobium* spp. characterized by allozyme electrophoresis, 16 different electrophoretic types (ETs) were identified (Table 2). More than one strain was observed for each of three ETs: CC2480a and CC2480c were observed for ET 2; TA1 and TA2 and their respective laboratory reisolates, SU489 and SU410, were observed for ET 3; and CC2238b and CC2247d were observed for ET 11.

Figure 1 summarizes estimates of the genetic divergence among the 21 strains of *Rhizobium* spp. The two species, *R.*

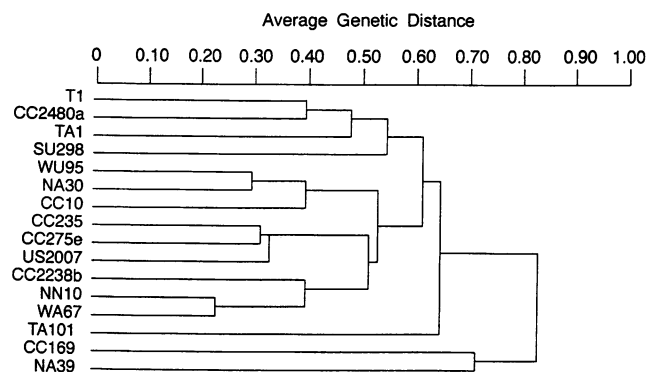

FIG. 1. Genetic relationships among the 16 ETs for 13 strains of *R. leguminosarum* bv. trifolii, 1 strain of *R. leguminosarum* bv. viciae (TA101) and 2 strains of *R. meliloti* (CC169 and NA39) based on allelic profiles at 28 loci. See Table 2 for the strains with the same allelic profile.

leguminosarum and *R. meliloti*, were clearly differentiated at an average genetic distance of 0.83. The two biovars, *R. leguminosarum* bv. trifolii and viciae, clustered at 0.65. All of the *R. leguminosarum* bv. trifolii strains were grouped in a large cluster within which there were further subclusters. The genetic distances between ETs of *R. leguminosarum* bv. trifolii strains ranged from a minimum of 0.23 to a maximum of 0.62.

RFLP analysis. Southern blots of *Hin*dIII-digested, total genomic DNAs were probed sequentially with two cloned fragments, pRt1013 and pIM178, of chromosomal DNA and three cloned DNA fragments, RtRS (pRt722), *nod* (pRt587), and *nif* (pRt585) gene probes, from the Sym plasmid of *R. leguminosarum* bv. trifolii ANU843 (Table 1).

(i) Chromosomal probes. Plasmid pRt1013 carries a reiterated sequence of DNA subcloned from the recombinant plasmid pRt654 (35). Hybridization analysis with the cloned fragment from pRt1013 revealed 10 different hybridization patterns each consisting of between two and five strongly hybridizing restriction fragments (Fig. 2). A similarly sized,

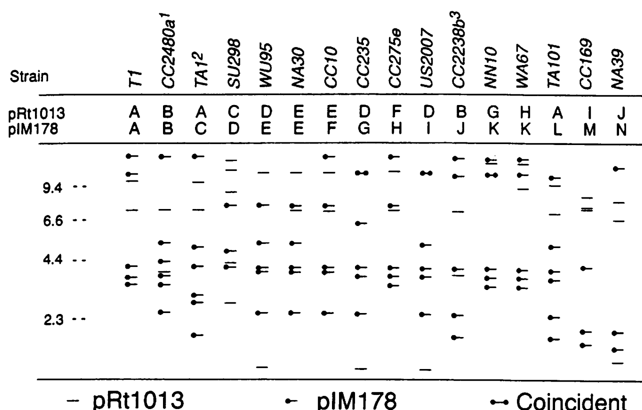

FIG. 2. Distribution of hybridizing restriction fragments detected by the two chromosomal DNA probes. Values on the left indicate the relative positions of size standards (in kilobases) derived by *Hin*dIII digestion of bacteriophage lambda *c*I857 DNA. [1]CC2480a and CC2480c hybridization profiles were identical. [2]TA1, TA2, SU410, and SU489 hybridization profiles were identical. [3]CC2238b and CC2247d hybridization profiles were identical.

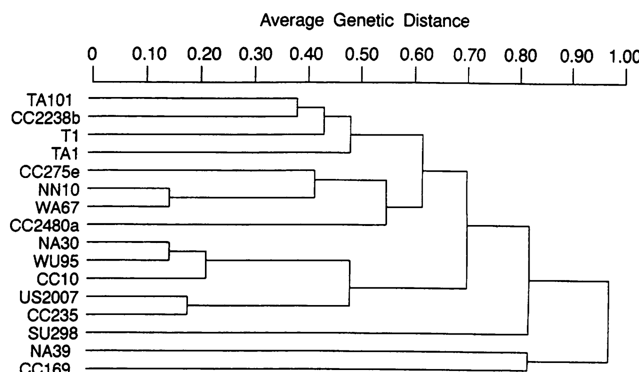

FIG. 3. Genetic relationships among 16 strains of *Rhizobium* spp.; 13 strains of *R. leguminosarum* bv. trifolii, 1 strain of *R. leguminosarum* bv. viciae (TA101), and 2 strains of *R. meliloti* (NA39 and CC169). The phenogram was constructed from the combined results of RFLP analyses around the two chromosomal DNA probes, pRt1013 and pIM178. See the legend to Fig. 2 for strains with the same hybridization profiles.

hybridizing restriction fragment of approximately 7.0 kb was observed in 13 of the 19 *R. leguminosarum* strains. Other variously sized restriction fragments were shared by one or more strains of *R. leguminosarum*. However, only 1 of the 6 hybridizing restriction fragments from the two *R. meliloti* strains was similar in size to 1 of the 11 such fragments observed among the *R. leguminosarum* strains. Strains of the same ET exhibited identical hybridization profiles. In four instances, strains of different ETs also exhibited very similar hybridization profiles (e.g., NA30 and CC10, NA30 and WU95, NN10 and WA67, and CC235 and US2007).

The same Southern blots were probed with the cosmid pIM178. Between three and six restriction fragments hybridized strongly with pIM178 (Fig. 2). A single hybridizing restriction fragment of ca. 4.2 kb was observed in 17 of the 19 *R. leguminosarum* strains. With pIM178 as the chromosomal probe, 14 RFLP patterns were observed. Again, strains of the same ET and, in two cases, strains of different ETs (WU95 and NA30, NN10 and WA67) exhibited identical hybridization profiles. The two *R. meliloti* strains hybridized weakly with pIM178, and only 1 of the 6 observed hybridizing fragments was similar in size to 1 of the 18 such fragments observed among the *R. leguminosarum* strains.

An RFLP distance matrix was computed from the DNA hybridization analyses (see Materials and Methods) and used to construct a phenogram, using UPGMA (Fig. 3). This analysis clearly differentiated between *R. leguminosarum* and *R. meliloti*; these two species differed at an average of 0.98. The *R. leguminosarum* strains formed a large diverse cluster within which there were many subclusters. The genetic distances between these strains ranged from 0 to a maximum of 0.85.

(ii) Symbiotic plasmid probes. The Sym plasmid of each strain was characterized by Southern blot hybridization analysis, using three cloned DNA fragments as probes: RtRS (pRt722), *nod* (pRt587), and *nif* (pRt585) gene probes (Table 1). Southern blots probed with RtRS revealed unique hybridization patterns for the 13 *R. leguminosarum* bv. trifolii strains, made up of between 1 and 10 hybridizing restriction fragments (Fig. 4). Only strains of *R. leguminosarum* bv. trifolii hybridized with RtRS under stringent experimental conditions, confirming the specificity of this probe (43). In contrast to the majority of *R. leguminosarum* bv. trifolii

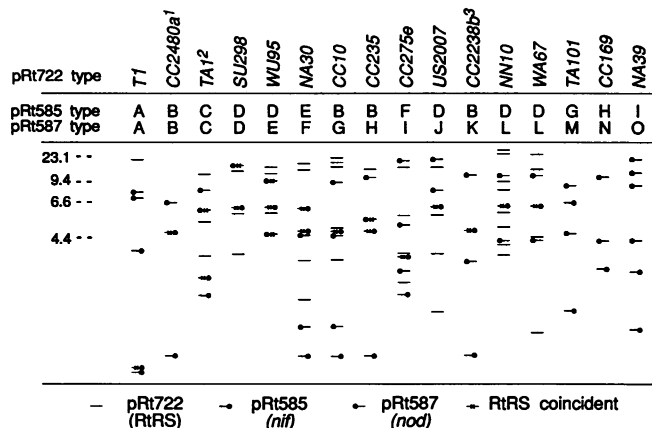

FIG. 4. Hybridization patterns of 13 strains of *R. leguminosarum* bv. trifolii, 1 strain of *R. leguminosarum* bv. viciae, and 2 strains of *R. meliloti* probed with a set of three Sym plasmid-specific (RtRS, *nif*, and *nod*) DNA probes. Values on the left indicate the relative positions of size standards (in kilobases) derived by *Hin*dIII digestion of bacteriophage lambda *c*I875 DNA. [1,2,3]See the legend to Fig. 2 for strains with the same hybridization patterns. The pRt722 (RtRS) type is named after the pre-eminent strain.

strains, which possessed five or six hybridizing restriction fragments, strains CC2480a, CC2480c, CC2238b, and CC2247d, the only isolates examined from the Mediterranean region, exhibited a single, similarly sized (ca. 4.5 kb), hybridizing restriction fragment. Strains TA1 and TA2 and their respective derivatives, SU489 and SU410, exhibited identical RtRS hybridization profiles.

The hybridization patterns to the *nif* and *nod* gene probes are also illustrated in Fig. 4. Among the *R. leguminosarum* bv. trifolii strains, there were 6 *nif* and 12 *nod* hybridization patterns. *nif* hybridization patterns B and D were the most frequently observed. Only one *nod* hybridization pattern, L, was observed for more than one strain of *R. leguminosarum* bv. trifolii. The *nif* and *nod* hybridization patterns of *R. leguminosarum* bv. viciae TA101 and the two *R. meliloti* strains were unlike any of the *R. leguminosarum* bv. trifolii hybridization patterns.

DISCUSSION

Allozyme electrophoresis has been used extensively to study genetic relationships in a wide range of eukaryotic organisms (32), but the technique has been applied to relatively few bacterial studies (37). Furthermore, with the exceptions of the studies of Pinero et al. (31) and Eardly et al. (11), the reported electrophoretic studies on *Rhizobium* species have been based on only a few enzyme loci. However, these studies have shown that numerous allelic variants exist for those loci examined (13, 14, 18, 27, 28, 45, 46). In this study, we examined 44 different enzyme loci, of which 28 loci provided staining of sufficient intensity and resolution for reliable genetic interpretation. All 28 enzyme loci were polymorphic and were, therefore, potentially useful for distinguishing between, and determining the genetic relationships among, the 21 *Rhizobium* strains examined.

Cluster analysis of the allozyme results revealed large genetic diversity among the strains of a single biovar, bv. trifolii (Fig. 1). This result was corroborated by cluster analysis of the RFLP data (Fig. 3). These results confirm an earlier conclusion, drawn from DNA-DNA homology analyses, that strains of *R. leguminosarum* bv. trifolii appear to belong to a genetically diverse group (20). Large genetic diversities within other cross-inoculation groups of *Rhizobium* spp. have been reported. Pinero et al. (31) examined 51 strains of *R. leguminosarum* bv. phaseoli isolated from a number of host plants including *Desmodium* sp. (wild tick clover) and *Leucaena leucocephala* (huaje). Among the collection of strains examined, some pairs of strains had no alleles in common. On the basis of their results, they concluded that *R. leguminosarum* bv. phaseoli may be a polyphylogetic group of isolates representing as many as seven different species. Eardly et al. (11) analyzed 232 strains of *R. meliloti* isolated from two perennial species of *Medicago*. Among these strains, there were two deeply divergent lineages clustering at a genetic distance of 0.83; they concluded that these two lineages represent different species. Large genetic diversity has been observed among strains of the soilborne bacteria *Pseudomonas syringae* (9) and *Clavibacter michiganese* (33).

Although the allozyme and RFLP data concur in showing that strains of *Rhizobium* spp. used in this study are genetically diverse, inspection of the phenograms show that the data from both methods were, not surprisingly, not in complete agreement in estimating genetic relationships among strains. However, the two approaches showed that (i) the two *R. meliloti* strains were different in many characteristics from the *R. leguminosarum* bv. trifolii strains, consistent with the recent taxonomic reappraisal of *Rhizobium* (21); (ii) the four pairs showing a low genetic difference by allozyme analysis (Fig. 1) also showed low genetic distance by analysis of hybridization profiles (Fig. 3); and (iii) strains of the same ET had the same hybridization profiles for both chromosomal and Sym plasmid probes. For the remaining relationships there was general agreement that these occur at large average genetic distances. Because of the large genetic distances involved between many of the strains used in this study, it would be inappropriate use of our data to resolve relationships between these strains (25). It should also be emphasized that the phenograms presented in Fig. 1 and 3 are not meant to imply phylogetic relationships, but are simply diagrammatic representations of the data.

RFLPs around the RtRS, *nif*, and *nod* gene probes provided useful genetic markers to characterize the Sym plasmid of *R. leguminosarum* bv. trifolii. These markers can facilitate studies of the ecology of Sym plasmids in nature. By relating Sym plasmid types with the host strain chromosomal type, it has been argued that Sym plasmids are transferred between strains in nature (35, 47). Species-specific, Sym plasmid-located probes could be obtained from other *Rhizobium* species and applied to the study of the ecology of their Sym plasmids.

An alternative approach to analyzing RFLPs around specific DNA probes is to examine the restriction pattern of the DNA after electrophoresis and staining with ethidium bromide (4, 17, 26). Although this method is very simple and does not require radioactive probes, there are several disadvantages. First, like protein pattern profiles, the restriction patterns can be very complex, which generally makes it very difficult to distinguish between closely related strains unless there are marked differences in their banding patterns. Second, without plasmid-specific DNA hybridization probes, it would not be possible to detect and characterize plasmid-linked sequences. The combination of these two factors would make it impossible to establish the relationship between the diversity of chromosome and Sym plasmid types by using RFLP analysis as demonstrated in this study.

It is highly improbable that two isolates would evolutionarily converge to the same ET over 28 loci. Therefore, strains of the same ET and hybridization profile can be regarded as clones of each other (38, 39). Thus, CC2480a and CC2480c, which were isolated from different plants growing in the same pasture (4a), are members of the same clone. Likewise, CC2238b and CC2247d, which were isolated from sites approximately 60 km apart (4a), are members of another clone. The RFLPs around the chromosomal probes and the three Sym plasmid probes support these conclusions. Young and Wexler (47) drew similar conclusions about apparently identical genotypes of *R. leguminosarum* bv. viciae isolated approximately 25 km apart. These results indicate that genetically related strains may be widely distributed. Widespread occurrences of the same genotypes of *Escherichia coli* have been reported (30). It appears that particular genotypes of *R. leguminosarum* bv. trifolii may also have widespread distributions.

In conclusion, the results of this study support the general view that allozyme electrophoretic and RFLP analyses are powerful techniques for discriminating between isolates of agriculturally significant bacteria (9, 13, 15, 23, 28, 33). The results presented here illustrate the ability of either technique to differentiate clearly between strains as well as to assess the genetic diversity among these strains. We are using the same techniques used in this study to examine the genetic diversity of, and incidence of genetic exchange of Sym plasmids between, naturally occurring nodule isolates of *R. leguminosarum* bv. trifolii.

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REFERENCES

1. **Benyon, J. L., and D. P. Josey.** 1980. Demonstration of heterogeneity in a natural population of *Rhizobium phaseoli* using variation in intrinsic antibiotic resistance. J. Gen. Microbiol. **118**:437–442.
2. **Beringer, J. E.** 1974. R. factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84**:188–198.
3. **Botstein, D., R. L. White, M. Skolnick, and R. W. Davis.** 1980. Construction of genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. **32**:314–331.
4. **Bouzar, H., and L. W. Moore.** 1987. Complementary methodologies to identify specific *Agrobacterium* strains. Appl. Environ. Microbiol. **53**:2660–2665.

4a. **Brockwell, J.** Personal communication.

5. **Bromfield, E. S. P., I. B. Sinha, and M. S. Wolynetz.** 1986. Influence of location, host cultivar, and inoculation on the composition of naturalized populations of *Rhizobium meliloti* in *Medicago sativa* nodules. Appl. Environ. Microbiol. **51**:1077–1084.
6. **Caugant, D. A., B. Levin, and R. K. Selander.** 1981. Genetic diversity and temporal variation in the *E. coli* population of a human host. Genetics **98**:467–490.
7. **Datta, N., R. W. Hedges, E. J. Shaw, R. B. Sykes, and M. H. Richmond.** 1971. Properties of an R-factor from *Pseudomonas aeruginosa*. J. Bacteriol. **108**:1244–1249.
8. **Demezas, D. H., J. M. Watson, T. B. Reardon, and A. H. Gibson.** 1988. A molecular approach to *Rhizobium* ecology, p. 196–197. *In* R. Palacios and D. P. S. Verma (ed.), Molecular genetics of plant-microbe interactions 1988. American Phytopathological Society Press, St. Paul, Minn.
9. **Denny, T. P., M. N. Gilmour, and R. K. Selander.** 1988. Genetic diversity and relationships of two pathovars of *Pseudomonas syringae*. J. Gen. Microbiol. **134**:1949–1960.

9a. **Downie, J. A.** Personal communication.

10. **Dughri, M. H., and P. J. Bottomley.** 1983. Complementary methodologies to delineate the composition of *Rhizobium trifolii* populations in root nodules. Soil Sci. Soc. Am. J. **47**:939–945.
11. **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.
12. **Edelstein, P. H., C. Nakahama, J. O. Tobin, K. Calarco, K. B. Beer, J. R. Joly, and R. K. Selander.** 1986. Paleoepidemiologic investigation of Legionnaires disease at Wadsworth Veterans Administration Hospital by using three typing methods for comparison of *Legionellae* from clinical and environmental sources. J. Clin. Microbiol. **23**:1121–1126.
13. **Engvild, K. C., and G. Nielsen.** 1985. Strain identification in *Rhizobium* by starch gel electrophoresis of isoenzymes. Plant Soil **87**:251–256.
14. **Fottrell, P. F., and A. O'Hara.** 1969. Multiple forms of D(-) 3-hydroxybutyrate dehydrogenase in *Rhizobium*. J. Gen. Microbiol. **57**:287–292.
15. **Gabriel, D. W., J. E. Hunter, M. T. Kingsley, J. W. Miller, and G. R. Lazo.** 1988. Clonal population structure of *Xanthomonas campestris* and genetic diversity among citrus canker strains. Mol. Plant Microbe Interact. **1**:59–65.
16. **Gibson, A. H.** 1965. Physical environment and symbiotic nitrogen fixation. III. Root temperature effects on the relative nitrogen assimilation rate. Aust. J. Biol. Sci. **18**:295–310.
17. **Glynn, P., P. Higgins, A. Squartini, and F. O'Gara.** 1985. Strain identification of *Rhizobium trifolii* using DNA restriction analysis, plasmid DNA profiles and intrinsic antibiotic resistances. FEMS Microbiol. Lett. **30**:177–182.
18. **Harrison, S. P., D. G. Jones, and J. P. W. Young.** 1989. *Rhizobium* population genetics: genetic variation within and between populations from diverse locations. J. Gen. Microbiol. **135**:1061–1069.
19. **Helentjaris, T., G. King, M. Slocum, C. Siedenstang, and S. Wegman.** 1985. Restriction fragment length polymorphisms as probes for plant diversity and their development as tools for applied plant breeding. Plant Mol. Biol. **5**:109–118.
20. **Jarvis, B. D. W., A. G. Dick, and R. M. Greenwood.** 1980. Deoxyribonucleic acid homology among strains of *Rhizobium trifolii* and related species. Int. J. Syst. Bacteriol. **36**:42–52.
21. **Jordan, D. C.** 1984. Family III. *Rhizobiaceae* Conn. 1938, p. 234–256. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
22. **Josey, D. P., J. L. Beynon, A. W. B. Johnston, and J. E. Beringer.** 1979. Strain identification in *Rhizobium* using intrinsic antibiotic resistance. J. Appl. Bacteriol. **46**:343–350.
23. **Lazo, G. R., R. Roffey, and D. W. Gabriel.** 1987. Pathovars of *Xanthomonas campestris* are distinguishable by restriction fragment-length polymorphism. Int. J. Syst. Bacteriol. **37**:214–221.
24. **Lesley, S. M.** 1982. A bacteriophage typing system for *Rhizobium meliloti*. Can. J. Microbiol. **28**:180–189.
25. **Maxson, L. R., and R. D. Maxson.** 1979. Comparative albumin and biochemical evolution in plenthodontid salamanders. Evolution **33**:1057–1062.
26. **Mielenz, J. R., L. E. Jackson, F. O'Gara, and K. T. Shanmugam.** 1979. Fingerprinting bacterial chromosomal DNA with restriction endonuclease *Eco*RI: comparison of *Rhizobium* spp. and identification of mutants. Can. J. Microbiol. **25**:803–807.
27. **Murphy, P. M., and C. L. Masterson.** 1978. Determination of multiple forms of esterase in *Rhizobium* by paper electrophoresis stains. J. Gen. Microbiol. **61**:121–129.
28. **Mytton, L. R., N. J. McAdam, and P. Portlock.** 1978. Enzyme polymorphisms as an aid to identification of *Rhizobium* strains. Soil Biol. Biochem. **10**:79–80.

29. **Noel, K. D., and W. J. Brill.** 1980. Diversity and dynamics of indigenous *Rhizobium japonicum* populations. Appl. Environ. Microbiol. **40:**931–938.
30. **Ochman, H., and R. K. Selander.** 1984. Evidence for clonal population structure in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **81:**198–201.
31. **Pinero, 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.
32. **Richardson, B. J., P. R. Baverstock, and M. Adams.** 1986. Allozyme electrophoresis: a handbook for animal systematics and population studies. Academic Press, Sydney, Australia.
33. **Riley, I. T., T. B. Reardon, and A. C. McKay.** 1988. Genetic analysis of plant pathogenic bacteria in the genus *Clavibacter* using allozyme electrophoresis. J. Gen. Microbiol. **134:**3025–3030.
34. **Schofield, P. R., M. A. Djordjevic, B. G. Rolfe, J. Shine, and J. M. Watson.** 1983. A molecular linkage map of nitrogenase and nodulation genes in *Rhizobium trifolii*. Mol. Gen. Genet. **192:**459–465.
35. **Schofield, P. R., A. H. Gibson, W. F. Dudman, and J. M. Watson.** 1987. Evidence for genetic exchange and recombination of *Rhizobium* symbiotic plasmids in a soil population. Appl. Environ. Microbiol. **53:**2942–2947.
36. **Schofield, P. R., R. W. Ridge, B. G. Rolfe, J. Shine, and J. M. Watson.** 1984. Host-specific nodulation is encoded on a 14 kb DNA fragment in *Rhizobium trifolii*. Plant Mol. Biol. **3:**3–11.
37. **Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whittman.** 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. **51:**873–884.
38. **Selander, R. K., and B. R. Levin.** 1980. Genetic diversity and structure in *Escherichia coli* populations. Science **210:**545–547.
39. **Selander, R. K., R. M. McKinney, T. S. Whittman, W. F. Bibb, D. J. Brenner, F. S. Nolte, and P. E. Pattison.** 1985. Genetic structure of populations of *Legionella pneumophila*. J. Bacteriol. **163:**1021–1037.
40. **Sneath, P. H., and R. R. Sokal.** 1973. Numerical taxonomy. The principles and practice of numerical classification. W. H. Freeman & Co., San Francisco.
41. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
42. **Thomas, C. M., and C. A. Smith.** 1987. Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation. Annu. Rev. Microbiol. **41:**77–101.
43. **Watson, J. M., and P. R. Schofield.** 1985. Species-specific, symbiotic plasmid-located repeated DNA sequences in *Rhizobium trifolii*. Mol. Gen. Genet. **199:**279–289.
44. **Whitfeld, P. L., P. H. Seeburg, and J. Shine.** 1982. The human proopiomelanocortin gene: organization, sequence and interspersion with repetitive DNA. DNA **1:**133–143.
45. **Young, J. P. W.** 1985. *Rhizobium* population genetics: enzyme polymorphism in isolates from peas, clover, beans and lucerne grown at the same site. J. Gen. Microbiol. **131:**2399–2408.
46. **Young, J. P. W., L. Demetriou, and R. G. Apte.** 1987. *Rhizobium* population genetics: enzyme polymorphism in *Rhizobium leguminosarum* from plants and soil in a pea crop. Appl. Environ. Microbiol. **53:**397–402.
47. **Young, J. P. W., and M. Wexler.** 1988. Sym plasmid and chromosomal genotypes are correlated in field populations in *Rhizobium leguminosarum*. J. Gen. Microbiol. **134:**2731–2739.