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Genotypic and Phenotypic Comparisons of Chromosomal Types within an Indigenous Soil Population of *Rhizobium leguminosarum* bv. trifolii†

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The relative genetic similarities of 200 isolates of *Rhizobium leguminosarum* bv. trifolii recovered from an Oregon soil were determined at 13 enzyme loci by multilocus enzyme electrophoresis (MLEE). These isolates represented 13 antigenically distinct serotypes recovered from nodules formed on various clover species. The MLEE-derived levels of relatedness among isolates of *R. leguminosarum* bv. trifolii were found to be in good agreement with the levels of relatedness established by using repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the PCR technique and with levels of relatedness from previously published DNA reassociation studies. BIOLOG substrate utilization patterns showed that isolates within an electrophoretic type (ET) were phenotypically more similar to each other than to isolates of other ETs. The soil isolates were represented by 53 ETs which could be clustered into seven groups (groups B, E, G, H1, H2, I, and J). Evidence for multilocus structure within the population was obtained, and group B was identified as the primary creator of the disequilibrium. Of 75 isolates belonging to the nodule-dominant serotype AS6 complex, 72 were found in group B. Isolates WS2-01 and WS2-02 representing nodule-dominant serotypes recovered from subclover grown at another Oregon site were also found in group B. Isolates representing the most numerous ETs in group B (ETs 2 and 3) were either suboptimally effective or completely ineffective at fixing nitrogen on six different clover species. Another four groups of isolates (groups A, C, D, and F) were identified when 32 strains of diverse origins were analyzed by MLEE and incorporated into the cluster analysis. Group A was most dissimilar in comparisons with other groups and contained strain USDA 2124 (T24), which produces trifolitoxin and has unique symbiotic characteristics.

For 70 years serotyping has been used to monitor root nodule occupancy by antigenically distinct strains of *Bradyrhizobium* and *Rhizobium* species (1, 5, 6, 18, 22, 39). Serotyping allows researchers to gain a superficial picture of the diversity within native rhizobial populations at specific sites, to track the occurrence and distribution of antigenically related bacteria across states, countries, and continents, and to study the autecology of these bacteria in soil (4, 7, 13, 15, 17, 30, 33, 36, 38, 40, 42, 49, 53). Studies carried out with *Bradyrhizobium japonicum* (24, 34, 35, 43, 50, 51, 54) and *Rhizobium leguminosarum* (8, 19, 55) have shown that individual serotypes can be composed of nonidentical strains. As a result, concerns have been raised about the limitations of serological approaches to address various rhizobial phenomena of potential agricultural and ecological significance. In this regard, several studies conducted with *Escherichia coli* and *Legionella* and *Salmonella* species have shown that specific antigenic signatures can be restricted to closely related strains. In other cases, however, chromosomally dissimilar strains had the same O antigens, and in some cases closely related strains had no antigens in common (2, 3, 11, 45, 59, 64).

In an accompanying paper (39a), we showed that members of serotype AS6 of *R. leguminosarum* bv. trifolii are the dominant nodule occupants of annual clover species grown in Abiqua soil in Oregon, even though the soil at this location contains at least 13 antigenically distinct serotypes. Since serotype AS6 is antigenically diverse, we wanted to clarify the genetic relatedness of members of serotype AS6 to one another and to members of other serotypes. There have been several previous reports of *E. coli* and *Salmonella* studies in which multilocus enzyme electrophoresis (MLEE) was used for this purpose (2, 3, 11, 45). Although this method has been used extensively by *Rhizobium* researchers (16, 20, 26, 48, 65, 66) to reveal diversity among strains of various species previously, no one has demonstrated the ability of MLEE to assess genetic distances between rhizobial strains. Although various researchers have examined relatedness among strains from either culture collections (16, 20, 48) or soil populations (26, 65, 66), no one has compared a soil population from one location with strains of diverse origins. In this study, we analyzed strains of *R. leguminosarum* bv. trifolii of diverse origins to determine their similarities with an *R. leguminosarum* bv. trifolii population recovered from one location.

MATERIALS AND METHODS

***R. leguminosarum* bv. trifolii.** A total of 32 strains were obtained from various culture collections, and the histories of these strains are described in Table 1. A total of 200 *R. leguminosarum* bv. trifolii isolates were chosen from isolates belonging to the 13 antigenically distinct serotypes isolated from root nodules of clover species grown in Abiqua soil. A detailed description of the site used and the characteristics of

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TABLE 1. Reference strains of *R. leguminosarum* bv. trifolii

Strain	Origin	Remarks	Reference
Strains from culture collections			
CC275e	Tasmania, Australia	New Zealand inoculant strain	32
TA1 (= ANU794)	Tasmania, Australia	Australian inoculant strain for white clover	32
TA2	Tasmania, Australia	Antigenically related to TA1	32
WU95	Australia	Australian inoculant strain for subclover	12
CC2480a	Greece		9
UNZ29	New Zealand	Australian inoculant strain	18
NZP549	New Zealand		32
NZP560	New Zealand	New Zealand inoculant strain	32
NZP5117	New Zealand		32
SU202	Wales, United Kingdom		32
NZP550/2	United States	Inoculant strain for arrowleaf clover, LiphaTech Co., Milwaukee, Wis.	32
NA30	Australia	Host-specific ineffectiveness on Howard subclover	23
SU298 (= ANU843)	Unknown	Molecular model strain of *R. leguminosarum* bv. trifolii	55
WA67	Australia	Successful subclover inoculant under extreme conditions	12
CC2238b	Israel	Competitive and persistent nodule occupant of subclover	9
RBL5280 (= RCR5 (= LPR5045)	Unknown	Molecular model strain of *R. leguminosarum*	29
USDA 2046 (= ATCC 14479)	Virginia, 1934	From red clover	37
USDA 2048 (= ATCC 10328)	Illinois, 1934	From red clover	37
USDA 2090a (= ATCC 14484)	Georgia, 1954	From crimson clover	37
USDA 2116	South Carolina, 1944	From subclover	37
USDA 2117	South Carolina, 1958	From subclover	37
USDA 2124	Florida, 1937	From *T. dubium*	37
USDA 2152	Texas, 1952	From subclover	37
USDA 2154 (= 162X42)	California, 1970	From subclover	37
USDA 2155 (= 162X43)	California, 1970	From subclover	37
USDA 2160 (= 162X95)	California, 1976	From subclover	37
USDA 2088	Georgia, 1954	From crimson clover	37
Oregon strains (non-Abiqua soil sites)			
WS2-01	Powers, Oreg.	Most nodule-dominant serotype of subclover	15
WS1-01	Powers, Oreg.	Secondmost dominant nodule occupant of subclover	15
WS2-02	Powers, Oreg.	Thirdmost nodule-dominant serotype of subclover	15
WR27	Powers, Oreg.	Nodule-dominant serotype of red clover	63
WR26	Powers, Oreg.	Secondmost nodule-dominant serotype of red clover	63

the soil is presented elsewhere (7, 39a). The isolates were obtained from field and greenhouse experiments carried out between 1981 and 1991; information about their histories will be provided to readers upon request.

MLEE. (i) Preparation of cell extracts. Each *Rhizobium* isolate was grown in 100 ml of a defined glutamate-mannitol nutrient broth for about 60 h at 28°C. The cells were harvested by centrifugation at 16,300 × *g* for 15 min, resuspended in 1 ml of ice-cold Tris-EDTA buffer (5 mM disodium EDTA, 10 mM Tris-HCl; pH 7.6), and transferred to a 1.7-ml microcentrifuge tube. A 30-μl portion of 100 mM dithiothreitol and 20 μl of a freshly prepared lysozyme solution (50 mg ml^{-1} in Tris-EDTA buffer) were added to each cell suspension, which was incubated on ice for 1 h with occasional mixing. Each sample was sonicated for three 10-s intervals with a Branson Sonifier 200 equipped with a step-down microtip (power setting 6, 50% duty cycle). Each cell extract was cooled in an ice bath for at least 10 min between sonication intervals. The cell debris was removed by centrifugation at 12,500 × *g* and 4°C for 20 min, and each supernatant was divided equally into three microcentrifuge tubes and stored at −70°C. Enzyme analyses were performed over the next 4 or 5 days, since the xanthine dehydrogenase (XDH), nucleoside phosphorylase (NSP), glucose-6-phosphate dehydrogenase (G6P), and β-hydroxybutyrate dehydrogenase (HBD) activities deteriorated during prolonged storage.

(ii) Preparation of starch gel. Starch gels were prepared 1 day before they were used. A 17-g portion of hydrolyzed potato starch (catalog no. S-4501; Sigma) was suspended in 150 ml of the appropriate gel buffer in a 500-ml thin-walled Erlenmeyer flask. The starch suspension was heated over a Bunsen burner to its boiling point with constant agitation. The gel was aspirated immediately until large bubbles appeared (ca. 10 s) and was poured quickly into a Plexiglas mold (13 by 10 by 0.6 cm) constructed on top of a glass plate. The gel was allowed to cool at room temperature for approximately 1 h, covered with plastic wrap, and refrigerated overnight.

(iii) Loading the gel and electrophoresis. A vertical slit was cut along the whole 13-cm length of the gel 1.5 cm from the edge with a single-edged razor blade, and the slit was teased open to produce a 2-mm gap. The gel was placed in a precooled (2°C) horizontal electrophoresis unit (Bio-Rad, Richmond, Calif.) with the slit toward the cathodal side. The cell extracts were partially thawed, and a 10-μl portion of each extract was pipetted onto individual Whatman no. 3 filter paper strips (5 by 10 mm). The strips loaded with extract were placed into the slit of the gel and butted up against the starch with 1-mm intervals between the strips. Extracts of specific isolates containing enzyme electromorphs having known mobilities were placed at each end of the sample set to serve as internal references. Two strips loaded with amaranth dye (catalog no. A-1016; Sigma) were placed at each end of the gel to track the migration front of the electrode buffer. The slit was closed gently but firmly to hold the paper strips in place. The gel was connected to the two electrode buffer reservoirs with strips of paper towel laid with one end immersed in the

electrode buffer and the other end overlapping the edge of the gel by 1 to 2 cm. The proteins were allowed to migrate into the gel for 10 min under the voltage specified for the buffer system used (see below). The paper strips were removed, the gel was covered with a plastic film, and electrophoresis was continued with constant voltage for 2 to 3 h depending on the buffer system. Following electrophoresis, the gel was sliced horizontally into three 2-mm slices with nylon fishing line. Each gel slice was placed in a plastic box (15 by 10 by 2 cm) for an enzyme assay. An electrode buffer solution was used for three electrophoretic runs before it was replaced with fresh buffer. The following three different electrode-gel buffer combinations were used routinely at specific voltages: 0.69 M Tris—0.16 M citrate (pH 8.0) was used as the electrode buffer in combination with a 1/30 dilution of the same buffer as the gel buffer at 130 V, 0.22 M Tris—0.09 M citrate (pH 6.3) was used as the electrode buffer in combination with 0.008 M Tris—0.003 M citrate (pH 6.7) as the gel buffer at 150 V, and 0.3 M boric acid neutralized with NaOH to pH 8.2 was used as the electrode buffer in combination with 10 mM Tris-HCl (pH 8.5) as the gel buffer at 200 V.

(iv) Enzyme assays. A total of 13 enzymes were assayed after electrophoresis in the following buffers: Tris-citrate electrode buffer (pH 8.0) (isocitrate dehydrogenase [IDH], β-galactosidase [BGA], malate dehydrogenase [MDH], phosphoglucoisomerase [PGI], superoxide dismutase [SOD], leucyl-glycine peptidase [PEP], xanthine dehydrogenase [XDH], glucose-6-phosphate dehydrogenase [G6P], and nucleoside phosphorylase [NSP]); Tris-citrate electrode buffer (pH 6.3) (6-phosphogluconate dehydrogenase [6PG] and β-hydroxybutyrate dehydrogenase [HBD]); and borate electrode buffer (pH 8.2) (adenylate kinase [ADK] and phosphoglucomutase [PGM]). The gel slices were incubated at room temperature with appropriate mixtures of reagents to locate the positions of specific enzymes. The reagents used for each enzyme were essentially those described elsewhere (57). In many cases, quantities of reagents were reduced considerably and enzyme activity was not affected. The gel slices were incubated in the dark, and the incubation periods ranged from 10 min to 4 h. When the bands were clear, the gel slices were rinsed with deionized water and fixed in a mixture containing acetic acid, methanol, and deionized water (1:4:5, vol/vol/vol).

(v) MLEE data analysis. Each isolate was characterized by its own combination of electromorphic variants of the 13 enzymes assayed. A distinct electromorphic profile was referred to as an electrophoretic type (ET). The genetic diversity (h) at an enzyme locus was calculated as follows: $h = (1 - \Sigma x_i^2)\,[n/(n-1)]$, where x_i is the frequency of the ith allele at the locus and n is the number of ETs in the population considered. The relative similarities among ETs were revealed by using a cluster analysis program designed by T. S. Whittam (Pennsylvania State University) and were displayed in the form of a dendrogram.

To determine the extent to which populations exhibit nonrandom combinations of alleles between loci, an observed allelic mismatch frequency distribution was obtained by comparing each ET with every other ET once (for a total of $n[n-1]/2$ comparisons, where n is the number of ETs), and for each paired comparison the number of dissimilar alleles (mismatches) was recorded. An equation for computing the variance of this distribution (V_o) has been derived previously (10) and is given by:

$$V_o = \sum_j h_j - \sum_j h_j^2 + 2\sum_j \sum_{l>j} \sum_i \sum_k (2p_{ji}\,p_{lk}\,D_{ik,jl} + D_{ik,jl}^2)$$

where h_j is the genetic diversity at the jth locus and p_{ji} and p_{lk} are the frequencies of the ith allele at the jth locus and the kth allele at the lth locus, respectively. $D_{ik,jl}$ is the coefficient of linkage disequilibrium for the specific alleles at the corresponding loci and equals the difference between the observed and expected ($p_{ji}\,p_{lk}$) frequencies of the diallelic genotype in the population. Summation is carried out over the range between 1 and the number of loci examined for the j and l indices (constraining $l > j$ ensures that each locus pair is considered only once) and between 1 and the number of alleles at the respective locus for the i and k indices. If a population has random genetic structure (linkage equilibrium), all of the diallelic disequilibrium values ($D_{ik,jl}$) are equal to zero. In this case, the equation reduces to the difference between the first two terms, which is the expected variance (V_e) of the distribution

$$(V_e = \sum_j h_j - \sum_j h_j^2)$$

and can be calculated directly from the single-locus diversity values (h) of the observed data set. Thus, the inflation of the observed variance over the expected variance is related to the extent to which the population exhibits nonrandom genetic structure. The ratio of V_o (the observed variance in the distribution of the number of mismatched loci over $n[n-1]/2$ paired ET comparisons) to V_e (the expected variance of such a distribution if all alleles were randomly associated) is a measure of the linkage disequilibrium in a population. With increasing linkage disequilibrium the ratio of V_o to V_e increases, and the inflation of V_o over V_e can be used to test the significance of linkage disequilibrium levels. In practice, the linkage disequilibrium statistics and allelic mismatch distributions described above were computed with the assistance of a program written by T. S. Whittam following translation by one of the authors (S.R.S.) from FORTRAN to the C programming language with minor modifications.

Comparison of MLEE with REP and ERIC PCR. To further determine whether MLEE data are good indicators of overall genetic relatedness among *Rhizobium* strains, multiple isolates were chosen from each of four ETs, and the profiles of DNA fragments generated by the PCR when repetitive extragenic palindromic (REP) and enterobacterial repetitive intergeneric consensus (ERIC) sequences were used as primers were determined as described elsewhere (14). Similarities among the isolates were evaluated by comparing the banding patterns observed for the isolates (both REP-specific and ERIC-specific patterns). Banding patterns were coded by determining the total number of unique bands observed in all of the isolates examined. For each isolate, each band position was assigned a 1 or a 0 to indicate the presence or absence of the band, respectively. For a pair of isolates, a simple matching coefficient was calculated as the sum of the number of bands present in both isolates and the number of bands absent from both isolates, divided by the total number of bands observed in all of the isolates examined. Similarity among the isolates was determined by constructing a dendrogram from the REP and ERIC PCR data by using the NTSYS-pc analysis package (version 1.50; Exeter Software, Setauket, N.Y.).

Symbiotic effectiveness of the major and minor nodule-occupying ETs on different clover species. Seeds of cultivar Nangeela subclover (*Trifolium subterraneum*), cultivar Florie red clover (*Trifolium pratense*), cultivar Tibbee crimson clover (*Trifolium incarnatum*), cultivar Yucchi arrowleaf clover (*Trifolium vesiculosum*), common white clover (*Trifolium repens*), and alsike clover (*Trifolium hybridum*) were surface disinfested

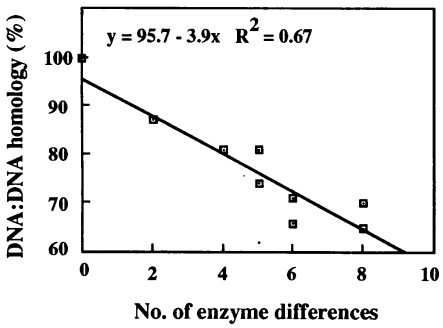

FIG. 1. Comparison of genetic relatedness among strains of *R. leguminosarum* bv. trifolii as determined by MLEE of 13 enzyme loci and by DNA reassociation (32). Strain CC275e was the reference strain.

by immersing them in 95% (vol/vol) ethanol for 30 s and in 25% (vol/vol) Clorox bleach for 5 min and then washing them in seven changes of sterile deionized water. The seeds were incubated on sterile water agar (1.5%, wt/vol) plates for 24 h at 4°C and then for another 24 h at room temperature. The seedlings were transplanted to N-free mineral salts agar slants as described elsewhere (19). Three ET 2 isolates and three ET 3 isolates were grown in 30-ml portions of glutamate-mannitol nutrient broth for 48 h at 28°C. Eight replicate seedlings of red clover, white clover, and alsike clover and five replicate seedlings of arrowleaf clover, crimson clover, and subclover were inoculated with 1-ml portions (approximately 5×10^8 cells) of each isolate. Uninoculated control seedlings received either 3-ml aliquots of sterile 18 mM KNO_3 at 7, 21, and 35 days after planting or 3-ml aliquots of deionized water. The seedlings were grown under greenhouse conditions as described previously (19). Herbage was removed from the plants 6 weeks after inoculation and dried at 55°C for 1 week, and then dry weights were determined. The yield data were analyzed by a one-way analysis of variance, and least-significant-difference values were calculated.

Determination of substrate utilization patterns with BIOLOG plates. BIOLOG plates (BIOLOG, Inc., Hayward, Calif.) were used to produce biotypic fingerprints for four ET 2 isolates, four ET 3 isolates, four ET 12 isolates, and four ET 33 isolates. The isolates were grown to the early log phase in defined glutamate-mannitol medium, harvested, washed twice in 50 mM K_2HPO_4 (pH 7.0), and resuspended to a density of 10^7 cells per ml in a solution containing (per liter) 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4$, and 0.08 g of $CaCl_2$. The cells were transferred to a multiwell inoculator plate, and then a 200-μl portion was inoculated into each well on the BIOLOG plates. The plates were incubated at 27°C for 24 h, and the respiratory responses were evaluated under subdued lighting. Either a negative respiratory response or a positive respiratory response was recorded for each of the substrates. The BIOLOG data were converted to two-dimensional binary matrix data (1, positive respiratory response; 0, no response) and were analyzed with the NYSYS-pc biostatistical analysis program (Applied Biostatistics, Inc.). Dendrograms were generated with the SIMQUAL (similarity for quantitative data) and SAHN (sequential agglomerative hierarchical and nested) clustering subprograms of NTSYS-pc.

RESULTS

Relatedness estimates obtained from MLEE, DNA-DNA hybridization, and REP ERIC PCR studies are correlated.

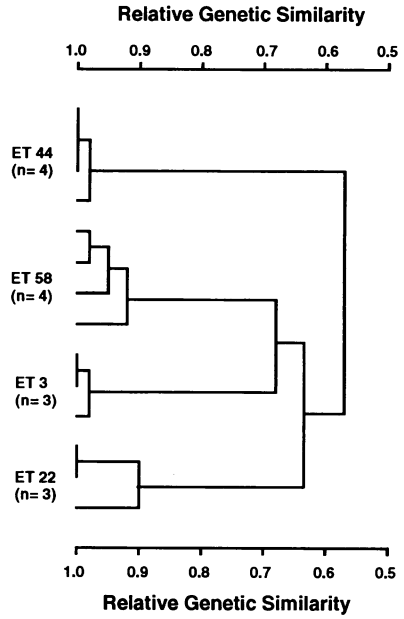

FIG. 2. Dendrogram of similarities between isolates of the same ETs. Data were derived from PCR fingerprints generated by using REP and ERIC PCR primers. The relationships were generated from combined REP and ERIC data sets.

Jarvis et al. (32) used DNA-DNA hybridization to determine the relatedness of several strains of *R. leguminosarum* bv. trifolii to a standard strain, strain CC275e. We conducted an MLEE analysis with 11 of the same strains and obtained a positive correlation ($r^2 = +0.67$; $P < 0.01$) between the strain similarities predicted by DNA-DNA hybridization and the strain similarities predicted by MLEE (Fig. 1). In addition, we performed an electrophoretic analysis of PCR products generated from chromosomal DNA by using reiterated DNA sequences as the primers (14, 34). Chromosomal DNAs were recovered from three or four rhizobial isolates representing each of four different ETs. Although isolates of the same ET were not always identical, all isolates of an ET were more tightly clustered with each other than with isolates of other ETs (Fig. 2). On the basis of these comparisons, we are confident that MLEE data provide a reasonable indicator of the overall genetic relatedness among the populations of *R. leguminosarum* bv. trifolii that we examined.

Nodule-dominant isolates from Oregon sites are clustered in 2 of 11 subpopulations of *R. leguminosarum* bv. trifolii identified by MLEE. A total of 53 ETs were identified among the 200 isolates of the 13 serotypes recovered from the Abiqua soil (Table 2), and the mean diversity value per enzyme locus was 0.46 (Table 3). The 32 strains obtained from diverse sources were represented by 29 ETs (Table 4), and the overall allelic diversity of these organisms (diversity value, 0.57) was not significantly different from that of the Abiqua soil population (Table 3). We found that two of the isolates obtained from diverse sources had ETs identical to those of Abiqua soil isolates. Isolates WS2-01 and WS2-02, which represent two serotypes that dominate nodules of subclover at the Powers site in southern Oregon, had the same ETs (ETs 7 and 8) as the majority of the isolates belonging to the nodule-dominant serotype in the Abiqua soil population (serotype AS6-c) (Tables 2 and 4).

The relative similarities among the 82 ETs studied were

TABLE 2. Distribution of 53 allelic profiles (ETs) among the serotypes of *R. leguminosarum* bv. trifolii recovered from Abiqua soil

Serotype	Group[a]	ET	No. of isolates[b]	Alleles at the following enzyme loci[c]:												
				IDH	BGA	G6P	MDH	PGI	PEP	XDH	NSP	6PG	HBD	ADK	PGM	SOD
AS6-a	B	2	23	1	6	1	1	2	4	6	8	15	11	2	2	2
AS6-b	B	3	18	1	6	1	1	2	4	6	8	15	11	2	2	4
	H1	1	1	4	2	1	1	2	2	2	4	2	8	4	2	2
	H1	13	1	4	2	1	1	2	2	2	2	2	7	4	2	2
AS6-c (AS21, unknown)[d]	B	7	13	1	6	1	1	2	4	6	8	2	11	2	2	4
	B	8	13	1	6	1	1	2	4	2	6	2	15	2	2	4
	B	9	1	1	6	1	1	3	4	6	6	2	11	2	2	2
	B	10	2	1	6	1	1	3	4	6	6	2	11	4	2	2
	B	32	2	1	6	1	1	2	4	1	6	2	15	2	2	4
	G	6	1	4	3	1	1	2	2	3	2	2	3	4	2	4
AP17	B	33	10	1	6	1	1	2	4	6	8	2	11	2	2	2
AS21 (AP11)[d]	G	12	31	2	3	1	1	2	4	4	2	2	4	4	2	4
	G	11	4	2	3	1	1	2	4	4	2	4	16	4	2	4
	G	15	1	4	3	1	1	2	2	4	2	2	4	4	2	4
AR23	H1	24	1	5	2	1	1	5	2	2	4	2	11	4	2	2
	H1	27	1	4	2	1	1	5	2	2	4	2	11	4	2	2
	J	28	2	3	3	1	1	4	6	2	4	2	11	2	2	4
	J	23	6	4	3	1	1	4	6	2	4	2	11	2	2	4
	J	53	1	4	7	1	1	4	6	2	4	2	4	2	2	4
	J	22	1	5	2	1	1	5	2	4	4	2	4	2	2	4
	J	25	1	5	2	1	1	2	6	2	2	2	3	2	2	4
AS27	H1	4	1	4	2	1	1	2	9	2	4	2	8	4	2	2
	H1	5	1	4	2	1	1	2	2	2	2	2	8	4	2	2
	H1	31	7	4	2	1	1	2	2	2	4	2	4	4	2	2
	I	29	2	4	7	1	1	4	2	3	4	4	0	2	2	2
	I	30	3	4	9	1	1	2	9	3	4	4	4	4	2	8
AS16	H1	34	1	4	6	1	1	2	9	2	2	2	7	4	2	2
	H1	35	3	4	7	1	2	2	9	2	2	2	8	4	2	2
	H1	36	1	4	7	1	2	2	9	2	2	2	8	4	2	2
	H1	37	1	4	7	2	1	2	6	2	2	2	8	4	2	2
AG4	H1	16	14	4	9	1	1	2	9	2	4	2	8	4	2	4
	H1	18	2	4	9	1	1	2	9	2	4	2	8	4	2	2
	I	17	3	4	9	1	1	4	9	3	4	4	4	2	2	4
	I	20	2	4	9	1	1	2	9	3	4	4	4	2	2	4
	I	19	1	4	9	1	1	2	9	2	2	4	4	2	2	4
	J	21	1	4	2	1	1	2	6	2	2	2	8	2	2	4
AS36	E	39	2	4	9	2	1	3	9	8	2	2	16	4	2	4
	H1	41	1	4	2	1	1	4	2	2	2	2	4	4	2	2
	H1	44	1	4	3	1	1	6	2	4	4	2	4	4	2	2
	I	40	2	4	2	1	1	2	2	2	4	4	4	4	2	4
	I	43	1	4	2	1	1	2	9	2	4	4	4	4	2	4
	I	38	1	4	2	1	1	2	2	2	4	4	4	2	2	2
	I	42	1	4	2	1	1	4	2	2	4	4	4	2	2	4
AR6	H1	45	3	4	2	10	1	2	2	2	4	2	8	4	2	2
	H1	46	1	4	2	10	1	2	2	2	4	4	8	4	2	2
AV5	H1	48	1	4	9	1	1	2	2	2	4	4	4	4	2	4
	H1	49	1	4	9	1	1	2	2	2	4	2	4	4	2	4
	H1	50	1	4	9	1	1	2	2	3	4	2	4	4	2	4

Continued on following page

TABLE 2—*Continued*

Serotype	Group[a]	ET	No. of isolates[b]	Alleles at the following enzyme loci[c]:												
				IDH	BGA	G6P	MDH	PGI	PEP	XDH	NSP	6PG	HBD	ADK	PGM	SOD
AP1	H1	51	1	4	9	1	1	4	2	2	2	2	4	4	2	2
	I	52	1	4	7	1	1	4	10	2	4	4	0	2	2	2
AWI	H2	54	1	4	1	1	1	2	4	2	8	2	2	4	2	2
Unknown[e]	H1	14	1	4	3	1	1	2	2	2	4	2	8	4	2	4
	G	47	2	2	3	1	1	2	4	4	2	2	16	4	2	4

[a] See Fig. 3 for descriptions of groups.
[b] A total of 200 isolates were examined.
[c] The numbers of alleles for the enzyme loci were as follows: IDH, 6; BGA, 6; G6P, 3; MDH, 2; PGI, 5; PEP, 5; XDH, 6; NSP, 4; 6PG, 3; HBD, 10; ADK, 2; PGM, 1; and SOD, 3. The locus diversity values for the enzyme loci were as follows: IDH, 0.48; BGA, 0.79; G6P, 0.14; MDH, 0.04; PGI, 0.48; PEP, 0.73; XDH, 0.60; NSP, 0.64; 6PG, 0.43; HBD, 0.77; ADK, 0.48; PGM, 0; and SOD, 0.53.
[d] Parentheses indicate that isolates of different serotypes had the same ET.
[e] Not related antigenically to any known serotype.

analyzed by an unweighted pair group method with averages cluster analysis and were expressed visually as a dendrogram (Fig. 3). The 232 isolates were divided into 11 distinct groups (groups A through J) which clustered with each other at relative dissimilarity values of >0.46; since the separation of the subgroup H1 and H2 isolates was not quite as large, we arbitrarily assigned these organisms to subgroups. The Abiqua soil isolates were found in 7 of the 11 groups (groups B, E, G, H1, H2, I, and J). Another four groups (groups A, C, D, and F) were identified when the 32 strains of diverse origins were taken into consideration. The isolates were distributed unevenly among the groups (Fig. 3). Although group B contained only 8 of the 53 ETs from Abiqua soil, it contained 82 (41%) of the 200 isolates from that soil population, and the majority of these (67 isolates) were in ETs 2, 3, 7, and 8 (Fig. 4). In addition, strains WS2-01, WS2-02, and WR27, which represent the nodule-dominant serotypes of red clover and subclover grown in soil from the Powers site in Oregon, were also found in group B. Only one isolate (CC2238b) from outside Oregon clustered with the group B isolates.

Although group G contained only five ETs from the Abiqua soil collection, one of them (ET 12) accounted for 31 isolates (Table 2) and belonged to serotype AS21 (the second most common serotype recovered from clover nodules at the Abiqua soil site). In addition, isolates WS1-01 and WR26 were found in group G (Table 4); these isolates also belonged to serotype AS21 and represented the second most dominant serotype recovered from nodules of subclover and red clover grown in soil from the Powers site. Group H1 contained a high percentage of the ETs from the Abiqua soil site (22 of 54 ETs) but only 47 of the 200 isolates. Only two ETs from this group were represented by substantial numbers of isolates (7 and 14 isolates of ETs 31 and 16, respectively).

TABLE 3. Genetic diversity within the local and diverse-origin populations of *R. leguminosarum* bv. trifolii

Origin of population	No. of isolates	No. of ETs	Average no. of alleles per locus	Diversity (ET basis)[a]
Abiqua soil	200	53	4.2	0.46 (0.07)
Diverse	32	29	4.7	0.57 (0.07)

[a] Overall genetic diversity was the average of the individual locus diversity values (h) and was calculated as follows: $(1 - \Sigma x_i^2) \cdot [n/(n-1)]$, where x_i is the frequency of the ith allele at the specific locus and n is the number of ETs in the population considered.

The MLEE analysis showed that several strains of *R. leguminosarum* bv. trifolii which have been used extensively (i) in studies of competitive nodulation (USDA 2124 [= T24], group A), (ii) in nodulation gene identification and regulation studies (ANU843, group D; RBL5280, group E), (iii) as commercial inoculant strains for subclover (WU95 and 162X95, group F), or (iv) as representatives of American Type Culture Collection type strains (USDA 2046, USDA 2048, and USDA 2090a, group H2) were dissimilar to members of the Abiqua soil population or were similar only to rare group E (ET 39, serotype AS36) and group H2 (ET 54, serotype AW1) isolates.

Multilocus structure within the Abiqua soil population of *R. leguminosarum* bv. trifolii. The Abiqua soil population showed evidence of statistically significant nonrandom association of alleles in multilocus combinations. The extent of the multilocus structure was assessed by comparing the variance in the distribution of the numbers of allelic mismatches between all pairs of the 53 ETs studied ($n[n-1]/2$ comparisons, where n is the number of ETs) with the variance expected if the associations among alleles at the different loci were completely random (10). The observed and expected variances of the mismatch distribution were 4.176 and 2.414, respectively, and the upper 95% confidence limit of the expected variance was 3.311. Since the observed variance was greater than expected, we inferred that there is a significant level of nonrandom multilocus structure in the Abiqua soil population. To determine which of the groups contributed to the overall genetic structure within the population, the multilocus structure was examined by using subsets of the Abiqua soil population and omitting each of the groups one at a time. Group B was identified as the major reason for the observed variance exceeding the expected variance of allelic mismatch distribution.

Serotype-ET relationships among the *R. leguminosarum* bv. trifolii isolates. The MLEE data show that 72 of 75 serotype AS6 isolates are found in group B (Table 2). All ET 2 and 3 isolates had the antigenic signatures of AS6-a and AS6-b, respectively, while 31 of 32 subtype AS6-c isolates were distributed among another five ETs within group B. Of the three AS6 isolates not found in group B, one subtype AS6-c isolate (88FL1,1) and two subtype AS6-b isolates (AS6-1 and

TABLE 4. Allelic profiles of the 32 isolates of *R. leguminosarum* bv. trifolii of diverse origins[a]

Isolate(s)	Group[b]	ET	Alleles at the following enzyme loci[c]:												
			IDH	BGA	G6P	MDH	PGI	PEP	XDH	NSP	6PG	HBD	ADK	PGM	SOD
USDA 2124	A	78	2	7	2	2	3	4	4	6	15	2	6	2	2
USDA 2152	A	80	2	7	2	2	2	4	5	8	15	2	6	2	2
WS2-02	B	7	1	6	1	1	2	4	6	8	2	11	2	2	4
WS2-01	B	8	1	6	1	1	2	4	2	6	2	15	2	2	4
WR27	B	57	1	6	1	1	2	5	6	6	2	11	2	2	2
CC2238b	B	60	1	7	1	1	2	4	6	8	2	2	2	2	4
USDA 2117	C	76	1	2	1	1	4	4	4	6	4	2	4	2	2
SU298 (= ANU843)	D	69	3	7	1	1	6	8	2	6	15	6	4	2	2
RBL5280 (= RCR5)	E	71	4	6	2	1	3	1	1	4	2	2	4	2	4
NZP549	F	63	2	7	1	1	3	6	4	0	2	17	6	2	4
NZP5117	F	66	2	3	1	1	3	5	3	1	2	2	6	2	2
WU95 and NA30[d]	F	70	2	3	1	1	3	5	5	2	2	2	6	2	2
USDA 2154	F	72	2	7	1	1	1	4	5	2	2	2	6	2	2
USDA 2155	F	75	4	3	1	1	3	4	4	2	2	2	6	2	2
USDA 2160 (= 162X95)	F	82	2	3	1	1	3	5	4	2	2	2	6	2	2
WS1-01	G	55	2	3	1	1	2	5	4	2	2	4	4	2	4
WR26 and CC275e[d]	G	56	2	3	1	1	2	5	4	2	2	16	4	2	4
SU202	G	61	2	3	1	1	2	5	5	0	5	17	6	2	4
NZP560	G	65	2	3	1	1	4	5	4	0	2	16	4	2	4
UNZ29	H1	67	4	6	1	1	2	6	2	2	2	7	4	2	2
CC2480a	H2	59	4	4	1	1	2	2	5	8	2	2	4	2	2
NZP550/2	H2	64	6	2	1	1	2	5	2	6	2	2	4	2	2
USDA 2116	H2	74	6	1	1	1	2	4	2	6	2	2	4	2	2
USDA 2090a (= ATCC 14484)	H2	77	5	1	1	1	3	4	2	8	2	2	4	2	2
USDA 2046 (= ATCC 14479)	H2	79	4	2	1	1	1	5	4	8	2	2	4	2	2
USDA 2048 (= ATCC 10328)	H2	81	5	2	1	1	3	5	2	8	2	2	4	2	2
TA1 and TA2[d]	I	58	4	2	1	1	2	2	3	4	5	4	2	2	2
WA67	J	68	5	2	1	1	2	2	3	2	2	4	2	2	4
USDA 2088	J	73	4	3	1	1	1	3	2	8	2	4	4	2	4

[a] See Table 1 for strain origins and histories.
[b] See Fig. 3 for group descriptions.
[c] The numbers of alleles for the enzyme loci were as follows: IDH, 6; BGA, 6; G6P, 2; MDH, 2; PGI, 5; PEP, 7; XDH, 7; NSP, 6; 6PG, 4; HBD, 9; ADK, 3; PGM, 1; SOD, 2; and SKD, 6. The locus diversity values for the enzyme loci were as follows: IDH, 0.78; BGA, 0.80; G6P, 0.19; MDH, 0.19; PGI, 0.68; PEP, 0.60; XDH, 0.82; NSP, 0.81; 6PG, 0.40; HBD, 0.70; ADK, 0.64; PGM, 0; and SOD, 0.48.
[d] Two isolates had the same ET.

AR18) were in groups G and H1, respectively. These isolates differed radically from their serotype relatives in group B by mismatches at 8 to 9 of the 13 loci.

Group B contained several isolates that did not possess antigens of the serotype AS6 complex (Tables 2 and 4). ET 33 isolates differ from AS6-a (ET 2) and AS6-b (ET 3) isolates at only one and two loci, respectively, and yet do not react with antisera that define the AS6 complex. Antiserum AP17 (raised against isolate ET33-1) reacts exclusively with ET 33 isolates. Although ET 7 consists primarily of isolates that have the AS6-c antigenic signature, two isolates were antigenically different. One isolate (88FL1,6/2) cross-reacted identically with antiserum AS21 (ET12-1 in group G). The other isolate (FL1,18) was not reactive with any of the antisera in our collection. Group B isolate WR27 (from the Powers site) belongs to serotype AS16, yet the AS16 isolates recovered from Abiqua soil clustered exclusively in group H1. Although isolates WS2-01 and WS2-02 had the same ETs as AS6-c isolates, they were antigenically unrelated to the AS6 complex.

Table 5 summarizes the distribution of the 13 serotypes found in the Abiqua soil population among the MLEE-derived groups. Serotype AP1, AW1, AR6, AV5, AP11, AS16, and AS17 isolates were restricted exclusively to specific groups. In the cases of serotypes AG4, AR23, AS27, and AS36, significant numbers of isolates with similar antigenic signatures were distributed in two or three of the groups. For example, 16 AG4 isolates belonged to ETs 16 and 18 in group H1, and there was only one allelic mismatch in these organisms (Table 2). A similar situation was observed with group I, in which six AG4 isolates belonged to ETs 17, 19, and 20, which mismatched with each other at one to three loci. However, there was an average of six allelic mismatches between the two clusters of ETs from serotype AG4 in groups H1 and I.

Phenotypic characteristics of ET 2 and 3 isolates. Although MLEE provided an overall picture of the chromosomal types found in the Abiqua soil isolates, we tried to obtain additional information concerning the phenotypic characteristics of isolates representing both major and minor nodule-occupying ETs. Despite the fact that ET 2 and 3 isolates were well represented in nodules of annual clovers and rare in nodules of perennials grown in Abiqua soil (39a), these isolates were either ineffective or suboptimally effective on both classes of clover species (Table 6). The symbiotic effectiveness properties of the three isolates of each ET were similar. Only in the case of white clover were ET 2 isolates more effective than ET 3 isolates.

BIOLOG data comparisons revealed that the isolates of an ET clustered together remarkably well (Fig. 5). The ET 33 isolates were most dissimilar from the other isolates and respired fewer (38 to 43) of the 95 substrates in the BIOLOG plates than the ET 2 isolates (45 to 48 substrates respired), ET 3 isolates (49 to 55 substrates respired), or ET 12 isolates (47 to 50 substrates respired). Differences in substrate utilization patterns indicated that the isolates of an ET were not identical

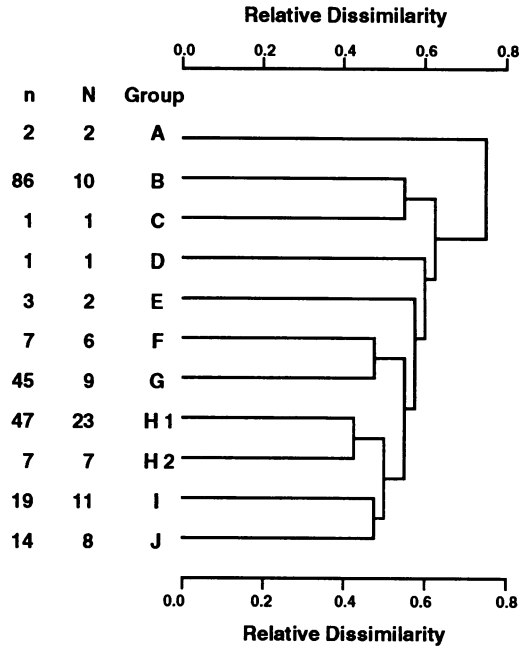

FIG. 3. Genetic relationships of 82 ETs of *R. leguminosarum* bv. trifolii. The dendrogram was generated by the average linkage method of clustering by using 13 enzyme loci. Each group represents a cluster of ETs that are ≥0.46 dissimilar. The number of ETs (N) and the number of isolates (n) in each group are indicated.

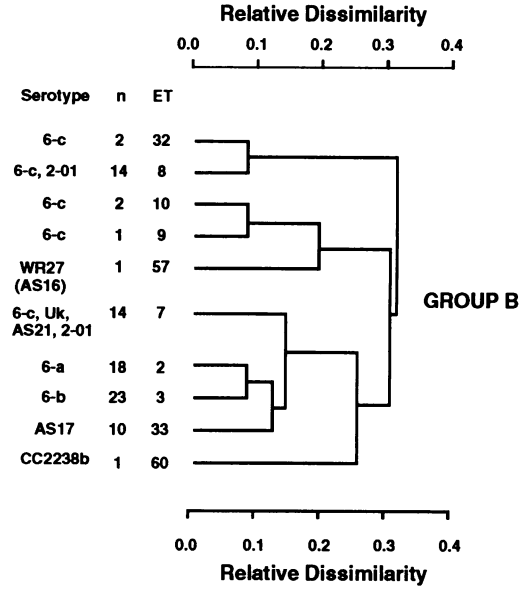

FIG. 4. Genetic relationships of 10 ETs of *R. leguminosarum* bv. trifolii clustered within group B. The number of isolates (n) within each ET and the serotypic affiliations are indicated. Uk, unknown.

and that the isolates of some ETs were more closely related than the isolates of other ETs (e.g., ET 12 compared with ET 33).

DISCUSSION

Confidence in the ability of MLEE to provide insight into intraspecies relationships among procaryotes improved when researchers established a positive correlation between the relatedness of *E. coli* strains established by DNA-DNA hybridization and the relatedness determined by MLEE (46). Since that time such relationships have been confirmed in *Legionella* spp. (59), *Streptococcus* spp. (25), and *Bacillus subtilis* (31). Although there have been several previous MLEE studies to reveal the diversity among strains of *R. leguminosarum* and *Rhizobium meliloti* (16, 20, 26, 48, 65, 66), the data in this paper are the first data which confirm that there is a positive correlation among overall relatedness of *Rhizobium* strains established by MLEE, relatedness determined by DNA-DNA hybridization (32), and relatedness determined by a repetitive sequence PCR technique (14).

Our findings place an indigenous soil population of *R. leguminosarum* bv. trifolii in perspective with strains that are well characterized. Obviously, neither extensively studied laboratory strains nor inoculant strains nor American Type Culture Collection type strains reflect the diversity within *R. leguminosarum* bv. trifolii as a whole. At this time it is unclear to what extent the diversity the Abiqua soil population represents other soil populations either in the immediate vicinity of this site or at other locations throughout the world. Our data reveal that the chromosomal types within groups A and B of *R. leguminosarum* bv. trifolii are genetically dissimilar from the other chromosomal types. Previous MLEE studies revealed that *R. meliloti* and *Phaseolus*-nodulating *Rhizobium* spp. are composed of strains so different that they must have been genetically separated for many years. It was hypothesized that such separation occurred when different species of *Medicago* and *Phaseolus* evolved in geographically separate locations (20, 41, 60). There is precedent for the same concept in *Trifolium* species, which are thought to have had multiple centers of origin (61). Unique host-*Rhizobium* idiosyncrasies exist be-

TABLE 5. Distribution of isolates from the 13 serotypes among the seven MLEE-derived groups of *R. leguminosarum* bv. trifolii found in Abiqua soil[a]

Group	No. of isolates in serotype:													
	AW1	AP1	AG4	AV5	AR6	AS6	AP11	AS16	AP17	AS21	AR23	AS27	AS36	Unknown
B						72			10	1				1
E													2	
G						1	1			35				2
H1			16	3	4	2		6			2	9	2	1
H2	1													
I		1	6									5	5	
J			1								11			

[a] Data summarized from Table 2.

TABLE 6. Symbiotic effectiveness of ET 2 and 3 isolates on perennial and annual clover species

Plant treatments	Shoot dry wt (mg/plant)[a]					
	Perennial species			Annual species		
	White clover	Alsike clover	Red clover	Subclover	Crimson clover	Arrowleaf clover
Nitrate	26.7	23.5	27.0	44.9	49.7	21.9
ET2-2	11.8[b]	13.8[b]	4.5[c]	29.5[b]	25.3[b]	3.4[c]
ET2-3	14.2[b]	18.4[b]	3.3[c]	29.2[b]	25.8[b]	2.7[c]
ET2-4	10.2[b]	17.7[b]	3.2[c]	30.2[b]	28.0[b]	3.2[c]
ET3-1	2.3[c]	10.3[b]	2.5[c]	36.5[b]	24.8[b]	3.1[c]
ET3-2	3.2[c]	11.3[b]	3.1[c]	28.0[b]	18.6[b]	2.6[c]
ET3-4	3.0[c]	11.4[b]	4.3[c]	36.1[b]	18.7[b]	3.2[c]
Uninoculated	1.3	1.6	4.8	18.7	15.1	3.3

[a] Means of five replicates per treatment for subclover, crimson clover, and arrowleaf clover and means of eight replicates per treatment for red clover, white clover, and alsike clovers.
[b] Mean shoot dry weight was significantly greater than that of uninoculated control plants but less than that of nitrate-supplemented plants.
[c] Shoot dry weight was not greater than that of uninoculated control plants.

tween *Trifolium* species and strains of *R. leguminosarum* bv. trifolii. For example, African species of clover do not form effective associations with strains of *R. leguminosarum* bv. trifolii that are of European and American origins (21, 44, 52), and Kura clover (*Trifolium ambiguum*) has a specific *Rhizobium* strain requirement (27, 47). Certainly, the MLEE data corroborate the unusual phenotypic characteristics reported for group A strain USDA2124 (56, 62) and indicate that this lineage merits further attention from both ecological and phylogenetic perspectives.

Although the group B strains were not as distinct as the group A strains, they nevertheless possessed allelic variants of IDH, BGA, and 6PG that were not found in other members of the Abiqua soil population. Furthermore, group B was identified as the primary creator of multilocus structure in the Abiqua soil population. It is interesting that CC2238b was the only strain examined from outside Oregon that belonged to group B. Demezas et al. (16) reported that CC2238b and CC2247d had the same ET and were recovered from different sites in Israel. Since subclover is a Mediterranean native which was introduced into North America about 70 years ago (61), it is entirely possible that members of group B are aliens to the western United States and arrived with their host plants. Equally plausible, however, is the possibility that group B members are native rhizobia adapted serendipitously to subclover because of their long association with native annual clover species (28). More research will be required to resolve this issue.

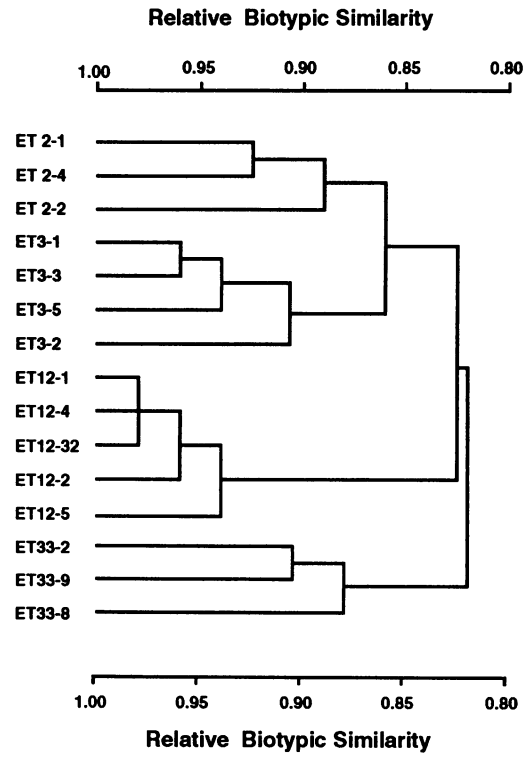

FIG. 5. Dendrogram showing the similarities between isolates of the same ETs derived from BIOLOG and substrate utilization patterns.

Our findings have significance for the use of serological techniques for studying *Rhizobium* ecology. Although antigens of the AS6 serotype complex were almost exclusively located within group B, it is significant that group B also contained nodule-dominant serotypes from another location and that these serotypes were antigenically unrelated to serotype AS6. Without the MLEE data, the connection between the two populations would have been overlooked. Our observation that some antigenic signatures are restricted to very similar ETs, whereas others are found in distinctly different groups, is entirely consistent with the findings obtained with *E. coli*, *Salmonella* spp., and *Legionella* spp. (2, 3, 11, 45, 59) and with *B. japonicum* (34). It is interesting that the 6-phosphogluconate dehydrogenase locus (*gnd*) was the only locus that differentiated between the antigenically distinct groups ET 2 and ET 33. A similar difference was observed for members of *E. coli* serogroups O1, O2, and O18. Since the *gnd* locus is closely linked to a region that encodes O-antigen biosynthesis on the *E. coli* chromosome, Selander et al. (58) speculated that a recombination event might have transferred both a novel O-antigen and a *gnd* locus simultaneously to another bacterial chromosome. The outcome of such an event was a novel serotype and a chromosome almost identical to the original serotype chromosome. In our work, this type of speculation must be tempered, since we have no knowledge concerning the proximity of the *gnd* locus and O-antigen genes on the *R. leguminosarum* chromosome, nor has a potential donor ET containing the antigen of AP17 been found elsewhere in the population.

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