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Variation of Clonal, Mesquite-Associated Rhizobial and Bradyrhizobial Populations from Surface and Deep Soils by Symbiotic Gene Region Restriction Fragment Length Polymorphism and Plasmid Profile Analysis

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Genetic characteristics of 14 *Rhizobium* and 9 *Bradyrhizobium* mesquite (*Prosopis glandulosa*)-nodulating strains isolated from surface (0- to 0.5-m) and deep (4- to 6-m) rooting zones were determined in order to examine the hypothesis that surface- and deep-soil symbiont populations were related but had become genetically distinct during adaptation to contrasting soil conditions. To examine genetic diversity, Southern blots of *Pst*I-digested genomic DNA were sequentially hybridized with the *nodDABC* region of *Rhizobium meliloti*, the *Klebsiella pneumoniae nifHDK* region encoding nitrogenase structural genes, and the chromosome-localized *ndvB* region of *R. meliloti*. Plasmid profile and host plant nodulation assays were also made. Isolates from mesquite nodulated beans and cowpeas but not alfalfa, clover, or soybeans. Mesquite was nodulated by diverse species of symbionts (*R. meliloti*, *Rhizobium leguminosarum* bv. phaseoli, and *Parasponia bradyrhizobia*). There were no differences within the groups of mesquite-associated rhizobia or bradyrhizobia in cross-inoculation response. The *ndvB* hybridization results showed the greatest genetic diversity among rhizobial strains. The pattern of *ndvB*-hybridizing fragments suggested that surface and deep strains were clonally related, but groups of related strains from each soil depth could be distinguished. Less variation was found with *nifHDK* and *nodDABC* probes. Large plasmids (>1,500 kb) were observed in all rhizobia and some bradyrhizobia. Profiles of plasmids of less than 1,000 kb were related to the soil depth and the genus of the symbiont. We suggest that interacting selection pressures for symbiotic competence and free-living survival, coupled with soil conditions that restrict genetic exchange between surface and deep-soil populations, led to the observed patterns of genetic diversity.

In order to function in legume symbiosis, bacteria must survive in soil, infect and nodulate the host, and reduce atmospheric dinitrogen. Genetic variation may help ensure the survival of a pool of competent microbes and facilitate symbiosis with the host under changing soil conditions. Studies of patterns of genetic variation in natural populations of root-nodulating bacteria are important to an understanding of symbiotic N_2 fixation in both natural and managed systems.

The *Prosopis glandulosa* (mesquite) symbiosis presents a natural model for testing ideas about how the host plant interacts with dramatically different soil environments to structure the phenotypic and genetic variation of microsymbiont populations (14, 15). Mesquite is a widespread desert woody legume that develops two lateral fine-root systems where deep groundwater is available. Steep gradients of soil chemistry, temperature, and available water occur from the surface soil zone to the deep, phreatic (groundwater-associated) rooting zone (23, 34, 35). The environmental conditions of both soil zones, extreme and fluctuating near the surface and moderate and constant at depth, support populations of fast-growing (FG) *Rhizobium* bacteria and slowly growing (SG) *Bradyrhizobium* bacteria. The phreatic zone, however, is probably more conducive to microbial survival and symbiosis, since symbiont population numbers are significantly greater there (15, 16, 35). A similar pattern has been observed for the West African tree legume *Acacia albida* (9).

Rhizobial and bradyrhizobial populations are found in both surface and phreatic rooting zones of a Sonoran Desert mesquite woodland near Harper's Well, Calif. These microbes occur in moist phreatic soil above permanent groundwater at 4 to 6 m and in the upper 0.5 m of soil. Jarrell and Virginia (13) hypothesized that the mesquite stand was established on a deep soil profile left temporarily moist during recession of the freshwater Lake Cahuilla 200 to 500 years ago (18). Since the 70 mm of annual rainfall (23) does not infiltrate below the 0.5-m surface rooting zone, a permanently dry intermediate soil layer formed following the gradual stabilization of the water table at approximately its current depth. Shortly after the establishment of the stand, this layer formed a physical barrier that was postulated to permanently restrict further movement of roots and symbionts between soil zones (13, 18). Specific adaptation of bacteria to the distinct soil environments that evolved over time was predicted to lead to genetic differences in the two isolated populations (14).

The study of mesquite microsymbionts isolated from surface- and deep-soil environments of Harper's Well (14) provided evidence for this hypothesis. Numerical taxonomic analysis (NT) quantified the phenotypic variations within this collection by measuring bacterial growth in culture as a function of temperature, salinity, pH, or specific nutrient or biochemical requirements. Five distinct phena of mesquite-associated isolates were identified (36). Mesquite-associated

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TABLE 1. Bacterial strains used in this study

Strain	Source or reference
Mesquite symbionts	
Rhizobium spp. strains HW1b, HW1e, HW8d, HW10a, HW10d, HW10i, HW17A, HW17c, HW22a, HW22b, HW22k, HW27a, HW27A, HW27e	14
Bradyrhizobium spp. strains HW8c, HW8e, HW8i, HW10b, HW10h, HW31a, HW43d, HW43e, HW44a	14
Other symbionts	
R. meliloti 102F34	G. Ditta
R. meliloti 1021	S. Long
R. meliloti AK631	A. Kondorosi
R. leguminosarum bv. phaseoli DB1	D. Berryhill
R. leguminosarum bv. phaseoli 8002	A. Johnston
Rhizobium sp. NGR234	B. Rolfe
Bradyrhizobium sp. BP501	D. Marvel
B. japonicum I110ARS	D. Kuykendall

bradyrhizobia formed the two most closely related groups: deep phreatic strains and a second phenon that included isolates from surface, intermediate, and phreatic zones. Rhizobia clustered by source soil zone. Deep phreatic and intermediate FG isolates were distinct, while two phena of surface strains were identified. These results imply that during the probable 200 to 500 years that mesquite has occupied the former lake bed, the surface and deep populations became genetically dissimilar as they adapted to separate soil conditions.

The objective of our study was to assess genetic diversity within a population of mesquite microsymbionts from the Harper's Well site and to relate this to the measures of phenotypic variation determined previously for the same isolates (36). We hypothesize that surface and deep populations were closely related at the time of stand establishment, diverged over time, and became genetically distinct populations. Multiple measures of diversity, including legume cross-inoculation (19), plasmid profile analysis (4), and restriction fragment length polymorphism (RFLP) (28, 37), were used to examine patterns of variation at the plasmid and chromosomal levels for regions associated with free-living bacterial survival and symbiotic performance. Genetic probes included *nif* and *nod* regions specific to symbiosis as well as the chromosomal *ndv* region, which is involved both in osmotic adaptation and in symbiosis. It was hypothesized that prolonged selection for the mesquite symbiosis would lead to conserved restriction fragments within rooting zones but that variation between surface and phreatic isolates would be evident because of the extreme differences in soil conditions between the two rooting zones.

MATERIALS AND METHODS

Strains, plasmids, and probes. Mesquite-associated strains, collected previously (14), are listed in Table 1. Probe DNA fragments were isolated, after *Eco*RI digestion, from the following sources. The *nod* probe carrying the 8.7-kb *nodDABC* region was isolated from pRmSL26 (21); the 6.7-kb *nifHDK* probe was isolated from pSA30 (5). The *ndvB* region fragment carries upstream flanking DNA and the 5′ coding portion of *ndvB* and was isolated from pTy20 (kindly provided by T. Dylan). General growth and maintenance of all symbiotic bacteria was on yeast extract mannitol (YM) (33) or tryptone yeast extract (3) medium at 30°C. Cultures were stored in 8.8% dimethyl sulfoxide at −70°C.

Host plant nodulation assays. Strains were tested for their abilities to form nodules on a variety of host legumes, including soybean (*Glycine max*), cowpea (*Vigna unguiculata*), bean (*Phaseolus vulgaris*), clover (*Trifolium subterraneum*), alfalfa (*Medicago sativa*), pea (*Pisum sativum*), and mesquite. Mesquite seeds from near Harper's Well, Calif., and other legume seeds were surface sterilized, planted in Leonard jars, and inoculated in accordance with standard protocols (33). Plants were grown under supplemental light with nutrient solution (29). After 4 to 6 weeks of growth, the roots were harvested and the host nodulation status was assessed.

Southern blots of rhizobial and bradyrhizobial genomic DNA. Small-scale DNA preparations were made from rhizobial and bradyrhizobial cultures by standard methods (38). DNA was digested with *Pst*I (Promega), and the fragments were separated on agarose gels. After alkaline denaturation, the DNA was transferred by Southern blotting onto GeneScreen Plus nylon membrane (GeneScreen) by protocols described elsewhere (25, 27).

Southern blots of genomic DNAs were probed with [α-^{32}P]dCTP-labeled (Prime-A-Gene labeling kit; Promega) *Eco*RI fragments isolated with the Gene Clean kit (Bio 101). Blots were hybridized with probe DNA at 42°C overnight under standard conditions (27). A second hybridization of the *ndv* probe to the SG strain blot was done at reduced stringency under the same salt conditions but with overnight hybridization at 37°C and 40°C washes. Autoradiographs were exposed at −70°C for 12 to 48 h. The probe was stripped from blots in accordance with the GeneScreen protocol for sequential reprobing of blots. The length of the hybridization probes was chosen to provide comparable signal sizes.

Visualization of mesquite-associated rhizobial and bradyrhizobial replicons. Plasmids of mesquite strains were isolated and visualized by using the modifications, related here, of a previously described technique (24). YM broth suspensions of 4- to 6-day-old YM plate-grown rhizobia and bradyrhizobia were stored at 4°C. Aliquots of cell suspensions (25 to 100 μl) were inoculated into 10 ml of YM broth and shaken at 30°C for 2 or 3 days, respectively, for rhizobia and bradyrhizobia. Cell pellets (10^8 to 5×10^8 cells) were disrupted with pipet tips. Rhizobia were washed successively with 300 μl of M9 salts (27) and 300 μl of 0.01% Tween, while bradyrhizobia were washed with M9 salts containing 0.5 M NaCl and then with 0.1% Sarkosyl. Residual wash solution was immediately removed, and the pellets were disrupted, resuspended in 50 μl of spheroplasting solution (24), and rapidly loaded into the anode-proximal well of a double-well gel. A continuous well (140 by 10 by 6 mm) was formed 0.8 mm to the cathode-proximal side of the cell-loading wells. At 15 min after the last suspension was loaded, 15 μl of pronase (5 mg/ml) in 10% Ficoll was overlaid and the wells were sealed. Molten sodium dodecyl sulfate-agarose mix was added to the cathode-proximal well 15 min after the addition of pronase to the mesquite rhizobia or 1 h prior to well loading of the bradyrhizobia. Electrophoresis was performed for times up to overnight at 10 mA before the current was increased to 50 mA for 10 h. Treatments of gels prior to photography were by standard methods (27). Plasmid molecular weight reference strains included *Rhizobium meliloti* 1021, which contains megaplasmids of approximately 1,700 and 1,400 kb (31) and *Rhizobium leguminosarum* bv. phaseoli DB1, which has three plasmids that are 881, 375, and 250 kb (3a).

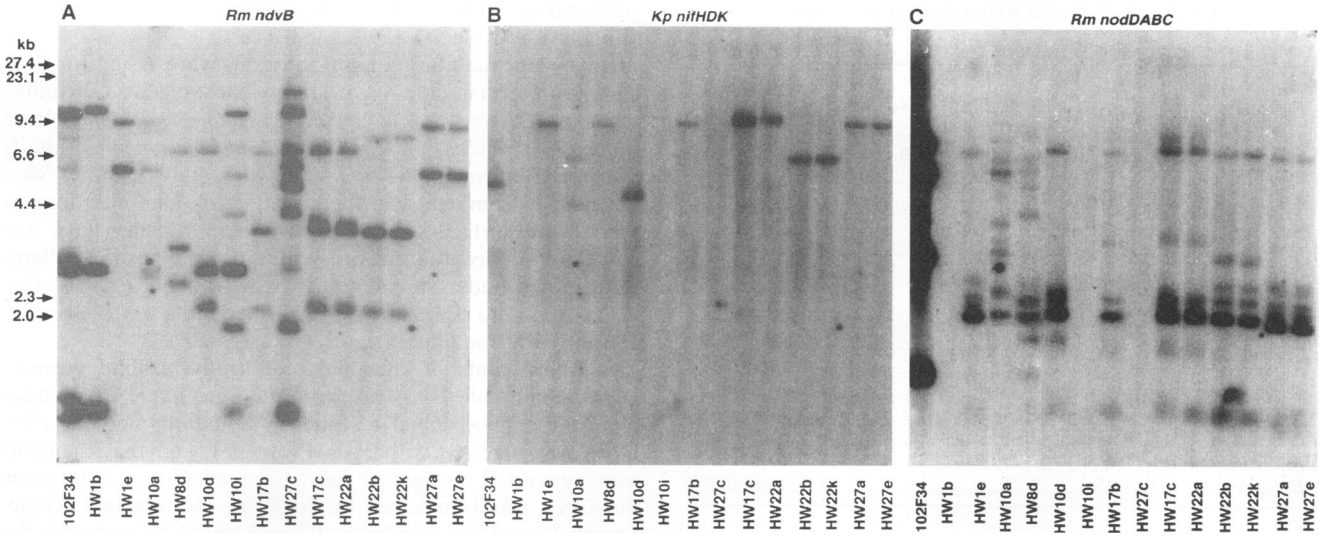

FIG. 1. RFLP analysis of Harper's Well (HW) rhizobia gene regions. The same electrophoretically resolved *Pst*I fragment blot of FG strain genomic DNA was sequentially hybridized with radiolabeled fragments of the *R. meliloti* (Rm) *ndvB* region (A), *K. pneumoniae* (Kp) *nifHDK* (B), and the *R. meliloti nodDABC* region (C). Genomic DNA of reference strain *R. meliloti* 102F34 was included in lane 1, and the remaining lanes contained DNA of the HW rhizobial isolates indicated below the autoradiographs. The positions and molecular sizes of bacteriophage λ *Hin*dIII DNA fragments are shown to the left of A. In C, lanes 10a and 8d were graphically enhanced to approximate the intensity of adjacent lanes in order to demonstrate weakly hybridizing bands only faintly evident in the original.

RESULTS

For plant host range testing, mesquite-associated isolates were chosen to represent the physiologically discrete phena of FG and SG strains identified by Waldon et al. (36). Both genera of mesquite-associated isolates nodulated (10 to nearly 100 nodules per plant) beans and cowpeas but not alfalfa, clover, or soybeans. Mesquite nodulation (seven or more nodules per plant) was deemed effective on the basis of nodule morphology, evidence of leghemoglobin (pink interior), and the plants' dark green foliage. Bean nodulation was judged ineffective on the basis of the same criteria, as was nodulation of most cowpea plants, but a few of the cowpeas had larger nodules with defined surface features and showed evidence of leghemoglobin production. Two FG isolates, 1b and 27c, did not nodulate any host plant, including mesquite.

Narrow- and broad-host-range microsymbionts nodulate mesquite. Mesquite seedlings were inoculated with several rhizobial and bradyrhizobial species to determine whether mesquite supports a diversity of symbionts. *R. meliloti* 1021 and 102F34, *R. leguminosarum* bv. phaseoli strain 8002, and broad-host-range *Parasponia* bradyrhizobial strain BP501 nodulated mesquite (more than eight nodules per plant). Broad-host-range *Parasponia* rhizobial strain NGR234, *Bradyrhizobium japonicum* I110ARS, and *R. meliloti* AK631 failed to nodulate mesquite.

***ndv* region hybridizes to different-sized fragments of rhizobial and bradyrhizobial DNA.** To examine the genetic diversity of the collection, Southern blots of *Pst*I-digested genomic DNA from mesquite-associated rhizobial or bradyrhizobial strains were sequentially hybridized with the *nodDABC* region of *R. meliloti* 1021 (21), the *Klebsiella pneumoniae nifHDK* region encoding nitrogenase structural genes (5), and the chromosome-localized *ndvB* region of *R. meliloti* 102F34 (11).

The results of probe hybridization to digested rhizobial DNAs are shown in Fig. 1. Hybridization to the *R. meliloti* chromosomal *ndvB* region revealed the greatest RFLPs within FG bacteria (Fig. 1A). Nine groups of strains with identical or unique fragment patterns were evident by *ndv* hybridization, and these are presented in Table 2. None of the hybridizing fragment sizes were observed in all strains, although all *ndv* groups shared subsets of fragments with other groups.

The standard hybridization conditions used revealed weak bradyrhizobial *ndv* hybridization signals (data not shown), the strengths of which increased after hybridization stringency was reduced (Fig. 2A). Five groups of SG strains with unique fragment patterns were observed (Table 2), and three *ndv* groups shared fragments among them. Two strains, 10b and 31a, shared no fragments with the other groups. The most diverse bradyrhizobial patterns of the three probes used were evident in *ndv* hybridization.

Mesquite-associated rhizobial and bradyrhizobial *nif* and *nod* RFLPs are less diverse than *ndv* RFLPs. Five *nif* groups of rhizobia were identified (Table 2). In contrast to the fragment length polymorphism evident by *ndv* hybridization, probing with *nifHDK* revealed that *nif* group 1 strains, including 7 of 11 strains from all soil depths, had the same hybridization pattern (Fig. 1B). Unique patterns were evident in three *nif* groups, while one group comprised three strains (1b, 10i, and 27c) that did not hybridize to the probe.

Seven groups of rhizobia were identified by *nod* hybridization (Fig. 1C), as shown in Table 2. Four groups including nine strains carried 2.3- and 6.6-kb fragments that hybridized to mesquite rhizobial *nodD* (data not shown). The strongest signal (2.0 kb) was evident in hybridizing strains of all soil zones whose weaker fragments varied in number and size. Patterns of strains 10a and 8d showed both unique higher-intensity signal sizes and increased numbers of weakly hybridizing bands. As with the *nif* probe, strains 1b, 10i, and 27c showed no *nod* hybridization.

The group of SG isolates showed less diverse *nif* hybridization patterns than those revealed by *ndv* hybridization to the same blot (Fig. 2B). Three SG *nif* groups were identified, with group 2 including a surface and a phreatic strain (Table 2),

TABLE 2. Mesquite isolate NT and RFLP groups

Strain	Soil depth[a]	NT phenon no.[b]	RFLP group no. with probe:		
			ndv	*nif*	*nod*
Rhizobial isolates					
1b	S	2	1[c]	1[d]	1[d]
1e	S	ND[e]	2	2	2
8d	P	4	3	2	3
10a	S	6	4	3	4
10d	S	6	5	4	5
10i	S	2	6	1	1
17b	I	3	7	2	6
17c	I	3	7	2	6
22a	I	3	7	2	6
22b	I	3	8	5	7
22k	I	3	8	5	7
27a	S	6	2	2	2
27c	S	2	9	1	1
27e	S	6	2	2	2
8c	P	2	1	1	1
8e	P	2	1	1	1
8i	P	2	1	1	1
10b	S	1	2	2	2
10h	S	1	3	3	2
31a	P	1	4	2	3
43d	P	2	5	1	4
43e	P	2	5	1	4
44a	P	2	5	1	4

[a] S, surface soil, 0 to 0.6 m; I, intermediate soil, 0.6 to 4.0 m; P, phreatic soil, 4.0 to 6.0 m.
[b] Phenotypically distinct groups were as defined previously (36).
[c] All strains in each group contained probe-hybridizing fragments of the same sizes.
[d] Strains 1b, 10i, and 27c in the *nif* 1 and *nod* 1 groups showed no hybridization to the probe.
[e] ND, not determined.

while all other phreatic strains had the same pattern (*nif* group 1).

Examination of the SG strains hybridized with the *nod* probe (Fig. 2C) revealed four groups (Table 2). Similar patterns were apparent in the two larger groups of phreatic strains, from which the less complex profiles of the remaining groups differed.

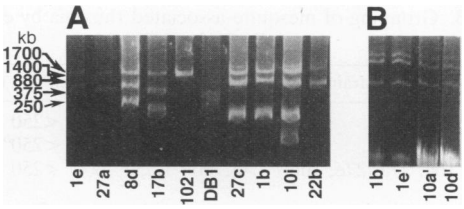

FIG. 3. Resolution of Harper's Well surface and subsurface rhizobial replicons. (A) DNA prepared by in-gel lysis of rhizobial strains (shown below the panel) was resolved by electrophoresis. Reference plasmids of known molecular size, shown to the left of the panel, were isolated from lysates of *R. meliloti* 1021 and *R. leguminosarum* bv. phaseoli DB1. (B) Examples of plasmid group 3 electrophoretic profiles exhibiting plasmids similar in size to the smallest of HW strain 1b. The sizes of strain 1b plasmids are the same as those shown in A.

Plasmid profiles distinguish rhizobial groups. Two larger replicons were observed in all rhizobial strains by in-well lysis (Fig. 3A), while differences in smaller plasmid sizes defined five groups (Table 3). Estimated sizes ranged from less than 250 kb to greater than 1,700 kb. The two megaplasmids of *R. meliloti* 1021 were not resolved in this gel (Fig. 3A) despite differing by 300 kb. The inflection of the standard curve near that size range and its steepness suggest that the slowest band was substantially larger than the 2,250-kb estimate obtained by straight-line extrapolation. In other gels, we observed a band larger than the two resident megaplasmids of *R. meliloti* 1021 (see the results discussed below) that must be its chromosome (31), and the slowest bands of mesquite-associated rhizobia migrate similarly, suggesting sizes of near 3.5 Mb. As shown in Fig. 3B, plasmid group 3 profiles varied with the appearance of a band similar in size to that of the smallest plasmid of strain 1b.

Bradyrhizobial soil zone populations differ in plasmid numbers. Extrachromosomal replicons distinguished surface and

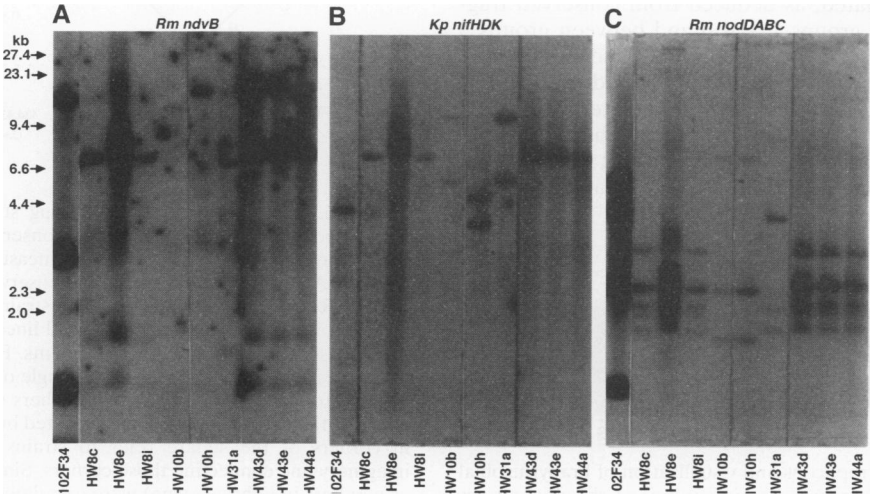

FIG. 2. RFLP analysis of Harper's Well bradyrhizobia gene regions. The same electrophoretically resolved *Pst*I genomic DNA fragment blot of SG strains was probed with radiolabeled DNA fragments, as used for the Fig. 1 autoradiographs: the *R. meliloti* (Rm) *ndvB* region (A), *K. pneumoniae* (Kp) *nifHDK* (B), and the *R. meliloti nodDABC* region (C). Genomic DNA of reference strain *R. meliloti* 102F34 was included in lane 1, and the remaining lanes contained DNA of HW bradyrhizobia as indicated below the autoradiographs. Positions and the molecular sizes of bacteriophage λ *Hin*dIII reference DNA fragments are shown to the left of A.

TABLE 3. Grouping of mesquite-associated rhizobia by estimated replicon size

Plasmid group and strain(s)	Estimated fragment size (kb)[a]				
FG 1: 1b	>1,700	1,550	800	<250	
FG 2: 10i, 27c	>1,700	1,550	600	<250[b]	<250[b,c]
FG 3[d]: 1e, 10a, 27a, 27e, 10d, 22b, 22k	>1,700	1,550	600	<250	
FG 4: 8d	>1,700	1,550	600	300	
FG 5: 17b, 17c, 22a	>1,700	1,550	600	250	<250

[a] Values preceded by ">" or "<" were for bands that fell outside of the standard range.
[b] There were two plasmids differing in size but less than 250 kb. The larger of the two <250-kb plasmids migrated as the same-sized plasmid in the two strains.
[c] Strain 10i was the only member of this group to retain the smallest plasmid.
[d] This group was characterized, in part, by the variable appearance of the <250-kb band.

phreatic bradyrhizobial populations, since surface strains contained one or two megaplasmids from 650 to 900 kb and up to two smaller plasmids (approximately 300 kb) while phreatic strains contained one, or no, plasmid smaller than 250 kb (Fig. 4A). At times, we observed much larger, 1,500-kb, bands such as those in strains 10b and 27b (Fig. 4B and C). The slowest-migrating bands of strain 10b (Fig. 4B) and *R. meliloti* 1021 (Fig. 4C) may be intact chromosomes. We defined five groups by distinctions in plasmids of 900 kb or smaller (Table 4).

DISCUSSION

Selective pressures of surface and subsurface rooting zones of deeply rooted plants, such as mesquite, differ where steep gradients of temperature, water availability, and nutrients occur with increasing depth. RFLP analyses provide evidence for the predicted relatedness of symbiont populations which numerical taxonomy had separated into diverse phena. The mesquite-associated rhizobial isolates in all *ndv* groups from all soil zones were related by conserved *ndv* fragments (Fig. 5): one fragment among groups 1, 4, 5, and 6; two fragments between groups 2 and 4; one fragment among groups 1, 6, and 9; one fragment among groups 3, 5, and 7; and one fragment among groups 5, 7, and 8. Similarly, all mesquite-associated bradyrhizobia were related, as deduced from conserved fragments of *ndv* between groups 1 and 3 and between groups 3 and 5 (Fig. 2A); of *nod* between *ndv* group 3 and *ndv* group 2 (Fig. 2C); and of *nif* between *ndv* groups 2 and 4 (Fig. 2B). These findings of the relatedness among all isolates within each genus are consistent with the nodulation assays that showed no differences among nodulating mesquite-associated isolates in cross-inoculation response despite the potential for diverse species to infect mesquite.

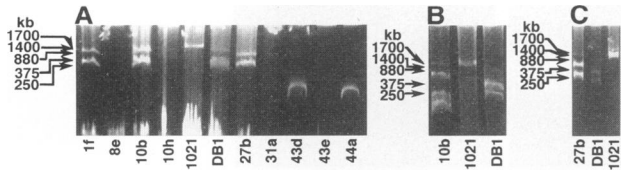

FIG. 4. Resolution of replicons and visualization of bradyrhizobial megaplasmids. (A) DNA prepared by in-gel lysis of rhizobial strains was resolved during agarose gel electrophoresis. Reference plasmids of known molecular size (shown to the left of panel) are in lysates of *R. meliloti* 1021 and *R. leguminosarum* bv. phaseoli DB1. Visualization of 1,500-kb megaplasmids of bradyrhizobial strains 10b (B) and 27b (C) showed plasmid profiles against reference plasmid profiles.

TABLE 4. Grouping of mesquite-associated bradyrhizobia by estimated replicon size

Plasmid group and strain(s)	Estimated fragment size (kb)		
SG 1: 1f, 10b	900	~275	~275
SG 2: 8e, 31a	—[a]	—	—
SG 3: 10h	900	650	
SG 4: 27b	650	~275	~275
SG 5: 43d, 43e, 44a	<250[b]		

[a] —, no hybridization detected.
[b] This band fell outside of the molecular weight standard size range.

RFLP results also support the hypothesis that rhizobial populations fall into groups (Table 2) that correspond to soil zones (Fig. 5). Four FG phena that were previously identified as phenotypically diverse by NT comprised a surface cluster and a subsurface cluster by *ndv* hybridization (summarized in Fig. 5). Further evidence for grouping according to soil zone is provided by plasmid profile analysis (Table 3).

Although neither NT (36) nor RFLP analysis entirely resolved bradyrhizobia into discrete soil zone-correlated groups (Table 2), other measures of diversity did. The SG isolates of phenon 2 were, except for one strain, phreatic isolates, whereas SG phenon 1 strains were of all depths. RFLP analysis revealed related genotypes that were diverse within and between rooting zones (Table 2; Fig. 5). Plasmid profile variation (Fig. 4), however, identified discrete groups of bradyrhizobia that differed by species and amounts of plasmid DNA as functions of soil depth (Table 4). Previous work showed that SG colony morphology type was correlated with soil depth (14). In summary, both rhizobia and bradyrhizobia fall into groups that correspond to distinct soil zones under the criteria of plasmid profile and colony morphology.

Even though the Harper's Well rhizobia and bradyrhizobia

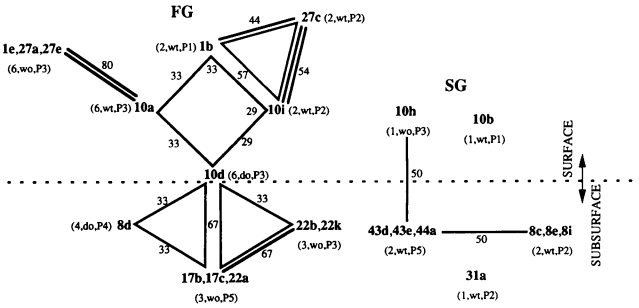

FIG. 5. Inferred relationships among strains of mesquite-associated rhizobia and bradyrhizobia by conservation of *ndv*-hybridizing restriction fragment lengths and other measures. The autoradiographs for Fig. 1A and 2A were analyzed for conservation of *ndv* hybridization signals. Relationships inferred among strains (boldface type) by that measure are indicated by as many solid lines between strains as there were conserved *ndv*-hybridizing fragments. Fragments shared by more than two strains are indicated by a triangle or modified square. Strains with no fragments in common with others stand detached. Numbers adjacent to lines refer to strains connected by those lines, and numbers in corners of the square refer to strains at opposite ends of an imaginary line connecting those corners. Similarity coefficients (smaller numbers in lightface type) were calculated according to the following equation: % similarity = $100 \times (2n_{XY})/(n_X + n_Y)$ (22). The phena to which strains were assigned previously by NT of growth-response phenotypes (36), strain colony morphology (do, dry and opaque; wo, wet and opaque; wt, wet and translucent [13a]), and plasmid groups as defined in this work are enclosed in parentheses.

TABLE 5. Similarity coefficient matrices for *ndv* (chromosomal)- and *nod* (plasmid)-region RFLPs

Replicon	*ndv* RFLP pattern similarity coefficient (%)[a]							
	1b	10i	1e, 27a, 27e	10a	10d	17b, 17c, 22a	22b, 22k	8d
27c chromosome	44	54	0	0	0	0	0	0
Plasmid	—[b]	—	—	—	—	—	—	—
1b chromosome		57	0	33	33	0	0	0
Plasmid		—	—	—	—	—	—	—
10i chromosome			0	29	29	0	0	0
Plasmid			—	—	—	—	—	—
1e chromosome				80	0	0	0	0
Plasmid				19	47	63	71	53
10a chromosome					33	0	0	0
Plasmid					44	44	40	9
10d chromosome						67	33	33
Plasmid						77	71	25
17b chromosome							67	33
Plasmid							80	35
22b chromosome								0
Plasmid								33

[a] % similarity coefficient = $100 \times (2n_{XY})/(n_X + n_Y)$, where n_{XY} is the number of fragments shared by genotypes X and Y, n_X is the total number of fragments in X and n_Y is the total number of fragments in Y (22).

[b] —, no *nod* region hybridization was detected in these genotypes.

were exposed over time to the same conditions within each of the two soil rooting zones, rhizobial populations at the Harper's Well site are more genetically diverse than bradyrhizobial populations. The two bradyrhizobial groups were the least distant phena identified. A subpopulation of the phreatic SG isolates was distinguished phenotypically in phenon 2, but phenon 1 SG isolates from both soil zones were taxonomically indistinct (36). The same measures identified diverse clusters of rhizobia that had been isolated from different soil depths. Perhaps, the original bradyrhizobial population was better adapted to both surface- and deep-soil environments and, therefore, less subsequent selection and genetic variation occurred.

Comparison of plasmid profile variation patterns also suggests a differential influence of root zone conditions on SG and FG strains (Fig. 3 and 4). Neglecting the 1,500-kb bands of surface SG strains, plasmid DNA contents of surface and phreatic strains varied by 1,000 to 1,500 kb (Table 4). Detectable differences of 300 kb or less in rhizobial plasmid DNA contents were found mostly in species smaller than 600 kb (Table 4). These results suggest that bradyrhizobial plasmids were not necessary for Harper's Well deep-soil survival but, consistent with other work, imply that functions important for survival in either soil zone may reside on rhizobial plasmids (1, 7). These observations probably reflect basic genetic and physiologic differences of the two symbiont genera.

The amounts of average sequence divergence of the two rhizobial replicons were similar (chromosome, 12.7%, versus plasmid, 12.4%) as calculated from *ndv* and *nod* hybridization results (Table 5 [22]), and this contrasts with previous indications of generally greater plasmid variation (chromosome, 5.7%, versus plasmid, 11.4% [37]). The selective factor of high salinity on mesquite symbionts (16, 34) is a possible contributor to this phenomenon, since concentrated variation within and around the large (9-kb [12]) *ndvB* region could affect its roles in osmoadaptation (10) or symbiosis (11), as shown by Dylan and coworkers. Phenotypically silent variation of the *ndvB* region might explain the more-than-twofold-greater diversity of rhizobial chromosome sequence than was shown in previous work (37), but a soil moisture regime (13, 18) that prevents microbial movement makes it unlikely that plasmid transfer between rooting zones was responsible for the observed patterns.

Both symbiont genera contained plasmids of up to 1.5 Mb (Fig. 3 and 4), and we are unaware of bradyrhizobial replicons larger than the 440-kb plasmids identified in *B. japonicum* (6). Mesquite-associated bradyrhizobia megaplasmids indicate a unique phylogeny, but the historic difficulty of SG plasmid isolation (8, 20) could explain why they have not been identified in other bradyrhizobia and suggests that not all SG mesquite-associated megaplasmids were isolated. We generally obtained 900-kb and smaller plasmids, but the reduced or variable yield of 900-kb and larger plasmids implied a threshold size for plasmid extraction efficiency. While the taxonomic standings of the mesquite symbionts are not entirely known, their carbon utilization patterns were characterized as intermediate to those of most rhizobia and bradyrhizobia (2).

Rhizobial pSym instability may have facilitated the numerical predominance of bradyrhizobia in deep-soil populations (14, 15). NT distinguished surface rhizobial phena 2 and 6 primarily by differences in carbon source utilization (36), which is sometimes plasmid encoded in rhizobia (1, 7, 32). Members of phenon 2 did not hybridize to *nod* and *nif* probes (Fig. 1) or, in plasmid blots, to the cloned *nodD* region of strain 17b (data not shown), while members of phenon 6 did hybridize to these probes. These results indicate that pSym deletion or curing in phenon 2 isolates is responsible for the in planta results (no nodulation of tested plant hosts) and could be responsible for the differences in carbon source utilization. Reports of nonsymbiotic rhizobial populations (30) probably arise because of pSym instability (8, 26). The lack of cloned bradyrhizobial *nodD* hybridization to 900-kb or smaller plasmids (data not shown) and the symbiotic stability of bradyrhizobia in our experiments implies *sym* linkage to an essential, stable bradyrhizobial replicon. The stability of bradyrhizobial *sym* regions, in combination with an enhancement of rhizobial pSym instability by high surface-soil temperatures (17), could structure phreatic populations by enrichment for bradyrhizobia.

Our results show lineages of related genotypes from the surface to the deep soil. The rhizobia and bradyrhizobia examined fall into soil zone-correlated groups of divergent but related isolates, suggesting a common progenitor for each

genus. We propose a scenario that may have led to the development of these genetically distinct, clonal populations at Harper's Well. The recession of ancient Lake Cahuilla left a deep column of moist soil that favored the expansion of existing mesquite stands to the newly exposed soil. The deep-reaching roots of establishing mesquite were likely vectors for the movement of microsymbionts to subsurface soil. Movement of rhizobia and bradyrhizobia from surface soil to deeper levels was along complex and steep selection gradients of decreasing temperature, salinity, and soil fertility but of increasing moisture. In addition to selection for saprophytic survival, populations were also selected for symbiotic capacity by mesquite. The release of nodulation-selected cells to soil upon nodule senescence further enriched adapted genotypes along the soil depth gradient. Development of the perennially dry intermediate soil layer formed a barrier preventing further downward movement of symbionts and effectively isolated the surface and deep populations for an extended time. We propose that interacting selection pressures for free-living competence and symbiotic proficiency coupled with soil physical conditions preventing genetic exchange between the spatially isolated microsymbionts led to the observed genetic divergence of populations.

We conclude that gradients of biological and environmental variables act to shape the genetic structure of symbiont populations. Our work suggests that analogous examples of microbial population divergence might be expected where genetically similar and metabolically active organisms become spatially isolated across steep environmental gradients over extended periods of time.

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