# *Rhizobium* gone native: Unexpected plasmid stability of indigenous *Rhizobium leguminosarum*

(plasmid transfer/*Trifolium*/population/nodulation/symbiosis)

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ABSTRACT     Lateral transfer of bacterial plasmids is thought to play an important role in microbial evolution and population dynamics. However, this assumption is based primarily on investigations of medically or agriculturally important bacterial species. To explore the role of lateral transfer in the evolution of bacterial systems not under intensive, human-mediated selection, we examined the association of genotypes at plasmid-encoded and chromosomal loci of native *Rhizobium*, the nitrogen-fixing symbiont of legumes. To this end, *Rhizobium leguminosarum* strains nodulating sympatric species of native *Trifolium* were characterized genetically at plasmid-encoded symbiotic (*sym*) regions (nodulation AB and nodulation CIJT loci) and a repeated chromosomal locus not involved in the symbiosis with legumes. Restriction fragment length polymorphism analysis was used to distinguish genetic groups at plasmid and chromosomal loci. The correlation between major *sym* and chromosomal genotypes and the distribution of genotypes across host plant species and sampling location were determined using $\chi^2$ analysis. In contrast to findings of previous studies, a strict association existed between major *sym* plasmid and chromosomal genetic groups, suggesting a lack of successful *sym* plasmid transfer between major *Rhizobium* chromosomal types. These data indicate that previous observations of *sym* plasmid transfer in agricultural settings may seriously overestimate the rates of successful conjugation in systems not impacted by human activities. In addition, a nonrandom distribution of *Rhizobium* genotypes across host plant species and sampling site demonstrates the importance of both factors in shaping *Rhizobium* population dynamics.

Transmissible plasmids are considered important in the divergence and adaptation of bacterial populations because they have several unique features that contribute to genomic plasticity and may affect the evolution of the loci they encode (1–3). They might: be lost and regained in populations, rapidly change in copy number, and undergo higher mutation rates because of the common occurrence of reiterated DNA (4, 5). In addition, transmissible plasmids are thought to undergo lateral transfer at rates higher than chromosomal genes, which may be particularly important in adaptation to variable environments (5, 6). To the extent that lateral transfer disrupts the linkage of plasmid and chromosomally encoded loci, strong selection at plasmid loci may not affect genetic divergence throughout the bacterial genome (5). Just as sexual recombination dilutes the effects of selection on particular loci in eukaryotic populations, plasmid transfer may mitigate the effects of selection for plasmid-encoded phenotypes on the

divergence of chromosomal loci and is thus considered an important force in prokaryotic divergence and speciation.

Our current view of the importance of plasmids is largely shaped by observations of those under intense selective pressures related to human activity. The global distributions and broad chromosomal host ranges of antibiotic resistance plasmids may be attributed to recent human selection (6). Isolates of *Escherichia coli* and *Salmonella* sampled before intensive use of antibiotics possess similar plasmids that lack antibiotic resistance genes and whose distributions argue for lower transfer rates than their modern counterparts (7, 8). Likewise, toxic pollution of aquatic and terrestrial habitats likely increases the rates of evolution and transfer of bacterial resistance plasmids. Pollution is correlated with increased plasmid diversity, frequency, and molecular weight (9–11), and a comparison of plasmid and chromosomal genotypes reveals significant lateral transfer of plasmids in toxin-containing habitats (12).

Although the transfer of plasmid-encoded traits is important in the adaptation of microbes to intensive selection, such strong selective pressures may not be typical of natural habitats. Observed frequencies and dynamics of plasmids under human-mediated selection may therefore not represent the role plasmids generally play in native bacterial populations. Plasmids not obviously under human selection, including the conjugative colicin plasmids of *E. coli* and the small cryptic plasmids of *Bacillus*, appear to undergo successful transfer only rarely in nature (13–15). In these cases, selective forces may rarely act independently on plasmid vs. chromosomally encoded phenotypes. In addition, plasmid transfer in native settings may be limited by ecological and physiological constraints, such as reduced opportunity for cell-to-cell contact because of low bacterial cell densities, a low metabolic rate of cells living in nutrient-poor habitats, layers of extracellular material deposited on cells in natural habitats, or shearing of sex pili because of physical disturbance (16).

One transmissible plasmid of particular ecological significance is the symbiotic (*sym*) plasmid of *Rhizobium leguminosarum*, which encodes loci that mediate the best studied eukaryote–prokaryote symbiosis: the nitrogen-fixing mutualism with legumes (17). Bacterial genes mediating root attachment, infection, nitrogen fixation, and, with one exception, host specificity are clustered in the plasmid-encoded *sym* region (18, 19). Although non-*sym* plasmids may participate in the symbiosis (20), the *sym* plasmid alone confers legume-specific symbiotic phenotypes (21). Survival of *Rhizobium* in the soil depends on tolerance of soil acidity, temperature, and moisture content (22). Phenotypes affecting growth and sur-

Table 1.    Derivation of DNA probes used in hybridization

| Genetic region | Plasmid | Manipulation of plasmid |
|---|---|---|
| *nodAB* of ANU843 | pRt572 (40) | 1.2-kb segment isolated from *Eco*RI and *Kpn*1 digestion |
| *nodCIJT* of ANU843 | pRt587 (40) | 4.2-kb segment isolated from *Hin*dIII, *Bam*H1, *Eco*RI digestion |
| 1-kb repeated chromosomal DNA | pRt1013 (39) | 1-kb segment isolated from *Pst*I-*Xho*I digestion |

vival in the soil are thought to be chromosomally encoded, as evidenced by loss of the *sym* plasmid in *Rhizobium* soil populations (23–25) and the significant main effect of chromosomal but not *sym* plasmid variation on competitive growth in the soil (ref. 26 but see ref. 27 for catabolic loss upon curing the *sym* plasmid). This apparent partitioning between the *sym* plasmid and the chromosome of loci involved in the legume symbiosis and survival in the soil, respectively, suggests that the two replicons may be affected by distinct suites of selective pressures.

In agricultural legume populations, lateral transfer of *sym* plasmids among *Rhizobium* strains is evidenced by disjunctures between genetic groups at *sym* and chromosomal loci (23, 28–32). In addition, strong correlations between agricultural legume species and *sym* genotypes but not nonsymbiotic genotypes (23, 28) argues that lateral transfer of *sym* genes may decouple the adaptation of loci mediating the interaction with the host plant from those loci involved in competitive growth in the soil (33). Establishing a monoculture of introduced host plants was shown recently to select for transfer of competitive *sym* genotypes into symbiotic and nonsymbiotic rhizobia in the field (34). That the symbiotic genes in this particular *Rhizobium* population were chromosomally encoded argues for a strong differential effect of host legume on symbiotic loci, relative to the effect at nonsymbiotic loci.

However, selective pressures in fields of introduced or agricultural legumes may differ considerably from those in native legume communities. Introduction of plants in monoculture, inoculation of *Rhizobium* at high densities, and lack of selection acting on host plants for symbiotic compatibility with indigenous *Rhizobium* may each affect the dynamics of the *Rhizobium*–legume symbiosis and alter rates of successful plasmid transfer across bacterial chromosomal groups. Patterns of high transfer rates of *sym* plasmids and apparent decoupling of *sym* and nonsymbiotic loci observed in human-impacted environments have shaped our view of *sym* plasmids, but these patterns may seriously misrepresent plasmid dynamics in populations of indigenous *Rhizobium* nodulating native legume species.

Given the potential importance of plasmid transfer in bacterial diversification and our limited understanding of plasmid dynamics in native populations, the goal of this study was to estimate levels of *sym* plasmid transfer within a *Rhizobium* population nodulating native legume species. Similar to previous studies of agricultural systems, *sym* plasmid transfer was determined from the correlation between major genotypes at nodulation and nonsymbiotic loci. Both host plant species (23, 28, 35) and sampling location (36, 37) are considered important in shaping genetic divergence of *Rhizobium*, so we used the unique strategy of sampling nodules of the same legume species across several sites to distinguish the effects of host plant and sampling location on the divergence of *Rhizobium* plasmid and chromosomal loci. In contrast to previous studies, we observed a strict correlation between genetic groups at symbiotic plasmid and chromosomal loci across several sampling locations and host plant species, which strongly argues for a surprising stability of *sym* plasmids within chromosomal lineages of native *Rhizobium* populations.

## MATERIALS AND METHODS

**Sampling Location and Strategy.** *Rhizobium* were sampled from nodules of four species of clover native to mountain meadows in the Sierra Nevada: *Trifolium bolanderi, T. longipes, T. monanthum,* and *T. wormskioldii. Trifolium* plants were sampled at two meadows, Fresno Dome and House meadows, located 66 km from each other. Relative to the California State University Fresno campus, House meadow is 64 km 10° north of west, and Fresno Dome meadow is 74 km 10° west of north. The meadows are ≈2130 m in elevation and are in separate watersheds. At both meadows, three *Trifolium* species were sampled (*T. bolanderi*, *T. longipes*, and *T. monanthum*), and at Fresno Dome meadow the fourth species, *T. wormskioldii*, also was sampled. All samples were taken in June and July, 1994.

Within each meadow, three 15 × 30-m plots were selected based on the presence of at least three of the four *Trifolium* species. Within each of the six plots, 10–12 plant individuals were sampled for each *Trifolium* species included in a given plot (≈30–48 total plant individuals per plot). Plant individuals were selected at random and were removed with ≈10 cm diameter × 10 cm depth of surrounding soil. A portion of soil core was removed for soil acidity and moisture analyses, and samples were returned to the laboratory on ice. Plants were stored in intact soil cores for up to 2 days at 4°C. Soil and other plant roots were gently removed, and the root system was rinsed in deionized water and stored at 4°C.

**Isolation of Bacterial Strains.** Five to eight arbitrarily selected nodules were removed from plant roots, treated with 1% hypochlorite for 2 minutes, rinsed three times in sterile deionized water, and then crushed and streaked on yeast mannitol agar (38). Plates were incubated for 3–5 days at 30°C and restreaked as necessary to obtain pure cultures. Isolates were stored frozen at −70°C in yeast mannitol broth plus 7% dimethyl sulfoxide.

**Restriction Fragment Length Polymorphism (RFLP) Analysis.** A subset of the *Rhizobium* strains was selected for genetic analysis such that isolates from each of the four *Trifolium* host plants were represented across several sampling plots. Total genomic DNA of 69 isolates was prepared as described (39). DNA samples (8–10 μg) were digested to completion (16 h) with *Hin*dIII and *Eco*RI separately and electrophoresed on a 0.7% agarose gel in TBE (89 mM Tris/689 mM boric acid/2 mM EDTA, pH 8.0). Gels were transferred to nylon membranes (Boehringer Mannheim). Labeled DNA probes were prepared by random priming, sequentially hybridized to the nylon membranes, and detected colorimetrically (Boehringer Mannheim Genius system). Only strongly hybridizing bands were scored. Lengths of hybridizing fragments were determined by probing nylons with labeled DNA of the size marker on the gel, a 1-kb ladder. Membranes were stripped of probe between hybridizations.

The probes used in this study were derived from ANU843, a reference strain of *R. leguminosarum* bv. *trifolii* (*R. leguminosarum* isolated from *Trifolium*), and include three regions: nodulation AB loci (*nodAB*), nodulation CIJT loci (*nodCIJT*), and a 1-kb repeated chromosomal sequence (Table 1).

**Analysis of RFLP Data.** Isolates were grouped according to their hybridization pattern at each of the three regions sampled (*nodAB*, *nodCIJT*, and the 1-kb reiterated chromosomal sequence). Nonrandom distribution of RFLP types across the two meadows and four host plants species was tested using $\chi^2$ analysis (JMP 3.1.5 program, SAS Institute, Cary, NC).

**DNA Sequencing.** Genealogies of the *16S rRNA* locus are considered representative of bacterial species phylogenies (41). To position *Rhizobium* isolates on a *16S rRNA* gene

phylogeny, two isolates from *T. longipes* and one isolate from *T. monanthum* were selected for sequence analysis at a region of the *16S rRNA* gene. A particularly informative region of the *16S rRNA* gene corresponds to *E. coli* gene codons 44–337 (42). This region was amplified by PCR using the primers (Y1 and Y2) and PCR conditions described previously (43). The PCR product was cycle-sequenced on both strands (Applied Biosystems Automated Sequencer). Sequences were submitted to the Ribosomal Database Project for comparison with all known prokaryotic *16S rRNA* sequences (44).

## RESULTS

**Identification of Genetic Groups.** Genetic groups of *Rhizobium* were distinguished based on patterns of hybridizing restriction fragments. At the chromosomal locus (probe pRt1013), two distinct RFLP groups (groups 1 and 2) were detected, which share only one fragment (Table 2). Groups 1 and 2 are each comprised of four subtypes, which have several fragments in common. Hybridization patterns at *nodAB* and *nodCIJT* reveal three main RFLP groups. Group A is comprised of subtypes FA1 to FA3 and HA1 to HA3, group B includes subtypes B1 to B4, and group C includes a single genotype. The lengths of hybridizing fragments suggest that rearrangements or duplications have occurred at each region probed. These patterns were not unexpected nor do they affect our ability to identify major genetic groups (see *Discussion*).

**Stable Associations Between Symbiotic Plasmids and Major Chromosomal Groups.** To estimate the level of *sym* plasmid transfer among different chromosomal lineages, the correlation between *nod* and chromosomal genetic groups was determined (Table 3). *Nod* group A occurred only in chromosomal group 1, and *nod* group B occurred in chromosomal group 2. The four strains with *nod* type C were also chromosomal group 2. This strict association between chromosomal

groups and the two dominant *nod* groups (A and B) indicates a lack of successful transfer of nodulation genes between chromosomal groups 1 and 2. Plasmid transfer within chromosomal groups 1 and 2 was not tested because of the small sample of rare subtypes.

**Associations Between *Rhizobium* Strains and *Trifolium* Host Plant Species.** At all sampling sites, nodule occupants of the clovers *T. bolanderi* and *T. longipes* were genetically distinct at *nod* and chromosomal loci from strains nodulating *T. monanthum* and *T. wormskioldii* (Table 4a). The single case of *Rhizobium* type 1/A (chromosomal group 1, *nod* group A) nodulating *T. monanthum* and the four cases of type 2/B nodulating *T. bolanderi* or *T. longipes* demonstrate rare instances of natural cross-infection. Within the *T. bolanderi* and *T. longipes* pair, there was no evidence for host–strain associations. However, host specificity within the *T. monanthum/T. wormskioldii* pair is suggested by the occurrence of *nod* group C only in nodules of *T. wormskioldii* collected from Fresno Dome site 1.

**Genetic Subdivision of *Rhizobium* Between Sampling Sites.** Distinct genotypes at *nod* and chromosomal loci were distributed nonrandomly across the two meadows sampled. This subdivision between meadows is most apparent at *nodCIJT* because Fresno Dome and House meadows had unique sets of genotypes at this locus within *nod* group A (Table 4b). To a lesser extent, genotypes within chromosomal type 2 also were distributed nonrandomly between meadows (Table 4c).

**Phylogenetic Position of Isolates.** Three isolates representing both chromosomal groups 1 and 2 were identical for a 296-bp segment of the *16S rRNA* gene (GenBank database accession nos. U72626 and U72627), indicating that the two chromosomal groups detected are considered the same species by conventional taxonomic methods. Based on a comparison with all known prokaryotic *16S rRNA* sequences, these native isolates cluster with the type strain *R. leguminosarum* LMG

Table 2.　Lengths of hybridizing fragments (in kilobases) of each *Rhizobium* genotype at the 1-kb reiterated chromosomal sequence (probe pRt1013), *nodAB*, and *nodCIJT*

| Genotype | | *Rhizobium* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Name | Isolates | *Eco*RI | | | | *Hin*dIII | | |
| | | 1-kb reiterated chromosomal locus (pRt1013) | | | | | | |
| 1a | 31 | | | 4.5 | 7 | 9 | 11.5 | |
| 1b | 1 | | | 4.5 | 8 | 9 | 11.5 | |
| 1c | 1 | | | 4.5 | 7 | 9 | 10 | |
| 1d | 1 | | | 4.5 | 8 | | 11.5 | |
| 2a | 7 | 2.3 | 4.3 | 4.5 | 4 | 6.8 | 7.2 | |
| 2b | 20 | | 4.3 | 4.5 | 4 | | 7.2 | |
| 2c | 6 | 3.3 | 4.3 | | 4 | | 7.2 | |
| 2d | 2 | | 4.3 | 4.5 | 4 | | 7.2 | 12 |

| Genotype | | *nodAB* | | | | *nodCIJT* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Name | Isolates | *Eco*RI | *Hin*dIII | | | *Eco*RI | | | *Hin*dIII | | |
| | | Nodulation loci | | | | | | | | | |
| FA1 | 16 | 2.9 | 1.5 | 3.3 | | 4.8 | 7.8 | 11.2 | 3.3 | 6.1 | 10 |
| FA2 | 1 | 2.9 | 1.5 | 3.3 | | 4.8 | 7.8 | | 3.3 | 8 | 10 |
| FA3 | 1 | 2.9 | 1.5 | 3.3 | 3 | 4.8 | | 11 | 3.3 | 7 | 11 |
| HA1 | 9 | 2.9 | 1.5 | 3.3 | | 4.8 | 6.8 | | 3.3 | 5.2 | |
| HA2 | 6 | 2.9 | 1.5 | 3.3 | | 4.8 | 10 | | 3.3 | 5.2 | |
| HA3 | 1 | 2.9 | 1.5 | 3.3 | | 4.8 | 10 | | 3.3 | 6.8 | |
| B1 | 28 | 7.2 | 1.9 | | 4.2 | 4.5 | | | 4.2 | | |
| B2 | 1 | 7.2 | 1.9 | | 4 | 5.2 | 13 | | 2.8 | 4 | |
| B3 | 1 | 3.3 | 1.9 | 3.3 | 4.2 | 4.5 | | | 4.2 | | |
| B4 | 1 | 8.5 | 1.9 | | 4.2 | 4.5 | | | 4.2 | | |
| C | 4 | 9 | 1.9 | 4.8 | 4 | 9 | 14 | 20 | 4.8 | 9 | 12 |

　　The number of *Rhizobium* isolates of each genotype is given in parentheses. In the text, *nod* genotypes FA1 to HA3 are considered "*nod* group A."

Table 3.    Number of *Rhizobium* isolates of each chromosomal genotype that carry a *sym* plasmid of a given *nod* genotype

| Chromosomal genotype | *nod* genotype | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | FA1 | FA2 | FA3 | HA1 | HA2 | HA3 | B1 | B2 | B3 | B4 | C |
| 1a | 15 | 1 | 1 | 9 | 5 | . | . | . | . | . | . |
| 1b | . | . | . | . | 1 | . | . | . | . | . | . |
| 1c | 1 | . | . | . | . | . | . | . | . | . | . |
| 1d | . | . | . | . | . | 1 | . | . | . | . | . |
| 2a | . | . | . | . | . | . | 7 | . | . | . | . |
| 2b | . | . | . | . | . | . | 15 | 1 | . | . | 4 |
| 2c | . | . | . | . | . | . | 5 | . | . | 1 | . |
| 2d | . | . | . | . | . | . | 1 | . | 1 | . | . |

., indicates zero isolates. *nod* genotypes were distributed nonrandomly across chromosomal genotypes ($\chi^2 = 87.4$, $df = 12$, $P < 0.000$). Chi-square analysis was performed after omitting cells with expected values <1.

9518 (45) with a sequence similarity of 97.3% and are identical to *R. leguminosarum* strain 8002 (46).

## DISCUSSION

The genetic plasticity conferred by transmissible plasmids is thought to be important in the diversification of bacterial populations. Relative to chromosomal regions, plasmids are easily lost and regained in populations, may change in copy number regulation as a mechanism for gene amplification, and potentially undergo elevated mutation rates (4, 5). The feature of plasmids most often emphasized as evolutionarily important is their lateral transfer among distinct bacterial chromosomal lineages, which has been demonstrated in several human-impacted environments (5, 6, 28). The significance of transmissible plasmids in adaptation to sporadic, or "local," selec-

Table 4.    Number of *Rhizobium* isolates in genetic groups, as distributed across (a) each of four *Trifolium* host plant species and (b and c) each of two sampling meadows

**a**

| *Rhizobium* genetic group (pRt1013/ *nod*) | Host plant species | | | |
|---|---|---|---|---|
| | *T. bolanderi* | *T. longipes* | *T. monanthum* | *T. wormskioldii* |
| 1/A | 16 | 17 | 1 | 0 |
| 2/B | 1 | 3 | 23 | 4 |
| 2/C | 0 | 0 | 0 | 4 |

**b**

| *nod* genotype | Sampling meadow | |
|---|---|---|
| | Fresno Dome | House |
| FA1 | 16 | 0 |
| FA2 | 1 | 0 |
| FA3 | 1 | 0 |
| HA1 | 0 | 9 |
| HA2 | 0 | 6 |
| HA3 | 0 | 1 |

**c**

| pRt1013 genotype | Sampling meadow | |
|---|---|---|
| | Fresno Dome | House |
| 2a | 0 | 7 |
| 2b | 17 | 3 |
| 2c | 6 | 0 |
| 2d | 1 | 1 |

Chromosomal and nodulation genotypes were distributed nonrandomly across host plant species (a, $\chi^2 = 85.0$, $df = 6$, $P < 0.000$). Each of House and Fresno Dome meadows had characteristic subtypes within *nod* type A (b, $\chi^2 = 39.9$, $df = 2$, $P < 0.000$). Likewise, subtypes within pRt1013 genotype 2 were distributed nonrandomly across meadows (c, $\chi^2 = 23.9$, $df = 2$, $P < 0.0005$). Chi-square analysis was performed after omitting cells with expected values <1.

tive pressures is suggested by the tendency for these replicons to encode locally adaptive traits such as virulence, antibiotic resistance, toxin production, and symbiotic phenotypes (2, 5). Divergence in prokaryotes is driven largely by ecological rather than sexual isolation (47, 48); therefore, the frequent transfer of these ecologically important traits may have profound implications for bacterial diversification and speciation. Conjugation of virulence, resistance, and symbiotic plasmids may mitigate the effect of plasmid level selection on chromosomal divergence and thus may allow genetic cohesion of chromosomal populations despite ecological and genetic differentiation of plasmid populations.

Given the potential importance of plasmids in bacterial evolution and the emphasis of previous studies on human-impacted populations, our objective was to explore the dynamics of an ecologically important plasmid within a native bacterial population. This study provides evidence for restricted successful transfer of plasmids among chromosomal groups within a population of *R. leguminosarum* isolated from nodules of four native, cooccurring species of *Trifolium.* The strict association of *nod* genotypes with distinct chromosomal genotypes strongly argues against any successful plasmid transfer between major chromosomal groups. This observed correlation stands in sharp contrast with previous field and laboratory studies of *Rhizobium*, which demonstrate *sym* plasmid transfer among divergent chromosomal lineages (23, 28–32). The novelty of our results is likely due to sampling *Rhizobium* indigenous to native legume species rather than strains associated with agricultural or introduced host plant species.

Although successful *sym* plasmid transfer clearly does not occur frequently between the major chromosomal lineages we detected, we cannot rule out the possibility that *sym* plasmids do transfer within those chromosomal groups. Such transfer would only be revealed by higher levels of genetic resolution. Probing with longer regions of DNA effectively samples more nucleotide sites and increases the likelihood of detecting closely related genotypes. However, for two reasons, we are confident that the 1-kb probe used here adequately distinguishes major chromosomal groups. First, this probe of a reiterated chromosomal locus samples a region greater than 1 kb; the lengths of hybridizing fragments indicate that the restriction site variation we detected occurs outside of the region probed. It is therefore not surprising that our relatively short chromosomal probe detected a similar number of genetic groups per isolate sampled as was found in previous studies [8 genetic groups/69 isolates sampled in this study compared with 10/56 (29) and 18/176 (39)]. Second, previous studies show that genetic groups distinguished by this 1-kb probe agree with genetic groups identified by much longer probes (26 kb) and multi-locus enzyme electrophoresis analysis (39, 49). Therefore, we are confident that the major chromosomal types distinguished with the 1-kb probe are genetically different groups within this population.

The observed stability of *sym* plasmids and these major chromosomal groups has several possible explanations, including physiological, genetic, and ecological constraints on conjugation in native bacterial populations. First, limited opportunities for lateral transfer may constrain conjugation in native settings. Plasmid transfer in soil environments requires high cell densities of both donor and recipient cells (50), a condition thought to exist only in the immediate vicinity of the root and inside the nodule. The amplified growth caused by specific plant exudates is often restricted to the preferred nodulating strain (ref. 51 but see ref. 52), so *Rhizobium* strains that specifically nodulate different plant species are not thought to cooccur at high densities near roots or inside nodules. However, the instances of natural cross-infection in this study (Table 4a) indicate an occasional close proximity of the two major strains, 1/A and 2/B, and suggest that physical opportunities for plasmid exchange exist in the field.

Second, genetic divergence among distinct chromosomal lineages may limit the transfer and maintenance of plasmids (32). Although previous studies attribute high local chromosomal divergence to migrant strains (52), the relative isolation of our sampling sites suggests that the observed divergence results from local diversification of indigenous *Rhizobium*. Our data are not amenable to estimates of nucleotide diversity, but the fact that chromosomal groups 1 and 2 share only one common fragment suggests that they are genetically distinct. This divergence may limit conjugation or the persistence of transconjugants in the field.

Finally, only a restricted set of plasmid–chromosome combinations may be competitive and able to persist in native populations. Like many plant species, the four *Trifolium* species we sampled are distributed nonrandomly across soil moisture and temperature (53), factors also shown to affect survival of *Rhizobium* in the soil (22). These legume soil preferences may cause the selective pressures shaping *sym* plasmid and chromosomal loci to overlap spatially and thus limit the range of viable plasmid–chromosome combinations; however, we find this alternative unlikely given the intimate sympatry of the *Trifolium* species sampled. Plants of different species occurred within 10-cm diameters, sometimes within 2 cm of each other (unpublished data).

Alternatively, transconjugants may be selected against if symbiotic phenotypes are encoded by chromosomal loci or if survival in the soil is influenced by *sym* plasmids. For example, symbiotic compatibility with native legume species may involve loci outside the well characterized nodulation region. This situation may arise if native host legumes themselves experience selection for symbiotic compatibility and are selected to associate with *Rhizobium* strains that are prevalent in the preferred soil type of the plant. To the extent that partitioning between replicons of symbiotic and soil-related phenotypes breaks down, transconjugants will be maladaptive.

The observed stability of symbiotic plasmids has important implications for the divergence and speciation of native *Rhizobium* populations. In agricultural systems, *sym* plasmid transfer may mitigate the ecological effect of the plant species in driving chromosomal divergence (29). However, in *Rhizobium* populations with restricted plasmid transfer such as we observed, host plant species may delineate ecological and genetic populations of both *sym* plasmid and chromosomal loci. *Rhizobium* strains that specifically nodulate different host plant species apparently constitute distinct ecological populations, and, in the absence of *sym* plasmid transfer, chromosomal divergence between these populations may occur more rapidly than previous studies suggest (23, 28–32).

Factors other than lateral transfer may differentially shape genetic divergence of plasmid and chromosomal loci. The occurrence of traits on plasmids may generally elevate genetic diversity and rates of evolution because of higher mutation rates or increased levels of reiterated DNA (5). Previous studies detecting a greater number of distinct genetic groups at *sym* than chromosomal loci suggest higher diversity at *nod* loci, with up to five distinct *sym* types per chromosomal group (31, 39, 49). This increased variability in the *sym* genome has been attributed to recombination within the *sym* plasmid, facilitated by the high frequency of reiterated sequences around the *sym* plasmid (18, 25, 31). In addition to higher mutation rates, increased population subdivision is known to elevate overall genetic divergence at a particular locus (54). Although our data do not allow direct comparisons of chromosomal and *sym* plasmid diversity levels, we found many more distinct genotypes at the *nod* region than at the chromosomal locus. Our results argue that subdivision between meadows contributes significantly to the observed variation within *nod* type A. Likewise, the presence of *nod* type C at Fresno Dome site 1 but not Fresno Dome site 2, sites within 100 m of each other, suggests a limited distribution of this *nod* group. Limited migration of *sym* plasmids between sites due to loss in soil populations (23–25), genetic drift of one variant into each population (55), or site-specific differences in soil profiles that select for alternative *nod* phenotypes (56) may contribute to population subdivision at symbiotic loci.

In summary, the plasmid stability observed in this study suggests that constraints on plasmid transfer, such as limited opportunity for transfer, chromosomal divergence, or the coupling of selective pressures shaping plasmid and chromosomal variation, may be especially important in indigenous microbial populations relative to agricultural systems. The maintenance of phenotypically important loci on plasmids may result from unique properties of plasmid genes other than transfer, including the ability to be lost and regained depending on the presence of a certain selective pressure or the potential to undergo higher rates of evolution due to relatively high frequency of reiterated DNA. These alternative forms of genetic plasticity may be important in the adaptation of native *Rhizobium* populations and may account for the maintenance of symbiotic genes on transmissible plasmids. The ability to lose nodulation and nitrogen-fixation genes may be adaptive to *Rhizobium* in the soil, where these loci are apparently detrimental and where *sym* plasmids are lost at high frequencies. In addition, the occurrence of *sym* genes on plasmids also may provide a mechanism for amplifying these loci by regulation of plasmid copy number. The stability of bacterial genomes depends on the strength of selection for transconjugants and opportunities for lateral transfer, both of which may be diminished in native microbial populations. A complete picture of the role of transmissible plasmids in bacterial diversification and genome plasticity will therefore require additional sampling of populations that do not experience human-mediated selection on plasmid phenotypes.

1. Levin, B. R. (1981) *Genetics* **99,** 1–23.
2. Reanny, D. (1976) *Bacteriol. Rev.* **40,** 552–590.
3. Levin, B. R. (1993) *Curr. Opin. Gen. Dev.* **3,** 849–854.
4. Modi, R. I. & Adams, J. (1991) *Evolution* **45,** 656–667.
5. Eberhard, W. (1990) *Q. Rev. Biol.* **65,** 3–22.
6. O'Brien, T. F., del Pilar Pla, M., Mayer, K. H., Kishi, H., Gilleece, E., Syvannen, M. & Hopkins, J. D. (1985) *Science* **230,** 87–88.
7. Hughes, V. M. & Datta, N. (1983) *Nature (London)* **302,** 725–726.
8. Jones, D. & Morley, S. (1981) *Ann. Appl. Microbiol.* **97,** 183–190.

9. Day, M. J., Burton, N. F. & Bull, A. T. (1988) *Lett. Appl. Microbiol.* **7,** 71–73.
10. Simon, R. D., Shilo, M. & Hastings, J. W. (1982) *Curr. Microbiol.* **7,** 175–180.
11. Glassman, D. L. & McNicol, L. A. (1981) *Plasmid* **5,** 231.
12. Sundin, G. W., Demezas, D. H. & Bender, C. L. (1994) *Appl. Environ. Microbiol.* **60,** 4421–4431.
13. Zawadzaki, P., Riley, M. A. & Cohan, F. M. (1996) *J. Bacteriol.* **178,** 191–198.
14. Riley, M. A. & Gordon, D. M. (1992) *J. Gen. Microbiol.* **138,** 1345–1352.
15. Mercer, A. A., Morelli, G., Heuzenroeder, M., Kamke, M. & Achtman, M. (1984) *Infect. Immun.* **46,** 649–657.
16. Trevors, J. T., Barkay, T. & Bourquin, W. (1987) *Can. J. Microbiol.* **33,** 191–198.
17. Young, J. P. W. & Haukka, K. (1996) *New Phytol.* **133,** 87–94.
18. Martinez, E., Romero, D. & Palacios, R. (1990) *Crit. Rev. Plant Sci.* **9,** 17–29.
19. Long, S. (1989) *Cell* **56,** 203–214.
20. Brom, S., Santos, A. G. D. L., Strepkowsky, T., Flores, M., Davila, G., Romero, D. & Palacios, R. (1992) *J. Bacteriol.* **174,** 5183–5189.
21. Hooykaas, P., van Brussel, A., den Dulk-Ras, H., van Slogteren, G. & Schilperoort, R. (1981) *Nature (London)* **291,** 351.
22. Lowendorf, H. (1980) *Adv. Microbiol. Ecol.* **4,** 87–124.
23. Laguerre, G., Bardin, M. & Amarger, N. (1993) *Can. J. Microbiol.* **35,** 1142–1149.
24. Segovia, L., Pinero, D., Palacios, R. & Martinez-Romero, E. (1991) *Appl. Environ. Microbiol.* **57,** 426–433.
25. Soberon-Chavez, G. & Najera, R. (1989) *J. Gen. Microbiol.* **35,** 464–468.
26. Brewin, N., Wood, E. & Young, J. (1983) *J. Gen. Microbiol.* **129,** 2973–2977.
27. Baldani, J. I., Weaver, R. W., Hynes, M. F. & Eardly, B. D. (1992) *Appl. Environ. Microbiol.* **58,** 2308–2314.
28. Laguerre, G., Mavingui, P., Allard, M. R., P. Charnay, M., Louvrier, P., Mazurier, S. I., Rigottier-Gois, L. & Amarger, N. (1996) *Appl. Environ. Microbiol.* **62,** 2029–2036.
29. Laguerre, G., Geniaux, E., Mazurier, S. I., Casartelii, R. & Amarger, N. (1993) *Can. J. Microbiol.* **39,** 412–419.
30. Geniaux, E., Laguerre, G. & Amarger, N. (1993) *Mol. Ecol.* **2,** 295–302.
31. Schofield, P., Gibson, A., Dudman, W. & Watson, J. (1987) *Appl. Environ. Microbiol.* **53,** 2942–2947.
32. Young, J. & Wexler, M. (1988) *J. Gen. Microbiol.* **134,** 2731–2739.
33. Sprent, J. I. (1994) *Plant Soil* **161,** 1–10.
34. Sullivan, J. T., Patrick, H. N., Lowther, W. L., Scott, D. B. & Ronson, C. W. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 8985–8989.
35. Harrison, S., Young, J. & Jones, D. (1987) *Plant Soil* **103,** 147–150.
36. Souza, V., Eguiarte, L., Avila, G., Cappello, R., Gallardo, C., Montoya, F. & Pinero, D. (1994) *Appl. Environ. Microbiol.* **60,** 1260–1268.
37. Souza, V., Nguyen, T. T., Hudson, R. R., Pinero, D. & Lenski, R. E. (1992) *Proc. Natl. Acad. Sci. USA* **89**.
38. Trayler, W. (1993) M.A. thesis (California State University, Fresno).
39. Demezas, D. H., Reardon, T. B., Watson, J. M. & Gibson, A. H. (1991) *Appl. Environ. Microbiol.* **57,** 3489–3495.
40. Innes, R., Hirose, M. & Kuempel, P. (1988) *J. Bacteriol.* **170,** 3793–3802.
41. Olsen, G. & Woese, C. R. (1993) *FASEB J.* **7,** 113–123.
42. Brosius, J., Dull, T. J., Sleeter, D. D. & Noller, H. F. (1981) *J. Mol. Biol.* **148,** 107–127.
43. Eardly, B. D., Young, J. P. W. & Selander, R. K. (1992) *Appl. Environ. Microbiol.* **58,** 1809-1815. (1995) *Appl. Environ. Microbiol.* **61,** 507–512.
44. Maidak, B. L., Larsen, N., McCaughey, M. J., Overbeek, R., Olsen, G. J., Fogel, K., Blandy, J. & Woese, C. R. (1994) *Nucleic Acids Res.* **22,** 3485–3487.
45. Willems, A. M. (1993) *Int. J. Syst. Bacteriol.* **43,** 305–313.
46. Young, J. P. W., Downer, H. L. & Eardly, B. D. (1991) *J. Bacteriol.* **173,** 2271–2277.
47. Cohan, F. M. (1995) *Evolution* **49,** 164–175.
48. Cohan, F. M. (1994) *Trends Ecol. Evol.* **9,** 175–180.
49. Demezas, D. H., Reardon, T. B., Strain, S. R., Watson, J. M. & Gibson, A. H. (1995) *Mol. Ecol.* **4,** 209–220.
50. Fernandez-Astorga, A., Muela, A., Cisterna, R., Iriberri, J. & Barcina, I. (1992) *Appl. Environ. Microbiol.* **58,** 392–398.
51. Moawad, H., Ellis, W. & Schmidt, E. (1984) *Appl. Environ. Microbiol.* **47,** 607–612.
52. Leung, K., Strain, S., Bruijn, F. D. & Bottomley, P. (1994) *Appl. Environ. Microbiol.* **60,** 416–426.
53. Ratliff, R. & Harding, E. (1993) in *Soil Acidity, Temperature, and Moisture Relationships of Four Clovers in Sierra Nevada Meadows* (Forest Service PSW Research Station, Albany, CA).
54. Simmons, G. M., Kreitman, M. E., Quattlebaum, W. F. & Miyashita, N. (1989) *Evolution* **43,** 393–409.
55. Hartl, D. & Clark, A. (1989) *Principles of Population Genetics* (Sinaur, Sunderland, MA).
56. McKay, I. & Djordjevic, M. (1993) *Appl. Environ. Microbiol.* **59,** 3385–3392.