Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 8985–8989, September 1995
Population Biology

Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment

JOHN T. SULLIVAN*, HEATHER N. PATRICK†, WILLIAM L. LOWTHER†, D. BARRY SCOTT‡, AND CLIVE W. RONSON*§

*Department of Microbiology, University of Otago, P.O. Box 56, Dunedin, New Zealand; †New Zealand Pastoral Agriculture Research Institute, Ltd., Invermay Agricultural Centre, Private Bag 50034, Mosgiel, New Zealand; and ‡Molecular Genetics Unit, School of Biological Sciences, Massey University, Private Bag 11222, Palmerston North, New Zealand

Communicated by Frederick M. Ausubel, Massachusetts General Hospital, Boston, MA, June 23, 1995 (received for review May 12, 1995)

ABSTRACT **Rhizobia were isolated from nodules off a stand of *Lotus corniculatus* established with a single inoculant strain, ICMP3153, 7 years earlier in an area devoid of naturalized *Rhizobium loti*. The isolates showed diversity in growth rate, *Spe* I fingerprint of genomic DNA, and hybridization pattern to genomic DNA probes. The 19% of isolates that grew at the same rate as strain ICMP3153 were the only isolates that had the same fingerprint as strain ICMP3153. Sequencing of part of the 16S rRNA gene of several diverse isolates confirmed that they were not derived from the inoculant strain. Nevertheless, all non-ICMP3153 strains gave *Eco*RI and *Spe* I hybridization patterns identical to ICMP3153 when hybridized to nodulation gene cosmids. Hybridization of digests generated by the very rare cutting enzyme *Swa* I revealed that the symbiotic DNA region (at least 105 kb) was chromosomally integrated in the strains. The results suggest that the diverse strains arose by transfer of chromosomal symbiotic genes from ICMP3153 to nonsymbiotic rhizobia in the environment.**

Bacteria of the genera *Rhizobium* and *Bradyrhizobium* are of agronomic importance because of their ability to form nitrogen-fixing symbioses with leguminous plants. However, attempts to use strains of rhizobia selected for their nitrogen-fixing ability as inoculants to increase symbiotic nitrogen fixation in crops or pastures are frequently frustrated by the presence of naturalized populations of rhizobia in the soil. These populations may be of inferior nitrogen-fixing ability but usually form the majority of nodules on field-grown plants (1–3). Even in situations where the naturalized population is small or undetectable, enabling inoculant strains to nodulate host plants in the first season, naturalized populations of unknown origin often dominate nodule formation within a few years (1, 4–6). The superior competitive ability of naturalized rhizobia is probably due to several factors including population size (7), distribution through the soil profile (8), and their adaptation to the local soil environment (1–3).

Long-established naturalized populations of various *Rhizobium* species are highly diverse and in many cases of clonal structure (9–13). However, the low linkage disequilibrium levels found in some populations suggest that chromosomal recombination can occur frequently enough, at least within some populations, to maintain the populations near linkage equilibrium (14–18). Evidence for lateral transfer and recombination of symbiotic plasmids in soil populations has also been obtained (19–21). While these studies document population diversity, the rate at which the populations diversify and the origin of the vast genetic diversity found in localized populations of rhizobia remain unknown.

We are working with *Rhizobium loti*, a species of fast-growing rhizobia able to form nodules on several *Lotus* species (22). Taxonomic studies based on 16S rRNA gene sequence indicate that *R. loti* and *Rhizobium huakuii* together constitute a separate line of descent from other fast-growing *Rhizobium* species (23, 24). *R. loti* also differs from other fast-growing rhizobia such as *Rhizobium leguminosarum* and *Rhizobium meliloti* in that its symbiotic information is chromosome rather than plasmid encoded (25–27). In this respect, it is more similar to the slow-growing species *Bradyrhizobium japonicum* (28).

All species of *Lotus* now in New Zealand were introduced from overseas and none is closely related to any native leguminous plants. Hence, it seems likely their rhizobia were similarly introduced (29). *Lotus corniculatus* is currently present only in a few localized areas and *R. loti* strains are still absent from most New Zealand soils (29–31). Here we report the isolation of diverse strains of *R. loti* from a stand of *L. corniculatus* established 7 years earlier with a defined inoculant strain at a site previously devoid of rhizobia capable of nodulating the legume. The diverse strains contain the identical symbiotic DNA region to the inoculant strain, suggesting they arose through symbiotic gene transfer from the inoculant strain to nonsymbiotic rhizobia.

MATERIALS AND METHODS

Strains and Growth Conditions. The inoculant was a culture of *R. loti* strain ICMP3153 (International Collection of Microorganisms from Plants, Landcare Research, Private Bag 92170, Auckland, New Zealand) commercially prepared in a peat base that had been sterilized by γ-irradiation. A portion of the culture assayed in 1993 was microbiologically pure. Strain ICMP3153 is derived from strain NZP2238 following plant passage. Other *R. loti* strains were available in our culture collection: NZP2014, NZP2037, NZP2213, NZP2234, and NZP2238 were described by Jarvis *et al.* (22), NZP2298 was originally strain L72M103C from Canada, and strain 8KC3 was a New Zealand soil isolate.

To isolate rhizobia from nodules, the nodules were surface-sterilized (27) and crushed individually in 50 μl of water, and the suspension was streaked onto G/RDM defined medium (32) for single colony purification. Plates were incubated at 26°C. Symbiotic properties of strains were assessed by inoculating *L. corniculatus* seedlings grown aseptically on slopes of nitrogen-free agar in test tubes (33). Rhizobia were isolated from nodules to confirm their identity.

Experimental Field Site. A stand of *L. corniculatus* was established in 1986 at a field site devoid of naturalized rhizobia capable of symbiotic association with the plant (30). The field site was undeveloped tussock (*Festuca novae-zealandiae* and *Chionochloa rigida*) grassland located at an elevation of 885 m in Lammermoor, Otago, New Zealand. The soil was a dark brown silt loam with a very acid pH (4.9) and a low (0.28%) total nitrogen content. The site was fertilized with molybdic superphosphate [200 kg/ha (1 ha = 10^4 m^2); 0.014% Mo, 7.6%

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

§To whom reprint requests should be addressed.

P, 10% S] and lime (500 kg/ha; 90% $CaCO_3$) before planting but was not otherwise cultivated. The lime was spread out and oven dried at 80°C for 2 days before use to kill any rhizobia (29). Seed was inoculated the day before sowing with the peat culture of *R. loti* strain ICMP3153 to give between 2.5×10^4 and 1.25×10^5 rhizobia per seed. The seed was sown by spreading it over the surface of the site. *L. corniculatus* seedlings that received insufficient inoculant failed to nodulate and perished through nitrogen deficiency within several weeks (30). Uninoculated seed planted under similar conditions adjacent to the field site in 1994 also failed to nodulate.

DNA Manipulations. Total genomic DNA was isolated as described (26) from 1.5-ml cultures grown in TY broth (34) at 28°C for 48 hr. Genomic DNA digested with *Eco*RI was separated on 1% agarose gels at 1 $V \cdot cm^{-1}$ for 17 hr.

For pulse-field gels, agarose plugs containing *R. loti* DNA were prepared (35) and the DNA was digested with *Spe* I or *Swa* I. DNA digests were separated in 1% LE agarose gels in TAFE II buffer using a Geneline II transverse alternating field system (Beckman). Electrophoretic conditions used for the *Spe* I digests were as follows: stage 1, 250 mA for 7 hr with 4-s pulse; stage 2, 250 mA for 7 hr with 16-s pulse; stage 3, 250 mA for 7 hr with 24-s pulse; stage 4, 300 mA for 10 hr with 32-s pulse. The conditions for *Swa* I digests were as follows: stage 1, 350 mA for 12 hr with 1-min pulse; stage 2, 370 mA for 12 hr with 2-min pulse; stage 3, 390 mA for 12 hr with 3-min pulse.

^{32}P-labeled probes were generated by random priming. DNA blots were hybridized for 24 hr at 65°C, washed at 65°C sequentially in 2× SSC/0.1% SDS, 1× SSC/0.1% SDS, and 0.1× SSC/0.1% SDS and then exposed to film for 6–18 hr. Cosmid DNA used to prepare probes was isolated using a plasmid preparation kit (Qiagen, Chatsworth, CA). The random fragments used as probes were cloned from a *Hin*dIII digest of NZP2238 DNA fractionated on a 1% agarose gel. Fragments about 4.5–5.5 kb in size were excised from the gel, purified, and cloned into pUC19. Plasmid DNA was isolated from five randomly chosen clones and pooled for use as a probe.

Plasmids in *Rhizobium* strains were visualized by agarose gel electrophoresis using a modified Eckhardt procedure (36).

For analysis of 16S rRNA gene sequence, a region of the 16S rRNA gene spanning nucleotides 44–337 in the *Escherichia coli* 16S rRNA sequence was amplified by PCR using primers Y1 and Y2 (37, 38). The PCR products were purified using a QIAquick kit (Qiagen) and sequenced using the Y1 and Y2 primers and an Applied Biosystems model 373A autosequencer. Data bases at the National Center for Biotechnology Information were searched using BLASTN (39) for similar DNA sequences via E-mail.

RESULTS

Genetic Diversity of Nodule Isolates. Rhizobial strains isolated from 320 nodules on plants selected from throughout the field site in 1993 showed heterogeneity in growth rates. The inoculant strain and 19% of the nodule isolates formed single colonies of about 1 mm in diameter on G/RDM medium within 4 days, whereas the remaining nodule isolates took between 5 and 8 days. Six groupings based on growth rate were apparent, with the most prevalent group taking 5 days to form 1-mm single colonies. Twelve isolates from various growth rate groups all formed nitrogen-fixing nodules when inoculated onto *L. corniculatus* seedlings.

In initial studies, the isolates were grouped by comparing fingerprints of genomic DNA digested with *Eco*RI. Twelve isolates with a growth rate identical to NZP2238 had a DNA fingerprint identical to NZP2238, whereas at least 10 different *Eco*RI fingerprints were apparent among 30 isolates from other growth rate groups. The genetic diversity of the isolates was confirmed by comparing *Spe* I DNA fingerprints of several isolates (Fig. 1*A*) and by Southern hybridization experiments. These experiments used either random cloned fragments of NZP2238 genomic DNA (Fig. 1*B*) or cosmid pPN32 (Fig. 1*C*) containing *R. loti* exopolysaccharide synthesis genes (40) as probes against *Eco*RI-digested DNA. Six isolates with a growth rate identical to NZP2238 had a DNA fingerprint identical to NZP2238 (except for a few bands due to a plasmid present in NZP2238 but not ICMP3153 or the nodule isolates; see below), whereas the isolates with different *Eco*RI fingerprints gave distinctive fingerprints. NZP2238 and the diverse

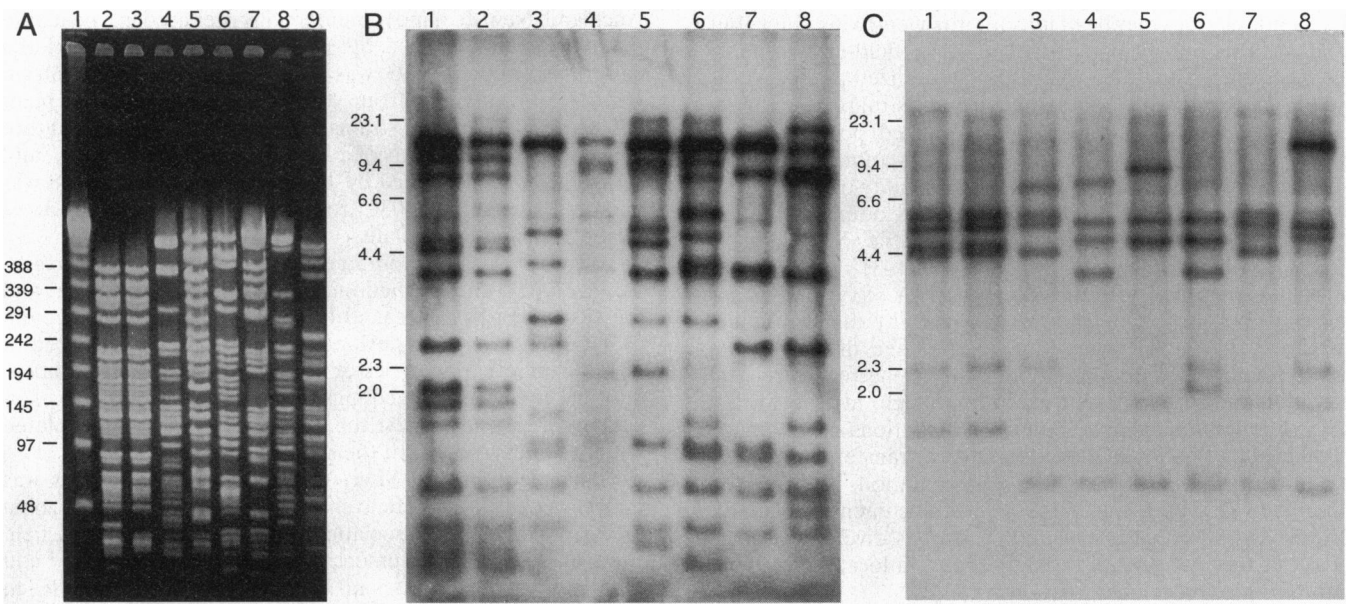

FIG. 1. Agarose gel and Southern hybridization analysis of genomic DNA from *R. loti* strains. (*A*) Pulse-field agarose gel of *Spe* I digests of NZP2238 and seven nodule isolates. Lanes: 1, λ concatemers; 2, NZP2238; 3, R7A, a nodule isolate similar to NZP2238; 4–9, nodule isolates R12C, R16C, R97A, R88B, R83C, and R8CS, respectively. The additional bands in lane 2 compared to lane 3 are due to the plasmid in NZP2238. (*B*) Southern blot of *Eco*RI digests of NZP2238 and seven nodule isolates hybridized to a collection of five *Hin*dIII fragments, each about 5 kb, randomly cloned from NZP2238. Lanes: 1, NZP2238; 2–8, nodule isolates R7A, R12C, R16C, R97A, R88B, R83C, and R8CS, respectively. (*C*) Southern blot of *Eco*RI digests of NZP2238 and seven nodule isolates hybridized to the *R. loti exo* gene cosmid pPN32. Lanes as in *B*.

nodule isolates shared few *Spe* I or hybridizing bands, suggesting that the isolates did not arise from the inoculant strain.

16S rRNA Gene Sequence. To determine whether the diverse nodule isolates were strains of *R. loti*, a 260-bp region of the 16S rRNA gene was sequenced for several isolates and the *R. loti* type strain NZP2213. The NZP2213 sequence was identical to the published sequence (GenBank accession no. X67229) of Willems and Collins (23). The majority of strains tested had a one-base mismatch to the NZP2213 sequence, NZP2238, and nodule isolates with similar DNA fingerprints to it had four single nucleotide mismatches; another nodule isolate, strain R88B, had three mismatches and was identical to a *R. huakuii* sequence (Fig. 2). All sequences had at least six mismatches to other bacterial 16S rRNA gene sequences in the data bases. These results confirm that the nodule isolates belong to the *R. loti*/*R. huakuii* lineage.

Plasmid Profiles. Plasmid profiling using a modified Eckhardt procedure (36) showed single large plasmids of different sizes in 3 of 40 isolates examined. Strain NZP2238 contained one plasmid as previously reported (27). However, nodule isolates similar to NZP2238 (e.g., R7A) were plasmid-free as were colonies recovered from the peat culture used to inoculate the site. The presence of a plasmid in NZP2238 but not R7A was also apparent from the *Spe* I digests (Fig. 1*A*) and from pulse-field gels of undigested DNA (not shown). An isolate of ICMP3153 obtained directly from the culture collection was plasmid-free, indicating that the plasmid was lost from NZP2238 prior to its being lodged in the ICMP culture collection and being provided for manufacture of the peat inoculant. ICMP3153 gave a *Spe* I fingerprint identical to R7A as expected.

Conservation of a Symbiotic DNA Region. The above results indicate that genotypically diverse strains of *R. loti* were present following growth of *L. corniculatus* in an environment previously devoid of naturalized strains capable of nodulating the legume. The symbiotic DNA of the strains was characterized by Southern hybridization analysis using two overlapping cosmids, pPN306 and pPN377, that encode several nodulation genes and span 39 kb of the *R. loti* strain NZP2037 genome (Fig. 3) (41, 42). All nodule isolates contained the symbiotic region identical to NZP2238. For example, in all strains pPN377 hybridized strongly to five bands in *Eco*RI digests (Fig. 4*A*). Both cosmids hybridized to a 105-kb fragment in *Spe* I digests (Fig. 4*B*) as did a *nifHD* gene probe pRt564 (43) (data not shown). No variation in the *Eco*RI hybridization pattern to pPN377 was found among >100 isolates tested. In contrast, 6 *R. loti* strains effective on *L. corniculatus* and originally isolated from diverse geographical areas produced different patterns when either *Spe* I (Fig. 4*C*) or *Eco*RI digests (not shown) were probed with pPN377.

```
NZP2213      AGGCTTAACACATGCAAGTCGAGCGCCTCGCAAGAGGAGCGGCAGACGGGTG
R huakuii                          C     G
NZP2238,R7A                        C     G
R12C,others
88B                                C     G

NZP2213      AGTAACGCGTGGGAATCTACCCATCTCTACGGAACAACTCCGGGAAACTGGA
R huakuii
NZP2238,R7A
R12C,others
R88B

NZP2213      GCTAATACCGTATACGTCCTTCGGGAGAAAGATTTATCGGAGATGGATGAGC
R huakuii
NZP2238,R7A                      T
R12C,others
R88B

NZP2213      CCGCGTTGGATTAGCTAGTTGGTGGGGTAATGGCCTACCAAGGCGACGATCC
R huakuii
NZP2238,R7A
R12C,others
R88B

NZP2213      ATAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAG
R huakuii                              C
NZP2238,R7A                            C
R12C,others                            C
R88B                                   C
```

FIG. 2. Alignment of 260 bp of the 16S rRNA gene sequence from several rhizobial strains. The *R. huakuii* sequence (GenBank accession no. D12797) was determined by Yanagi and Yamasato (24). Blanks indicate sequence that is identical to the *R. loti* NZP2213 sequence. Nine other nodule isolates with different DNA fingerprints had the same sequence as R12C.

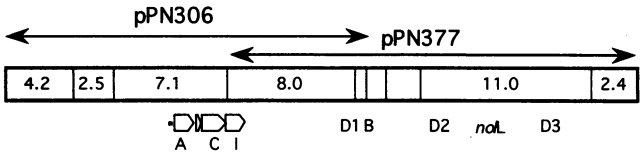

FIG. 3. *Eco*RI physical map of nodulation gene cosmids pPN306 and pPN377 derived from *R. loti* strain NZP2037 (41, 42). Nodulation genes indicated by arrows have been sequenced, while genes marked by name only were identified by hybridization.

Genomic Integration of the Symbiotic DNA Region. The above results suggest that the diverse nodule isolates acquired their symbiotic DNA region by gene transfer from the inoculant strain. To determine whether the symbiotic DNA was integrated into the genome of the diverse isolates, *Swa* I digests of genomic DNA separated on pulse-field gels were Southern blotted and hybridized to pPN377. A fragment of about 760 kb hybridized in NZP2238 and R7A, whereas fragments between 1000 kb and 1300 kb hybridized in each of the diverse nodule isolates (Fig. 5).

DISCUSSION

Genotypically diverse strains of *R. loti* were found nodulating a stand of *L. corniculatus* that was inoculated with a single strain of *R. loti* and planted 7 years earlier in an area devoid of naturalized rhizobia capable of nodulating the legume. Despite their diversity, all strains contained a symbiotic region identical to the original inoculant strain. This region of at least 105 kb was probably integrated into the chromosome of the strains. These findings suggest that diverse populations of nodulating strains of *R. loti* arise by lateral transfer of chromosomal symbiotic genes from symbiotic to nonsymbiotic rhizobia in the field.

The diversity of the strains and their distinct origin from the inoculant strain were shown by restriction fragment length polymorphisms (RFLPs) (Fig. 1) and by sequencing of a region of the 16S rRNA gene that is sufficiently variable to allow classification of Rhizobiaceae (23, 38) (Fig. 2). The RFLP analysis showed very few bands in common between the diverse isolates and the inoculant strain, both at the whole genome level as revealed by *Spe* I digests and in localized regions of the chromosome as revealed by hybridizations of *Eco*RI digests to cosmids containing genes involved in exopolysaccharide synthesis and to randomly cloned fragments. *R. loti* is a heterogeneous species with some isolates that nodulate *L. corniculatus* sharing as little as 47% DNA–DNA homology (22) and little phenotypic similarity (44). This heterogeneity is reflected in 16S rRNA gene sequence. Willems and Collins (23) found the entire 16S rRNA gene sequence from two *R. loti* strains differed by three single nucleotide substitutions, and we found the sequence from inoculant strain ICMP3153 differed by four single nucleotide substitutions from the *R. loti* type strain within the 260-bp region sequenced. Sequences from most nodule strains had a 1-bp substitution from the type strain but one had three substitutions and was identical to sequence from the type strain of *R. huakuii*. There is normally little or no variation in 16S sequence within a bacterial species (45, 46) and so the degree of variability in 16S sequence observed, together with the range in overall relatedness (22, 44), suggests that strains able to nodulate *L. corniculatus* may in fact comprise several species able to receive and express the

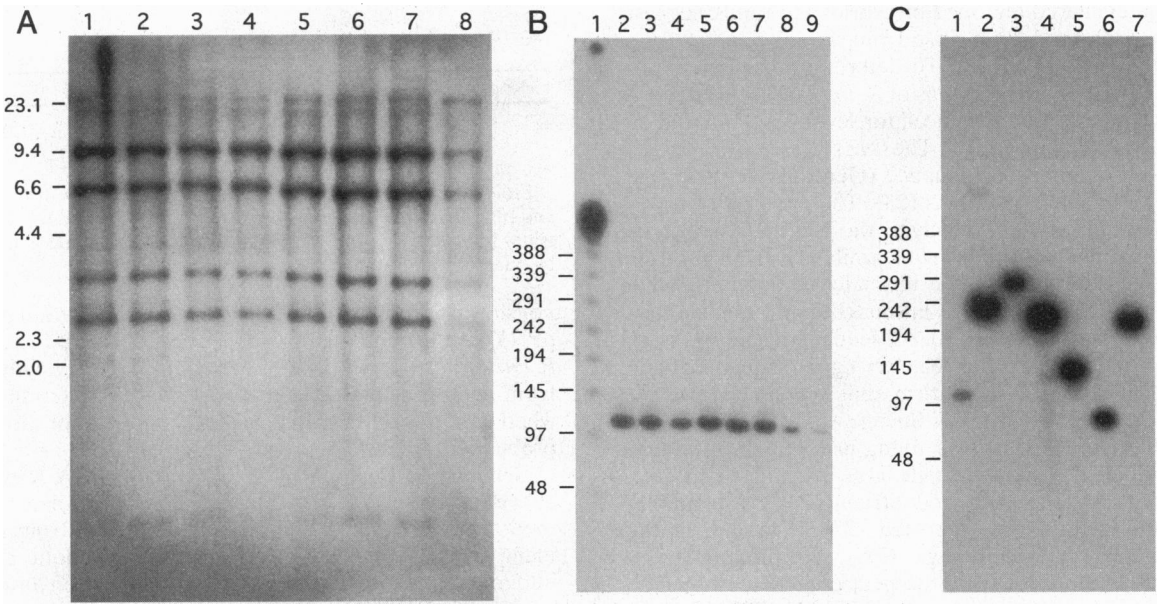

FIG. 4. Southern blots hybridized to the *R. loti nod* cosmid pPN377. (*A*) *Eco*RI-digested DNA. Lanes: 1, NZP2238; 2–8, nodule isolates R7A, R12C, R16C, R97A, R88B, R83C, and R8CS, respectively. (*B*) *Spe* I-digested DNA (gel shown in Fig. 1*A*). Lanes: 1, λ concatemers; 2, NZP2238; 3–9, nodule isolates R7A, R12C, R16C, R97A, R88B, R83C, and R8CS, respectively. (*C*) *Spe* I-digested DNA of NZP2238 and *R. loti* strains of diverse geographical origin. Lanes: 1, NZP2238; 2, cc811; 3, L72M103C; 4, 8KC3; 5, NZP2014; 6, NZP2213; 7, NZP2037.

particular symbiotic DNA region. Certainly the differences in 16S sequence between the diverse nodule isolates and ICMP3153 indicate the strains are not closely related. Since the symbiotic regions are identical, the diverse isolates must have received their symbiotic regions by lateral transfer.

It seems most likely that the lateral transfer of the conserved symbiotic region occurred from the inoculant strain to nonsymbiotic progenitors of the diverse strains that were already present as saprophytes in field site soil. The absence of naturalized symbiotic *R. loti* at the field site is shown by the findings that aseptically grown *L. corniculatus* seedlings inoculated with soil from the site fail to nodulate and uninoculated seedlings planted at the site fail to nodulate and perish from nitrogen deficiency by 16 weeks after germination (refs. 30 and 31; unpublished data). The area of the field site is devoid of legumes, except for occasional patches of clover, and there are no *L. corniculatus* stands within several kilometers. If the nonsymbiotic progenitors were carried into the field site, carriage must have been selective for nonsymbiotic strains of several different genotypes, as *R. loti* strains with diverse *nod* genes were not found in the site. Different *nod* hybridization patterns are found in symbiotic *R. loti* strains of different geographical origins (Fig. 4*C*). The alternative explanation to the lateral transfer occurring at the site—namely, that the diverse symbiotic strains were carried in on the seed—still requires that the strains acquired their symbiotic region by gene transfer from a common donor.

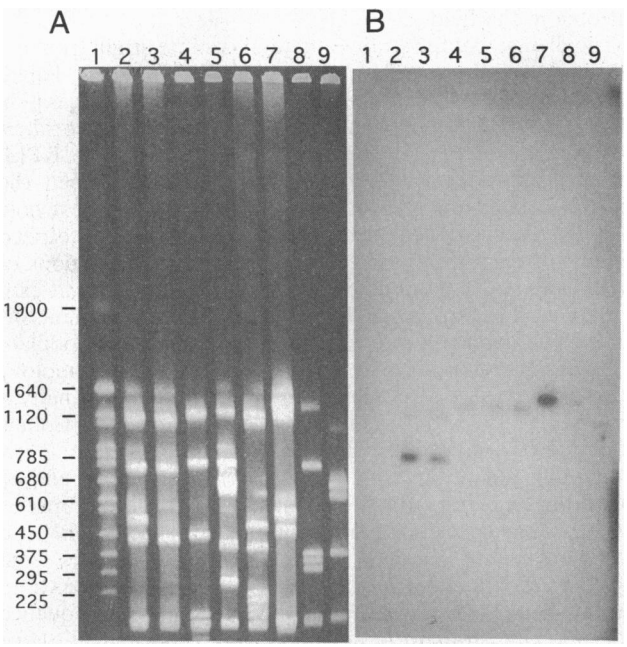

FIG. 5. (*A*) Pulse-field agarose gel of *Swa* I-digested DNA of NZP2238 and seven nodule isolates. Lanes: 1, *Saccharomyces cerevisiae* chromosomes (New England Biolabs); 2, NZP228; 3–9, nodule isolates R7A, R12C, R16C, R97A, R88B, R83C, and R8CS, respectively. (*B*) Southern blot of gel shown in *A* hybridized to the *nod* cosmid pPN377.

Nonsymbiotic strains of *R. leguminosarum*, a species with plasmid-encoded symbiotic genes, have been isolated from soil from areas with a history of legume growth (47–49) and may be abundant (50), suggesting that nonsymbiotic strains may play a more important role in the ecology of rhizobia than previously realized. Our results suggest that rhizobia may exist as nonsymbiotic saprophytes in soil even in the absence of a legume host and acquire symbiotic genes by lateral transfer from introduced symbiotic rhizobia when a legume host is present. These "new" strains may account for the rapid diversification of naturalized populations of rhizobia in the field. In addition, the strains are likely to be adapted to the local environment and out-compete the inoculant strain for nodule formation.

The lateral transfer of the symbiotic DNA region does not seem to involve nonintegrative plasmids. Previous studies have shown that *R. loti* strains contain at most a single large indigenous plasmid that does not hybridize to symbiotic genes (25, 26) and can be cured without adversely affecting nodule formation (26, 27), inferring a chromosomal location for the symbiotic genes. We did not detect plasmids in the inoculant strain or most nodule isolates and the transferred DNA seemed to be chromosomally integrated, as shown by the strain-specific pattern obtained from hybridization of *nod* gene probes to genomic DNA digests generated by the rare-cutting

enzyme *Swa* I (Fig. 5). The nature of the process mediating chromosomal symbiotic gene transfer between these bacteria in the natural environment remains to be determined but it seems unlikely to be transformation or transduction given the minimum 105-kb size of the transferred DNA segment.

Lateral transfer of symbiotic genes is thought to have played an important role in the evolution of *Rhizobium* and *Bradyrhizobium* species (51, 52). Our results show that lateral symbiotic gene transfer accounts for the evolution of new rhizobial strains in the field and is not limited to rhizobia with plasmid-encoded symbiotic genes. Characterization of the mechanisms involved should shed new light on the role of gene transfer in bacterial ecology and evolution in soil.

We thank V. Trainor for technical assistance. This work was supported by a grant from the New Zealand Foundation for Research, Science and Technology.

1. Dowling, D. N. & Broughton, W. J. (1986) *Annu. Rev. Microbiol.* **40,** 131–157.
2. Triplett, E. W. & Sadowsky, M. J. (1992) *Annu. Rev. Microbiol.* **46,** 399–428.
3. Streeter, J. G. (1994) *Can. J. Microbiol.* **40,** 513–522.
4. Chatel, D. A., Shipton, W. A. & Parker, C. A. (1973) *Soil Biol. Biochem.* **5,** 815–824.
5. Roughley, R. J., Blowes, W. M. & Herridge, D. F. (1976) *Soil Biol. Biochem.* **8,** 403–407.
6. McLoughlin, T. J., Hearn, S. & Alt, S. G. (1990) *Can. J. Microbiol.* **36,** 839–845.
7. Thies, J. E., Bohlool, B. B. & Singleton, P. W. (1992) *Can. J. Microbiol.* **38,** 493–500.
8. Kamicker, B. J. & Brill, W. J. (1987) *Appl. Environ. Microbiol.* **53,** 1737–1742.
9. Young, J. P. W. (1985) *J. Gen. Microbiol.* **131,** 2399–2408.
10. Young, J. P. W., Demetriou, L. & Apte, R. G. (1987) *Appl. Environ. Microbiol.* **53,** 397–402.
11. Piñero, D., Martínez, E. & Selander, R. K. (1988) *Appl. Environ. Microbiol.* **54,** 2825–2832.
12. Strain, S. R., Leung, K., Whittam, T. S., Debruijn, F. J. & Bottomley, P. J. (1994) *Appl. Environ. Microbiol.* **60,** 2772–2778.
13. Martínez, E., Romero, D. & Palacios, R. (1990) *Crit. Rev. Plant Sci.* **9,** 59–93.
14. Eardly, B. D., Materon, L. A., Smith, N. H., Johnson, D. A., Rumbaugh, M. D. & Selander, R. K. (1990) *Appl. Environ. Microbiol.* **56,** 187–194.
15. Souza, V., Nguyen, T. T., Hudson, R. R., Piñero, D. & Lenski, R. E. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 8389–8393.
16. Maynard Smith, J., Smith, N. H., O'Rourke, M. & Spratt, B. G. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 4384–4388.
17. Souza, V., Eguiarte, L., Avila, G., Cappello, R., Gallardo, C., Montoya, J. & Piñero, D. (1994) *Appl. Environ. Microbiol.* **60,** 1260–1268.
18. Strain, S. R., Whittam, T. S. & Bottomley, P. J. (1995) *Mol. Ecol.* **4,** 105–114.
19. Schofield, P. R., Gibson, A. H., Dudman, W. F. & Watson, J. M. (1987) *Appl. Environ. Microbiol.* **53,** 2942–2947.
20. Young, J. P. W. & Wexler, M. (1988) *J. Gen. Microbiol.* **134,** 2731–2739.
21. Laguerre, G., Mazurier, S. I. & Amarger, N. (1992) *FEMS Microbiol. Ecol.* **101,** 17–26.
22. Jarvis, B. D. W., Pankhurst, C. E. & Patel, J. J. (1982) *Int. J. Syst. Bacteriol.* **32,** 378–380.
23. Willems, A. & Collins, M. D. (1993) *Int. J. Syst. Bacteriol.* **43,** 305–313.
24. Yanagi, M. & Yamasato, K. (1993) *FEMS Microbiol. Lett.* **107,** 115–120.
25. Pankhurst, C. E., Broughton, W. J. & Weineke, U. (1983) *J. Gen. Microbiol.* **129,** 2535–2543.
26. Chua, K.-Y., Pankhurst, C. E., MacDonald, P. E., Hopcroft, D. H., Jarvis, B. D. W. & Scott, D. B. (1985) *J. Bacteriol.* **162,** 335–343.
27. Pankhurst, C. E., MacDonald, P. E. & Reeves, J. M. (1986) *J. Gen. Microbiol.* **132,** 2321–2328.
28. Kündig, C., Hennecke, H. & Göttfert, M. (1993) *J. Bacteriol.* **175,** 613–622.
29. Greenwood, R. M. (1964) *Proc. N.Z. Grassl. Assoc.* **26,** 95–101.
30. Chapman, H. M., Lowther, W. L. & Trainor, K. D. (1990) *Proc. N.Z. Grassl. Assoc.* **51,** 147–150.
31. Patrick, H. N. & Lowther, W. L. (1992) *Proc. N.Z. Grassl. Assoc.* **54,** 105–109.
32. Ronson, C. W., Nixon, B. T., Albright, L. M. & Ausubel, F. M. (1987) *J. Bacteriol.* **169,** 2424–2431.
33. Vincent, J. M. (1970) *A Manual for the Practical Study of Root Nodule Bacteria*, International Biological Programme Number 15 (Blackwell Scientific, Oxford).
34. Beringer, J. E. (1974) *J. Gen. Microbiol.* **84,** 188–198.
35. Borges, K. M. & Bergquist, P. L. (1992) *BioTechniques* **12,** 222–223.
36. Hynes, M. F., Simon, R., Müller, P., Niehaus, K., Labes, M. & Pühler, A. (1986) *Mol. Gen. Genet.* **202,** 356–362.
37. Eardly, B. D., Young, J. P. W. & Selander, R. K. (1992) *Appl. Environ. Microbiol.* **58,** 1809–1815.
38. Young, J. P. W., Downer, H. L. & Eardly, B. D. (1991) *J. Bacteriol.* **173,** 2271–2277.
39. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410.
40. Hotter, G. S. & Scott, D. B. (1991) *J. Bacteriol.* **173,** 851–859.
41. Scott, D. B., Young, C. A., Collins-Emerson, J. M., Terzaghi, E. A., Rockman, E. S., Lewis, P. E. & Pankhurst, C. E. (1995) *Mol. Plant Microbe Interact.*, in press.
42. Scott, D. B., Chua, K.-Y., Jarvis, B. D. W. & Pankhurst, C. E. (1985) *Mol. Gen. Genet.* **201,** 43–50.
43. Schofield, P. R., Djordjevic, M. A., Rolfe, B. G., Shine, J. & Watson, J. M. (1983) *Mol. Gen. Genet.* **192,** 459–465.
44. Novikova, N. I., Pavlova, E. A., Vorobjev, N. I. & Limeshchenko, E. V. (1994) *Int. J. Syst. Bacteriol.* **44,** 734–742.
45. Woese, C. R. (1987) *Microbiol. Rev.* **51,** 221–271.
46. Young, J. P. W. (1994) in *Advances in Molecular Genetics of Plant-Microbe Interactions*, eds. Daniels, M. J., Downie, J. A. & Osbourn, A. E. (Kluwer, Dordrecht, The Netherlands), Vol. 3, pp. 73–80.
47. Laguerre, G., Bardin, M. & Amarger, N. (1993) *Can. J. Microbiol.* **39,** 1142–1149.
48. Jarvis, B. D. W., Ward, L. J. H. & Slade, E. A. (1989) *Appl. Environ. Microbiol.* **55,** 1426–1434.
49. Soberón-Chávez, G. & Nájera, R. (1989) *Can. J. Microbiol.* **35,** 464–468.
50. Segovia, L., Piñero, D., Palacios, R. & Martínez-Romero, E. (1991) *Appl. Environ. Microbiol.* **57,** 426–433.
51. Young, J. P. W. (1993) in *New Horizons in Nitrogen Fixation*, eds. Palacios, R., Mora, J. & Newton, W. E. (Kluwer, Dordrecht, The Netherlands), pp. 587–592.
52. Martínez-Romero, E. (1994) *Plant Soil* **161,** 11–20.