APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2000, p. 2445–2450 Vol. 66, No. 6
0099-2240/00/$04.00+0


# Higher Diversity of *Rhizobium leguminosarum* Biovar viciae Populations in Arable Soils than in Grass Soils

K. M. PALMER AND J. P. W. YOUNG*

*Department of Biology, University of York, York, United Kingdom*

Received 23 December 1999/Accepted 10 April 2000

**The bacterial genetic diversity after long-term arable cultivation was compared with that under permanent grassland using replicated paired contrasts. Pea-nodulating *Rhizobium leguminosarum* populations were sampled from pairs of arable and grass sites at four locations in Yorkshire, United Kingdom. Isolates were characterized using both chromosomal (16S-23S ribosomal DNA internal transcribed spacer PCR-restriction fragment length polymorphism) and plasmid (group-specific *repC* PCR amplification) markers. The diversities of chromosomal types, *repC* profiles, and combined genotypes were calculated using richness in types (adjusted to equal sample sizes by rarefaction), Shannon-Wiener index, and Simpson's index. The relative differences in diversity within each pair of sites were similar for all three diversity measures. Chromosomal types, *repC* profiles, and combined genotypes were each more diverse in arable soils than in grass soils at two of the four locations. The other comparisons showed no significant differences. We conclude that rhizobial diversity can be affected by differences between these two management regimens. Multiple regression analyses indicated that lower diversity was associated with high potential nitrogen and phosphate levels or with acidity.**

The effects of land management practices on bacterial diversity are not known. It has been suggested that agriculture creates highly selective and homogeneous environments that reduce bacterial diversity (21), and it has been shown that artificial fertilizer application in previously unfertilized soil led to decreased rhizobial diversity in symbiotic nodules (2). Conversely, it has been argued that cultivation results in more diverse populations, and greater diversity of substrate utilization by total microbial communities from cultivated fields than from pastures has been reported previously (13). Lower rates of plasmid transfer have been inferred in natural pastures in comparison with other studies in arable fields (32). A study of *Rhizobium leguminosarum* populations from a grazed pasture and an ungrazed open woodland revealed similar levels of diversity (28).

Our study compares the diversity of pea-nodulating *R. leguminosarum* biovar viciae in arable and grassland soils. Arable soils are frequently disturbed by plowing, monoculture rotation, and harvest by denudation. Grass soils harbor a relatively stable plant population which is grazed or mowed. Arable soils are also subject to higher levels of soil amendments, fertilizers, herbicides, and pesticides than are grass soils. It is known that rhizobial numbers are affected by soil amendments, such as manure, lime, and phosphate (see reference 19 for a review), and by levels of fertility (10).

We have used replicate pairs at four locations, sites within each pair being as close to each other as possible. The replication allows us to assess the difference in rhizobial diversity between arable and grass management systems while taking into account the inevitable variation from site to site. *R. leguminosarum* genotypes are not evenly distributed across the United Kingdom (7, 36). The soil type can affect microbial activity (6), possibly due to differences in the distribution of suitable niches (22) and predation pressure by protozoa (3). Cultivation of the host plant has been shown to have a homogenizing effect on rhizobial populations (10), but none of the arable sites in this study had cropped peas within the past 5 years and host plants of *R. leguminosarum* biovar viciae were very uncommon at the pasture sites.

Peas (*Pisum sativum* cv. Kelvedon Wonder) were used as a uniform trap host to sample the rhizobial populations at each site. It has been demonstrated that peas are nodulated by a wide range of *R. leguminosarum* genotypes (e.g., see reference 34), and we have data demonstrating that peas and wild *Vicia* and *Lathyrus* species select a similar range of genotypes from a natural population (L. A. Mutch and J. P. W. Young, unpublished data).

The chromosomal portion of the *R. leguminosarum* genome was characterized using the internal transcribed spacer (ITS) between the 16S and 23S ribosomal DNA (rDNA) genes. The ITS can be interpreted as an indicator of chromosomal variation: *rrn* genes have been located only on the chromosome in *R. leguminosarum* (12). Restriction fragment length polymorphism analysis provides a level of resolution of intraspecific diversity in *R. leguminosarum* that is comparable to that of other DNA methods (17). The plasmid portion of the genome has been characterized using six sequence groups of the plasmid replication gene, *repC*, which are postulated to belong to different incompatibility groups (24, 30; K. M. Palmer, S. L. Turner, and J. P. W. Young, unpublished data). This method is independent of the traits borne by the plasmids and has the potential to characterize more than one plasmid in a strain. As well as comparing diversity measures between each pair of arable and grass sites, we tested the response of heterogeneity indices in multiple regression analyses with the soil factors analyzed at each site.

## MATERIALS AND METHODS

**Collection of soil samples.** Pairs of sites were selected from four farms in Yorkshire with good records of their management regimens, the grass sites being permanent or semipermanent. The sites from each location were of the same soil type, with the exception of the Bishop Burton sites. All of the arable sites received herbicides, pesticides, and treatments necessary for their crop. High Mowthorpe is situated on chalk uplands. The arable site (with winter wheat) was directly above the grass site, a permanent dairy pasture (grid reference SE898685). Manure from the dairy unit was spread on the arable field in the

* Corresponding author. Mailing address: Department of Biology, University of York, P. O. Box 373, York YO10 5YW, United Kingdom. Phone: (44) 1904-432914. Fax: (44) 1904-432860. E-mail: jpy1@york.ac.uk.

previous fall. Bishop Burton is situated on loam over chalk. The arable site (winter wheat, SE977406) was 1.5 km distant from the grass site, a horse paddock for several years (SE986415). Piggery manure was spread on the arable site in the previous fall. Headley Hall is situated on calcareous loam over clay. The arable site (potatoes) and the grass site (permanent dairy pasture) were situated on slopes facing each other (SE440420). Askham Bryan is situated on noncalcareous water gley. The arable site (potatoes) was subject to a phosphorus fertilizer trial and 0.2 km distant from the grass site, a permanent hay and sheep grazing meadow (SE547467).

Soils were collected between 5 and 7 August 1996 by removing the top 5 cm of soil from 20 places in each field, the subsamples being mixed before isolation of rhizobia.

***R. leguminosarum* bv. viciae isolation.** Pea seeds (Kelvedon Wonder) were surface sterilized in 2% hypochlorite, rinsed twice in sterile water, and incubated on solidified TY (1) at 26°C for 3 days. Pots (12-cm diameter) were sterilized in 0.1% sodium hypochlorite, rinsed in sterile water, and autoclaved. Autoclaved Terragreen soil conditioner, a calcined attapulgite clay (Oil Dri UK Ltd.), was placed in the bottom 4 cm, followed by a 5-cm layer of fresh sample soil (stored at 4°C for 1 to 3 days) into which were placed sterile, germinated peas. The soil surface was covered with a layer of sterile Terragreen to prevent splash-over during watering. Two pots for each soil sample were each seeded with four peas, placed on an open mesh in a greenhouse, and watered with sterile water. All of the plants were equally well nodulated. Nodules were collected 4 to 5 weeks later, and isolates were obtained from sterilized (cleaned in Tween, sterilized in 0.1% sodium hypochlorite for 15 min, and rinsed twice in sterile water) and crushed nodules on TY agar plates. After incubation at 26°C for 3 days, a well-separated colony from each nodule was subcultured onto fresh TY plates. Isolates were stored at −80°C in TY broths containing 20% glycerol. Ten isolates were collected from each plant and coded as follows: High Mowthorpe (M), Bishop Burton (B), Headley Hall (H), or Askham Bryan (A); arable (A) or grass (G); pot (1 or 2); plant (a, b, c, or d); and number (1 to 10). A total of 285 isolates were characterized: 47 from MA, 50 from MG, 44 from BA, 49 from BG, 35 from HA, 14 from HG, 20 from AA, and 26 from AG.

Peas in a control pot of sterile Terragreen became nodulated with about a quarter of the number of nodules observed on the peas in soil. The genotypes of nine control pot isolates were identical to the most common genotype observed in the collection, suggesting that the source of contamination was the adjacent pots of soil. We concluded that the incidence of cross-contamination was insignificant since the level of nodulation in the control pot was low, even though the nutrient-free conditions in Terragreen are highly conducive to nodulation. Any nodulation by contaminants would tend to obscure differences in diversity, and so differences can be regarded as genuine.

In order to check that the population sampling had not been biased from pot to pot, the numbers of the genotypes that were obtained from each site were compared between the two replicate pots. All of the dominant genotypes from each site were detected in both pots in largely similar proportions, and so the isolate collection from each site was treated as one sample.

**PCR and restriction digest conditions.** PCRs were performed using *Taq* polymerase (Promega) with total DNA extracts or fresh cells as template. 16S-23S rDNA ITS PCR used the primers FGPS1490 and FGPL132′ and conditions previously described (17). *Hae*III (Promega) digestions in buffer C at 37°C were performed after ammonium acetate-ethanol precipitation and resuspension. Conditions for PCR amplification of *repC1* to *repC6* sequence groups have been described previously (30; K. M. Palmer, S. L. Turner, and J. P. W. Young, unpublished data). The presence (+) or absence (−) of each *repC* group was scored for each isolate. A *repC* profile of −−−−−− does not mean that the isolate is barren in plasmids but shows that there are no plasmids bearing *repC* sequences from any of the six sequence groups. Other *repC* sequences have been found in *R. leguminosarum* strains (K. M. Palmer, S. L. Turner, and J. P. W. Young, unpublished data).

Streptomycin-resistant derivatives were generated in some cases to demonstrate culture purity. Washed cell pellets from TY broths were resuspended and spread onto TY agar containing 100 μg of streptomycin $ml^{-1}$. After 3 to 5 days of incubation, single colonies were subcultured onto 200 μg of streptomycin $ml^{-1}$.

**Diversity measures.** The estimated numbers of types in each site were calculated by rarefaction with an estimation of the variance using Simberloff's (26) computer program modified by Krebs (14). The use of rarefaction allows comparison of the number of types in samples of different sizes by limiting the sample to the smallest size in the set of populations and calculating the richness in types. We have used 2 √ variance as an estimate of the 95% confidence interval. Excel worksheet formulae (Microsoft) were used to calculate the Shannon-Wiener index, $H' = -\Sigma p_i \cdot \ln p_i$, where $p_i$ is proportion of the $i$th type in the population (20), and Simpson's index of diversity with Pielou's estimator for finite populations, $1 - D = 1 - \Sigma[n_i(n_i - 1)/N(N - 1)]$, where $n_i$ is number of the $i$th type and $N$ is number of individuals in the population (23). The Shannon-Wiener index calculates the uncertainty of predicting the type of another isolate from that population, and Simpson's index of diversity is based on the probability of picking two different individuals. Both the Shannon-Wiener and Simpson indices measure heterogeneity by incorporating both richness and distribution (evenness) of types, but the Simpson index reflects differences in the dominant types more than the Shannon-Wiener index does.

TABLE 1. Distribution of *R. leguminosarum* biovar viciae genotypes, composed of ribosomal ITS types and plasmid *repC* profiles, from arable lands and grasslands

| Genotype | | No. of isolates from site[a]: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ITS type | *repC* profile[b] | MA | MG | BA | BG | HA | HG | AA | AG |
| A | −−+−−− | 8 | 48 | 18 | 16 | | 4 | 10 | 14 |
| | +−+−−− | | | 4 | 10 | | | | 1 |
| | −−+−+− | | | 4 | 1 | | | | 7 |
| | −−+−−− | 4 | | | | | | | |
| | −−+−−+ | | | 1 | | | | | 1 |
| F | −−+−−− | | | 9 | 6 | | | 4 | 3 |
| | +−+−−− | | | 5 | 8 | | | 3 | |
| | −−+−−+ | | | 1 | 3 | | | | |
| Z | −−+−−− | | | | | 10 | 7 | | |
| | −−+−−− | 5 | 1 | | | 1 | | | |
| | −−−+−− | | | | | 1 | | | |
| | +−+−−− | 1 | | | | | | | |
| W | +−−−−− | | | | | 7 | | | |
| | +−+−−− | | | | | 6 | | | |
| | −−+−−− | | | | | 2 | | | |
| | −−−−−− | | | | | 1 | | | |
| Y | +−+−−− | 3 | | | | | | | |
| | −−−−−− | | | | | | 2 | | |
| | −−+−−− | | | | | 1 | | | |
| B | −−−+−− | 2 | | | | | | | |
| | −−+−−− | 2 | | | | | | | |
| | +−+−−− | 1 | | | | | | | |
| X | −−−−−− | | | | | 5 | | | |
| C | −−+−−− | 2 | | | | 1 | | | |
| | −−+−−− | 1 | | | | | | | |
| E | −−+−−− | 2 | | | | | | | |
| | −−+−−+ | 2 | | | | | | | |
| H | −−+−−− | | | | | | | 2 | |
| | −−+−−− | | | | 1 | | | | |
| I | −−+−−− | 3 | | | | | | | |
| S | −−+−−− | 2 | | | | | 1 | | |
| G | +−+−−− | | | 2 | | | | | |
| P | −−+−−− | | | | 2 | | | | |
| R | −−+−−− | 2 | | | | | | | |
| Q | −+−+−− | 2 | | | | | | | |
| D | −−−+−− | 1 | | | | | | | |
| J | −−+−−− | | 1 | | | | | | |
| K | −−+−−− | 1 | | | | | | | |
| L | −−−−−− | 1 | | | | | | | |
| M | −−−+−− | | | | 1 | | | | |
| N | −−−+−− | 1 | | | | | | | |
| T | −−−−−− | | | | 1 | | | | |
| U | −−−−−− | 1 | | | | | | | |
| V | −−+−+− | | | | | | | 1 | |

[a] First letter: M, High Mowthorpe; B, Bishop Burton; H, Headley Hall; A, Askham Bryan; second letter: G, grass; A, arable.
[b] Score for the presence (+) or absence (−) of *repC*1 to *repC*6, respectively.

**Soil analysis.** Two replicate samples from each mixture of subsamples were taken for chemical analysis. Fresh soil-water (1:2) mixtures were used to estimate pH (4). All other analyses used air-dried (30°C for 12 h) and sieved (2-mm-pore-size) soil. Conductivity at 25°C (25) and water-extractable phosphate, calcium, and magnesium were measured with 1:2 soil-water mixtures. Phosphate content was determined by the measurement of molybdenum blue by ascorbic acid reduction (0.5 M sulfuric acid, 0.0024% ammonium molybdate, 0.01375% potassium antimony tartrate, 0.53% ascorbic acid), calibrated with known phosphate concentrations (4) at 660 nm. Calcium and magnesium concentrations were determined by atomic absorption spectrometry in the presence of 1% lanthanum (18). Mineral nitrogen (nitrate, nitrite, and ammonium) was extracted in potassium chloride and distilled with a modification of Kjeldahl digestion (5) with Devarda's alloy, collecting the distillate into boric acid indicator solution (50 g of boric acid $liter^{-1}$, 4.5 μg of methyl red $liter^{-1}$, and 7.5 μg of bromocresol green $liter^{-1}$) (H. Vallack, personal communication). The content of organic matter was estimated by loss on ignition at 550°C (4), which also included loss due to heat decomposition of carbonates. As each pair of soils from High Mowthorpe, Headley Hall, and Askham Bryan were the same soil type, differ-

TABLE 2. Diversity measures for eight *R. leguminosarum* populations based on ITS type, *repC* profile, and combined genotype data sets

| Site[a] | ITS type | | | *repC* profile | | | Genotype | | |
|---|---|---|---|---|---|---|---|---|---|
| | E (ITS)[b] | *H'*[c] | 1 − *D*[d] | E (*repC* profile) | *H'* | 1 − *D* | E (genotype) | *H'* | 1 − *D* |
| MA | **8.52 (2.52)**[e] | **3.46** | **0.90** | **5.15 (1.88)** | **2.29** | **0.76** | **10.37 (2.58)** | **4.08** | **0.95** |
| MG | **1.56 (1.26)** | **0.28** | **0.08** | **1.20 (0.90)** | **0.14** | **0.04** | **1.56 (1.26)** | **0.28** | **0.08** |
| BA | 2.54 (1.00) | 1.16 | 0.52 | 3.33 (1.26) | 1.45 | 0.56 | 5.62 (1.92) | 2.43 | 0.78 |
| BG | 3.35 (1.78) | 1.54 | 0.59 | 4.00 (1.96) | 1.82 | 0.65 | 6.09 (2.16) | 2.69 | 0.82 |
| HA | 3.74 (1.42) | 1.74 | 0.67 | **4.72 (1.44)** | **2.16** | **0.76** | **6.53 (2.18)** | **2.79** | **0.85** |
| HG | 4.00 (0.00) | 1.69 | 0.69 | **2.00 (0.00)** | **0.59** | **0.26** | **4.00 (0.00)** | **1.69** | **0.69** |
| AA | **3.62 (1.04)** | **1.58** | **0.65** | 3.60 (0.53) | 1.32 | 0.50 | 4.60 (1.06) | 1.92 | 0.71 |
| AG | **1.92 (0.56)** | **0.52** | **0.21** | 3.08 (1.38) | 1.27 | 0.52 | 3.99 (1.46) | 1.54 | 0.62 |

[a] First letter: M, High Mowthorpe; B, Bishop Burton; H, Headley Hall; A, Askham Bryan; second letter: G, grass; A, arable.
[b] Expected number of types in a sample of 14 isolates, calculated by rarefaction, with 2× standard deviation in parentheses.
[c] Shannon-Wiener index.
[d] Pielou's estimator for finite populations.
[e] Diversity measures in boldface are significantly different between arable land and grassland.

ences in this weight loss can be attributed to differences in organic content at these sites. The potential mineral nitrogen in the soil samples (i.e., that in the organic fraction of the soil) was assessed using an incubation technique (4; H. Vallack, personal communication): 20 g of air-dried soil was moistened to 25% water content and inoculated with a drop of garden soil water to provide a bacterial population for the decomposition of organic material. The samples were incubated at 27°C in the dark for 6 weeks, moisture was replenished weekly by weight, the mineral nitrogen content was determined as described above, and the potential nitrogen values were determined by subtraction of the original mineral nitrogen value. The clay composition was measured with a Bouyoucos hydrometer, essentially according to reference 4 using 5% sodium hexametaphosphate and 0.7% sodium carbonate as a dispersal agent. Water-soluble calcium and magnesium concentrations were determined by atomic absorption spectrometry in the presence of 1% lanthanum (18).

**Data analysis.** All analyses were performed using SPSS 8.0 for Windows. The relationship between the soil factors was determined using principal components analysis. Stepwise multiple regression analyses were performed between the set of analyzed soil parameters and each of the Shannon-Wiener and Simpson diversity indices. The default criterion probabilities of *F* for inclusion and removal (0.05 and 0.1, respectively) did not allow the inclusion of any parameters in many analyses, so the criterion probability for inclusion was increased to 0.25, and that for exclusion was increased to 0.255.

## RESULTS AND DISCUSSION

**Genotypes of *R. leguminosarum* bv. viciae.** Table 1 shows the genotypes of the *R. leguminosarum* bv. viciae isolates at each of the sites, based on the ITS typing of the chromosome and the *repC* profiles in the plasmid portion of the genome.

Twenty-five *Hae*III restriction patterns of the 16S-23S ITS (data not shown) were observed from the collection of 285 isolates. This is in the range of variation that can be expected for *R. leguminosarum* populations. Using three loci in multilocus enzyme electrophoresis, reports have been made of 15 electrophoretic types (ETs) from 439 isolates within one field, 7 ETs from 85 isolates from two Norfolk (United Kingdom)

TABLE 3. Soil analysis

| Site[a] | pH | Conductivity[b] | Mineral nitrogen[c] | Loss on ignition[d] | Potential nitrogen[c] | Phosphate[c] | Clay[d] | Calcium[c] | Magnesium[c] |
|---|---|---|---|---|---|---|---|---|---|
| MA | 6.6[e] | 540 | 74 | 6.8 | 62 | 1 | 31 | 71 | 1 |
| | 6.7[e] | 520 | 69 | 6.4 | 66 | 1 | 28 | 78 | 0 |
| MG | 6.2 | 690 | 120 | 14.7 | 284 | 4 | 17 | 98 | 7 |
| | 6.2 | 690 | 135 | 16.2 | 286 | 4 | 26 | 111 | 8 |
| BA | 6.4 | 240 | 20 | 5.2 | 64 | 20 | 23 | 26 | 6 |
| | 6.5 | 250 | 21 | 4.6 | 58 | 20 | 23 | 27 | 6 |
| BG | 6.4 | 200 | 3 | 10.4 | 138 | 7 | 16 | 21 | 4 |
| | 6.3 | 210 | 3 | 9.4 | 145 | 7 | 14 | 20 | 4 |
| HA | 6.4 | 1,140 | 275 | 4.6 | 133 | 8 | 27 | 105 | 67 |
| | 6.4 | 1,160 | 295 | 4.0 | 80 | 7 | 26 | 108 | 67 |
| HG | 6.8 | 380 | 30 | 7.0 | ND[f] | 5 | 16 | 17 | 25 |
| | 6.8 | 360 | 29 | 6.9 | 120 | 4 | 15 | 18 | 21 |
| AA | 5.8 | 1,060 | 129 | 4.7 | 40 | 14 | 20 | 40 | 29 |
| | 5.8 | 1,060 | 150 | 4.5 | 10 | 21 | 17 | 48 | 37 |
| AG | 6.2 | 200 | 13 | 6.2 | 136 | 39 | 16 | 6 | 3 |
| | 6.0 | 210 | 16 | 10.5 | 129 | 46 | 18 | 8 | 4 |

[a] First letter: M, High Mowthorpe; B, Bishop Burton; H, Headley Hall; A, Askham Bryan; second letter: G, grass; A, arable.
[b] Conductivity units; microsiemens per centimeter.
[c] Micrograms per gram of oven-dried soil, after calculating the moisture content from the sand-silt-clay proportions of air-dried soil.
[d] Percentage of oven-dried (105°C) weight.
[e] Each soil was analyzed in duplicate; the two results are shown.
[f] ND, not determined.

populations, and at least 12 ETs from 721 isolates across the United Kingdom (7, 35, 36). Using a 26.2-kb hybridization probe, 17 restriction patterns were detected in 85 isolates in two Norfolk populations (36). Considering the smaller target with 16S-23S rDNA ITS characterization, a high level of resolution has been obtained. Some of the restriction patterns have also been found in French soils (G. Laguerre, personal communication).

Seventeen patterns appeared to represent single PCR products, 1,100 to 1,400 bp, as their restriction fragments summed to no more than the size of the PCR product. The other patterns (Z, W, Y, X, I, S, P, and R) were generated from a mixture of two or more PCR products. In these cases, the isolate cultures were demonstrated to be pure by the generation of identical ITS restriction patterns from streptomycin-resistant derivatives. We assume that the amplification of multiple 16S-23S ITSs reflects heterogeneity among the three *rrn* operons in the *R. leguminosarum* genome (11, 17).

Each isolate was scored for the presence or absence of each *repC* group. *repC3* was the most prevalent group (in 259 isolates), followed by *repC*1 (51 isolates), *repC*4 (37 isolates), *repC*5 (13 isolates), *repC*6 (8 isolates), and *repC*2 (2 isolates). Eleven *repC* profiles were observed out of a potential 64 ($2^6$).

The choice of pairs of sites as close to each other as possible has enabled us to compare the different land management histories at each location. The genotypes within each pair of sites are more similar to each other than to those at any other site, even though one pair, at Bishop Burton, was separated by 1.5 km. Samples of a population in an arable field, separated by 20 m, have been shown not to differ (35), and it appears that the scale of similarity can extend beyond this range.

There is a significant correlation between ITS type W and *repC*1 plasmids in the arable site at Headley Hall ($\chi^2 = 24.4$, 1 df, $P < 0.001$). This association might be due to coadaptation leading to enhanced symbiotic (36) or saprophytic abilities. Another possible explanation is that there has been insufficient time to distribute the plasmid among the population after a recent founder event or that there are restrictions on plasmid transfer. Correlations have previously been observed between chromosomal backgrounds and pSym genotypes (16, 36) or plasmid profiles (16).

**Comparison of diversity levels between arable and grass sites.** The rarefaction of the samples to 14 isolates with an estimate of the 95% confidence intervals allows comparison of the number of ITS types, *repC* profiles, and combined genotypes within each pair of sites (Table 2). The arable sites have a significantly higher expected number of types than the grass sites at High Mowthorpe (all three character sets), Headley Hall (*repC* profiles and combined genotypes), and Askham Bryan (ITS types). These observations are supported by the differences in the Shannon-Wiener and Simpson diversity indices.

Our results demonstrate that rhizobial genetic diversity can be as high, or higher, in arable fields subjected to repeated cultivation as in relatively undisturbed grasslands. It has been reported previously (13) that a cultivated wheat field harbored more phenotypically diverse microbial communities than did a pasture, a result which was linked to the diversity of stress responses. This is in contrast to an observed decrease in arbuscular mycorrhizal genetic diversity between woodland and arable soils (8). It is possible that many mycorrhizal fungi cannot maintain their hyphal structure in a disturbed soil environment, while discrete rhizobial cells are able to survive. We suggest that arable lands create conditions that favor the introduction of diverse rhizobial types or their diversification or that conditions in grasslands select for more uniform rhizobial populations. Studies that encompass more than one season will be able to determine the relative stabilities of arable and grass soil populations, which can be affected by founder events and cycles of bacteriocin production and resistance (33).

TABLE 4. Scores for the first four principal components of the soil analysis results, extracting 96.6% of the variance

| Soil factor | Value for principal component: | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| pH | 0.730 | $-7.26 \times 10^{-3}$ | −0.602 | −0.228 |
| Conductivity | 0.862 | $-6.15 \times 10^{-2}$ | 0.414 | $9.486 \times 10^{-3}$ |
| Mineral nitrogen | 0.915 | $3.521 \times 10^{-2}$ | 0.393 | $3.609 \times 10^{-2}$ |
| Loss on ignition | −0.344 | 0.923 | 0.137 | $1.762 \times 10^{-2}$ |
| Potential nitrogen | −0.128 | 0.924 | 0.264 | $-8.05 \times 10^{-3}$ |
| Phosphate | −0.498 | −0.529 | 0.482 | 0.431 |
| Clay (%) | 0.703 | $1.848 \times 10^{-2}$ | −0.446 | 0.549 |
| Calcium | 0.835 | 0.492 | $4.000 \times 10^{-2}$ | 0.240 |
| Magnesium | 0.782 | −0.288 | 0.414 | −0.309 |
| % Variance extracted | 47.78 | 25.73 | 15.35 | 7.71 |

**Relationship between soil parameters.** We analyzed each soil sample in duplicate in order to determine whether there are specific soil parameters that influence rhizobial diversity. Factors were chosen that have been shown to influence rhizobial diversity (pH [7] and magnesium [15]), cell functioning (calcium [31] and magnesium [29]), or bacterial survival (clay [3]) and that can be expected to change according to the land management (conductivity, mineral nitrogen, organic content, potential nitrogen, phosphate, and clay). The results are shown in Table 3. The duplicate results largely agreed with each other. Some consistent differences between the soil factors at the two types of site can be observed. The arable sites contained larger proportions of clay and smaller proportions of organic matter and potential nitrogen than the grass sites.

The means of the duplicate results were analyzed using principal components analysis. The component scores for each soil factor are shown in Table 4. The first two components were the most significant, extracting 73.5% of the variance. The first component shows correlations between the ionic factors, including clay which acts as an ionic sink, and between the organic factors, including phosphate. The second component analyzes the organic factors in more depth, indicating an inverse relationship between phosphate levels and both organic content and potential nitrogen levels. Calcium and magnesium also vary with the organic factors. The strong relationships that are indicated with the ionic and organic factors provide insights into the multiple regression analyses that were subsequently performed between the soil factors and diversity measures.

**The response of diversity measures to soil parameters.** The general trend of increased diversity in arable soils compared with that in grass soils, described above, has been detected by comparing contrasting pairs of sites. To test the response of the Shannon-Wiener and Simpson indices to the soil parameters, multiple regression analyses were performed (SPSS 8.0 for Windows). The most significant models are shown in Table 5.

Analyses with the ITS diversity measures indicate a negative relationship between rhizobial diversity and potential nitrogen-phosphate levels. Potential nitrogen and phosphate levels are maintained by continuous release from organic substrates or clay particles and so are more stable than the soluble nutrient status, which varies with fertilization regimens. The continuous high levels of the nutrients may have an effect similar to that of high levels of fertilization, which is known to decrease both

TABLE 5. Most significant models found by stepwise multiple regression between soil parameters and diversity measures

| Data set | Diversity index[a] | Model[b] | Analysis of variance[c] | | | | | | Unstandardized coefficients[d] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Source of variation | Sums of squares | df | Mean square | Variance ratio | *P* | Parameter in model | β | SE | *t* value | *P* |
| ITS type | $H'$ | PotN[e] and P | Regression | 5.286 | 2 | 2.643 | 10.791 | 0.015 | Constant | 3.339 | 0.434 | 7.688 | 0.001 |
| | | | Residual | 1.225 | 5 | 0.245 | | | PotN | $-9.37 \times 10^{-3}$ | 0.002 | −3.824 | 0.012 |
| | | | Total | 6.511 | 7 | | | | P | $-6.36 \times 10^{-2}$ | 0.018 | −3.520 | 0.017 |
| ITS type | $1 - D$ | PotN and P | Regression | 0.479 | 2 | 0.240 | 50.525 | 0.000 | Constant | 1.092 | 0.060 | 18.056 | 0.000 |
| | | | Residual | $2.373 \times 10^{-2}$ | 5 | $4.745 \times 10^{-3}$ | | | PotN | $-3.09 \times 10^{-3}$ | 0.000 | −9.047 | 0.000 |
| | | | Total | 0.503 | 7 | | | | P | $-1.64 \times 10^{-2}$ | 0.003 | −6.529 | 0.001 |
| Combined genotype | $H'$ | pH | Regression | 7.932 | 1 | 7.932 | 11.202 | 0.015 | Constant | −19.563 | 6.423 | −3.046 | 0.023 |
| | | | Residual | 4.248 | 6 | 0.708 | | | pH | 3.385 | 1.011 | 3.347 | 0.015 |
| | | | Total | 12.180 | 7 | | | | | | | | |
| Combined genotype | $1 - D$ | PotN | Regression | 0.345 | 1 | 0.345 | 13.919 | 0.010 | Constant | 1.015 | 0.104 | 9.735 | 0.000 |
| | | | Residual | 0.149 | 6 | $2.477 \times 10^{-2}$ | | | PotN | $-2.81 \times 10^{-3}$ | 0.001 | −3.731 | 0.010 |
| | | | Total | 0.493 | 7 | | | | | | | | |

[a] $H'$, Shannon-Wiener index; $1 - D$, Pielou's estimator for finite populations.
[b] Interaction between soil factors.
[c] df, degrees of freedom; *P*, probability of obtaining or exceeding the variance ratio under the null hypothesis that all coefficients are simultaneously equal to zero.
[d] β, partial regression slope coefficients; SE, standard error of the coefficient; *P*, probability of obtaining the *t* value under the null hypothesis of zero coefficient.
[e] PotN, potential nitrogen.

nodulation (27) and symbiont diversity (2). We did not observe variable nodulation levels, but it remains to be confirmed whether nutrient levels affect rhizobial diversity during saprophytic growth rather than the symbiotic process.

Analyses with the combined genotype diversity measures show a positive relationship between diversity and pH, despite a relatively narrow range of pH values (5.8 to 6.8). Low diversity has been correlated with low pH previously (7). The reason for the effect of acidity on rhizobial diversity is unknown, although rhizobia suffer acid stress below pH 5 (31). Heavy metals have increased mobility in acidic soils and have a negative relationship with diversity (9). Decreases in diversity in acidic and metal-polluted soils have been accompanied by reductions in rhizobial density (7, 9).

Analysis of the diversity measures from the *repC* profile character set produced significant models only with five or six parameters (data not shown). Such complex models are not robust given the available data and were not considered further. A positive relationship has previously been observed between the number of plasmid profiles and pH (7).

Because of the extreme diversity measures at the High Mowthorpe sites, the regression analyses were repeated excluding these sites (data not shown). Models for each character set were similar with either diversity index and similar to the analyses with all of the sites, a pH interaction being the most significant model in three analyses with the combined genotypes. Analyses with the diversity measures from the *repC* profiles produced significant models with an interaction between calcium and magnesium ($P = 0.042$ with Shannon-Wiener index; $P = 0.038$ with Simpson index). An influence of magnesium concentration on the set of rhizobial genotypes present in a population has been recorded previously (15).

The use of multiple regression analyses has allowed correlation of combinations of independent soil factors with the diversity measures, the best models of which are reported here. Other soil factors that are correlated with potential nitrogen, phosphate, or acidity may also have an effect on rhizobial diversity, and the effect of the soil may be a result of the combination of soil factors. Environmental manipulation experiments are needed to test the influence of individual and combined soil factors and hence to establish the causes of the higher diversity in arable populations than in grassland populations.

## ACKNOWLEDGMENTS

This work was supported by a Ph.D. studentship awarded to K.M.P. by the Science and Engineering Research Council.

We thank Harry Vallack, University of York, for advice on soil analysis, and Gisèle Laguerre, INRA Dijon, for information on ITS profiles.

## REFERENCES

1. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84:**188–198.
2. **Caballero-Mellado, J., and E. Martínez-Romero.** 1999. Soil fertilization limits the genetic diversity of *Rhizobium* in bean nodules. Symbiosis **26:**111–121.
3. **England, L. S., H. Lee, and J. T. Trevors.** 1993. Bacterial survival in soil: effect of clays and protozoa. Soil Biol. Biochem. **25:**525–531.
4. **Grimshaw, H. M.** 1989. Analysis of soils, p. 7–45. *In* S. E. Allen (ed.), Chemical analysis of ecological materials. Blackwell Scientific Publications, Oxford, United Kingdom.
5. **Grimshaw, H. M., S. E. Allen, and J. A. Parkinson.** 1989. Nutrient elements, p. 81–159. *In* S. E. Allen (ed.), Chemical analysis of ecological materials. Blackwell Scientific Publications, Oxford, United Kingdom.
6. **Groffman, P. M., P. Eagan, W. M. Sullivan, and J. L. Lemunyon.** 1996. Grass species and soil type effects on microbial biomass and activity. Plant Soil **183:**61–67.
7. **Harrison, S. P., D. G. Jones, and J. P. W. Young.** 1989. *Rhizobium* population genetics: genetic variation within and between populations from diverse locations. J. Gen. Microbiol. **135:**1061–1069.
8. **Helgason, T., T. J. Daniell, R. Husband, A. H. Fitter, and J. P. W. Young.** 1998. Ploughing up the wood-wide web? Nature **394:**431.
9. **Hirsch, P. R., M. J. Jones, S. P. McGrath, and K. E. Giller.** 1993. Heavy metals from past applications of sewage sludge decrease the genetic diversity of *Rhizobium leguminosarum* biovar trifolii populations. Soil Biol. Biochem. **25:**1485–1490.
10. **Hirsch, P. R.** 1996. Population dynamics of indigenous and genetically modified rhizobia in the field. New Phytol. **133:**159–171.
11. **Huber, I., and S. Selenska-Pobell.** 1994. Pulsed-field gel electrophoresis-fingerprinting, genome size estimation and *rrn* loci number of *Rhizobium galegae*. J. Appl. Bacteriol. **77:**528–533.
12. **Jumas-Bilak, E., S. Michaux-Charachon, G. Bourge, M. Ramuz, and A. Allardet-Servent.** 1998. Unconventional genomic organization in the alpha subgroup of the *Proteobacteria*. J. Bacteriol. **180:**2749–2755.
13. **Kennedy, A. C., and K. L. Smith.** 1995. Soil microbial diversity and the sustainability of agricultural soils. Plant Soil **170:**75–86.
14. **Krebs, C. J.** 1989. Species diversity measures, p. 328–370. *In* C. J. Krebs (ed.), Ecological methodology. Harper Collins, New York, N.Y.
15. **Labes, G., A. Ulrich, and P. Lentzsch.** 1996. Influence of bovine slurry

deposition on the structure of nodulating *Rhizobium leguminosarum* bv. viciae soil populations in a natural habitat. Appl. Environ. Microbiol. **62:** 1717–1722.
16. **Laguerre, G., E. Geniaux, S.-I. Mazurier, R. R. Casartelli, and N. Amarger.** 1993. Conformity and diversity among field isolates of *Rhizobium leguminosarum* bv. viciae, bv. trifolii, and bv. phaseoli revealed by DNA hybridization using chromosome and plasmid probes. Can. J. Microbiol. **39:**412–419.
17. **Laguerre, G., P. Mavingui, M.-R. Allard, M.-P. Charnay, L. Louvrier, S.-I. Mazurier, L. Rigottier-Gois, and N. Amarger.** 1996. Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars. Appl. Environ. Microbiol. **62:**2029–2036.
18. **Lanyon, L. E., and W. R. Heald.** 1982. Magnesium, calcium, strontium and barium, p. 247–262. *In* A. L. Page, R. H. Miller, and D. R. Keeney (ed.), Agronomy no. 9, 2nd ed. Methods of soils analysis. Part 2: chemical and microbiological properties. American Society of Agronomy, Soil Science Society of America, Madison, Wis.
19. **Lowendorf, H. S.** 1980. Factors affecting survival of *Rhizobium* in soil. Adv. Microb. Ecol. **4:**87–124.
20. **Margelef, D. R.** 1958. Information theory in ecology. Gen. Syst. **3:**36–71.
21. **Martínez-Romero, E., and J. Caballero-Mellado.** 1996. *Rhizobium* phylogenies and bacterial genetic diversity. Crit. Rev. Plant Sci. **15:**113–140.
22. **Mendes, I. C., and P. J. Bottomley.** 1998. Distribution of a population of *Rhizobium leguminosarum* bv. trifolii among different size classes of soil aggregates. Appl. Environ. Microbiol. **64:**970–975.
23. **Pielou, E. C.** 1969. An introduction to mathematical ecology. Wiley, New York, N.Y.
24. **Rigottier-Gois, L., S. L. Turner, J. P. W. Young, and N. Amarger.** 1998. Distribution of *repC* plasmid-replication sequences among plasmids and isolates of *Rhizobium leguminosarum* bv. viciae from field populations. Microbiology **144:**771–780.
25. **Rowland, A. P., and H. M. Grimshaw.** 1989. Analysis of waters, p. 62–80. *In* S. E. Allen (ed.), Chemical analysis of ecological materials. Blackwell Scientific Publications, Oxford, United Kingdom.
26. **Simberloff, D. S.** 1978. Use of rarefaction and related methods in ecology, p. 150–165. *In* K. L. Dickson, J. Cairns, Jr., and R. J. Livingston (ed.), Biological data in water pollution assessment: quantitative and statistical analyses. American Society for Testing and Materials, Philadelphia, Pa.
27. **Sprent, J. I.** 1979. The biology of nitrogen-fixing organisms, p. 75–113. McGraw Hill Book Co, Maidenhead, United Kingdom.
28. **Strain, S. R., K. Leung, T. S. Whittam, F. J. de Bruijn, and P. J. Bottomley.** 1994. Genetic structure of *Rhizobium leguminosarum* biovar trifolii and viciae populations found in two Oregon soils under different plant communities. Appl. Environ. Microbiol. **60:**2772–2778.
29. **Stryer, L.** 1995. Biochemistry, 4th ed. Freeman, New York, N.Y.
30. **Turner, S. L., L. Rigottier-Gois, R. S. Power, N. Amarger, and J. P. W. Young.** 1996. Diversity of *repC* plasmid-replication sequences in *Rhizobium leguminosarum*. Microbiology **142:**1705–1713.
31. **Watkin, E. L. J., G. W. O'Hara, and A. R. Glenn.** 1997. Calcium and acid stress interact to affect the growth of *Rhizobium leguminosarum* bv. trifolii. Soil Biol. Biochem. **29:**1427–1432.
32. **Wernegreen, J. J., E. E. Harding, and M. A. Riley.** 1997. Rhizobium gone native: unexpected plasmid stability of indigenous *Rhizobium leguminosarum*. Proc. Natl. Acad. Sci. USA **94:**5483–5488.
33. **Wilson, R. A., B. A. Handley, and J. E. Beringer.** 1998. Bacteriocin production and resistance in a field population of *Rhizobium leguminosarum* biovar viciae. Soil Biol. Biochem. **30:**413–417.
34. **Young, J. P. W.** 1985. *Rhizobium* population genetics: enzyme polymorphism in isolates from peas, clover, beans and lucerne grown at the same site. J. Gen. Microbiol. **131:**2399–2408.
35. **Young, J. P. W., L. Demetriou, and R. G. Apte.** 1987. *Rhizobium* population genetics: enzyme polymorphism in *Rhizobium leguminosarum* from plants and soil in a pea crop. Appl. Environ. Microbiol. **53:**397–402.
36. **Young, J. P. W., and M. Wexler.** 1988. Sym plasmid and chromosomal genotypes are correlated in field populations of *Rhizobium leguminosarum*. J. Gen. Microbiol. **134:**2731–2739.