Genetic relationships among rhizopine-producing *Rhizobium* strains

M. Wexler,^{1†} D. M. Gordon² and P. J. Murphy¹

Author for correspondence: M. Wexler. Tel: +44 1603 592195. Fax: +44 1603 592250.
e-mail: m.wexler@uea.ac.uk

¹ Department of Crop Protection, University of Adelaide, Glen Osmond, SA 5064, Australia

² Division of Botany and Zoology, Australian National University, Canberra, ACT 0200, Australia

Chromosomal and symbiosis-related genotypes of rhizopine-producing and non-producing isolates of *Rhizobium meliloti* and *Rhizobium leguminosarum* were examined by multilocus enzyme electrophoresis and RFLP. The distribution of rhizopine production in both species was found to be independent of host genotype. Conversely, rhizopine production was associated with particular symbiotic plasmid types. This association may explain the observed distribution of rhizopine production in *R. leguminosarum* and *R. meliloti*. Rhizopine synthesis (*mos*) genes showed greater sequence divergence than rhizopine catabolism (*moc*) genes in both *R. meliloti* and *R. leguminosarum*. Furthermore, *mos* and *moc* genes were less divergent in *R. leguminosarum* than *R. meliloti*, suggesting a more recent evolution in the former species.

Keywords: rhizopine, rhizobia, restriction fragment length polymorphisms (RFLP), multilocus enzyme electrophoresis (MLEE)

INTRODUCTION

The symbiosis between nitrogen-fixing *Rhizobium* and leguminous plants confers considerable economic benefit in a variety of agricultural environments. Rhizobia are commonly used as inoculants where legume crops or pastures are sown. In the search for better inoculants, natural or genetically modified rhizobia are selected for their efficient ability to fix nitrogen under specific environmental conditions. A drawback to these developments is that often inoculants do not persist when introduced into a new environment. It is therefore important to identify traits that enable a strain to compete successfully in the diverse microbial community of the rhizosphere.

The production of rhizopines by some species of *Rhizobium* is thought to be one of these traits (Tempé & Petit, 1983; Murphy *et al.*, 1987, 1995). Rhizopines are inositol-like compounds synthesized by the bacteroid within the nodule. Only free-living rhizobia are able to catabolize these substances (Murphy *et al.*, 1988; Saint *et al.*, 1993). The abilities to synthesize and to catabolize rhizopines are co-occurring traits encoded by the symbiotic plasmid (Saint *et al.*, 1993; Wexler *et al.*, 1995). Although the exact

nature of the competitive advantage conferred by rhizopine production is unknown, *in vitro* experiments, pot trials and field testing have shown that rhizopine-producing rhizobia tend to have a competitive advantage and persist in the environment longer compared to non-producers (Jensen, 1987; Evans & Howieson, 1993; Murphy *et al.*, 1995; P. J. Murphy & D. M. Gordon, unpublished data).

Not all species of *Rhizobium* produce rhizopines. In a survey of over 330 *Rhizobium* isolates, Wexler *et al.* (1995) detected the ability to catabolize rhizopines in *Rhizobium meliloti* and *Rhizobium leguminosarum* bv. *viciae*. Rhizopine catabolism has not been detected in *Rhizobium etli*, *Rhizobium tropici* or *R. leguminosarum* bvs *trifolii* and *phaseoli*. Also, not all strains of a species produce rhizopines; 11% of *R. meliloti* strains and 12% of *R. leguminosarum* bv. *viciae* strains were found to produce rhizopines (Wexler *et al.*, 1995).

Our goal in this study was to examine two issues related to the distribution of rhizopine genes in two species of *Rhizobium*. Is rhizopine production restricted to a closely related subset of strains within a species? Does the occurrence of rhizopine production correlate with the degree of similarity among symbiotic plasmids? To examine these issues, we investigated the chromosomal and symbiotic plasmid genotype of representative rhizopine-producing and non-producing strains of *R. leguminosarum* and *R. meliloti*. We also investigated the relationships between the genes responsible for rhizopine

† Present address: School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK.

Abbreviations: MLEE, multilocus enzyme electrophoresis; UPGMA, unweighted pair group method.

synthesis (*mos*) and catabolism (*moc*) in rhizopine-producing strains.

METHODS

Bacterial strains. The *R. meliloti* and *R. leguminosarum* bv. *viciae* strains selected for this study were a subset of those examined by Wexler *et al.* (1995). The non-rhizopine-producers were chosen such that the various geographic and host plant origins of the strains were represented. Details concerning the strains are given by Wexler *et al.* (1995). This study included all of the rhizopine-producing *R. meliloti* strains and all but one of the *R. leguminosarum* bv. *viciae* strains. Strain numbers are shown in the figures. Forty-eight strains of *R. meliloti*, of which 13 produced rhizopines, and 40 strains of *R. leguminosarum*, of which 8 produced rhizopines, were studied.

Strain characterization. Multilocus enzyme electrophoresis (MLEE) was the technique used to determine the degree of genotypic similarity among the strains. It is generally assumed that this method detects allelic variation at chromosomally determined structural gene loci (Selander *et al.*, 1986). Allelic profiles at 14 loci were determined for each species. Full details of the methods and results of the MLEE analysis are presented by Gordon *et al.* (1995). Only data for the unique electrophoretic types were presented in Gordon *et al.* (1995); in this study we have included all of the strains examined regardless of their MLEE genotype.

Symbiotic plasmid characterization. RFLP analysis of the *nod* region was the method used to assess the genetic similarity of the symbiotic plasmids in each of the host strains. Genomic DNA was extracted (Wilson, 1991), digested with *Bam*HI, *Eco*RI, *Hind*III or *Sal*I, and subjected to electrophoresis on 0.7% agarose gels. Southern blotting and hybridizations were performed using standard methods (Sambrook *et al.*, 1989). Blots were washed under high stringency conditions [$0.1 \times$ SSC ($1 \times$ SSC is 0.15 M sodium chloride and 0.015 M sodium citrate), 0.1% SDS at 65 °C]. Probe DNA was labelled using digoxigenin-labelled dUTP according to the manufacturer's instructions (Boehringer Mannheim). The *R. meliloti* strains were probed with pGMI149, which contains a 29 kb insert (comprising *nod* genes and flanking sequences) cloned into pRK290 (Truchet *et al.*, 1985). The *R. leguminosarum* bv. *viciae* strains were probed with pIJ1089, which contains a 30 kb insert (comprising most of the *nod/nif* region and flanking sequences) cloned into pLAFR1 (Downie *et al.*, 1983).

Characterization of the rhizopine genes. RFLP analysis was used to determine the genetic relationships of the *moc* genes and *mos* gene regions of the rhizopine producers. Genomic DNA was digested with *Pst*I, *Bam*HI, *Eco*RI, *Hind*III or *Sal*I. The following probes were used. pPM1031 (Murphy *et al.*, 1987) contains the *R. meliloti* L5-30 *moc* genes (15.1 kb) cloned into pLAFR1, and pPM1242 (Rao *et al.*, 1995) contains a 5.7 kb *Apa*I–*Nde*I fragment carrying the L5-30 *mos* genes cloned into pGEM-7Zf(+). These probes were hybridized to all rhizopine-producing strains. The probe pPM1213 (Wexler, 1994) contains the *R. leguminosarum* strain 1a *moc* genes (13.7 kb) cloned into pVK102. pPM1302 (Wexler, 1994) contains a 5.3 kb 1a *Hind*III fragment cloned into pUCD2608 (this fragment has homology to the *R. meliloti* L5-30 *mosB* gene). These probes were hybridized to the *R. leguminosarum* bv. *viciae* strains.

Statistical analysis. The genetic distance between the isolates was estimated as $2m_{xy}/(m_x + m_y)$, where m_{xy} is the number of restriction fragments (or alleles) shared by isolates x and y , while

m_x and m_y are the total number of fragments (or alleles) present in isolates x and y . Phenograms were constructed from the distance matrices using the unweighted pair group method (UPGMA) and consensus trees were determined using the Strict method (Rohlf, 1993). The degree of DNA sequence divergence was calculated by the method of Nei & Li (1979).

Distance matrices were compared using a Mantel test. The comparison of distance matrices, in essence, asks the following question: do pairs of chromosomally similar strains (MLEE) also have genetically similar symbiotic plasmids (RFLP)? The statistical significance of the resulting matrix correlation coefficients was determined using a permutation approach, i.e. the observed coefficient was compared to the distribution of coefficients generated when one matrix is repeatedly compared with the other matrix. For each comparison, every element of the second matrix is randomly replaced by another element. Phenograms were compared by first calculating the matrix of cophenetic values for each phenogram produced by the UPGMA analysis. The similarity of the resulting cophenetic matrices was then quantified using a Mantel test and the significance of the correlation was determined in the same manner as for the distance matrix comparisons. Comparisons between cophenetic matrices ask a similar question to that asked when distance matrices are compared. In the case of the cophenetic matrices it is the phenograms produced by the UPGMA analysis of the distance matrices that are being compared and not the distance matrices themselves. Analyses were carried out with the software NTSYS (Rohlf, 1993).

Randomization tests were performed in order to determine whether rhizopine producers were, on average, significantly more similar than other isolates regardless of their rhizopine status (Sokal & Rohlf, 1981), e.g. to determine whether the symbiotic plasmids of the 13 *R. meliloti* rhizopine producers were, on average, more alike than the symbiotic plasmids of the other 35 isolates the following steps were performed. First, from the RFLP distance matrix the observed mean pairwise genetic distance between the rhizopine producers was calculated. There are 78 such distances [$13(13-1)/2$]. A random sample of size 78 was then drawn from the entire distance matrix of 1128 [$48(48-1)/2$] values between all 48 strains of *R. meliloti* and the mean of these 78 distances was calculated. This procedure was repeated 250 times and the probability of drawing a sample as extreme as the observed estimate was determined. In essence, this procedure poses the question: does any set of 78 distance estimates produce a mean genetic distance as small as that observed for the 13 rhizopine strains?

RESULTS

Rhizopine production in relation to chromosomal and symbiotic plasmid genotypes

Analysis of the MLEE data partitioned the 48 *R. meliloti* isolates into two divisions (A and B) with a further subgrouping occurring within division A (subgroups A1 and A2; Fig. 1a) (Gordon *et al.*, 1995). All the rhizopine-producing isolates were in subpopulation A1. The mean genetic distance between the 13 rhizopine-producing isolates, together with the mean genetic distance between all strains, division A strains and group A1 strains is presented in Table 1. In terms of chromosomal similarity, the rhizopine isolates were more similar, on average, than division A strains but were about as similar as the group A1 strains.

Fig. 1. Phenograms depicting the genetic relationships among *R. meliloti* strains in terms of (a) chromosomal genotypes and (b) the genotypes of the symbiotic plasmids. Chromosomal genotypes were identified by MLEE at 14 loci (Gordon *et al.*, 1995). The symbiotic plasmid genotypes were identified by RFLP analysis of *nod* gene regions and phenograms were constructed from the distance matrices using the UPGMA method. Asterisks indicate the rhizopine-producing isolates.

Table 1. Genetic similarity of rhizopine-producing isolates in relation to all isolates in *R. meliloti* and *R. leguminosarum* bv. *viciae*

Estimates of chromosomal similarity are based on MLEE (Gordon *et al.*, 1995). Estimates of symbiotic plasmid similarity are based on RFLP analysis of the *nod* region.

Isolate	Chromosome mean genetic distance	Symbiotic plasmid mean genetic distance
<i>R. meliloti</i>		
Rhizopine producers	0.179	0.372
All isolates	0.465*	0.568*
Division A isolates	0.294*	0.556*
Group A1 isolates	0.190†	0.485*
<i>R. leguminosarum</i> bv. <i>viciae</i>		
Rhizopine producers	0.600	0.667
All isolates	0.501†	0.779*

* Mean genetic distance of the rhizopine producers is significantly lower than expected by chance, $\alpha = 0.005$.

† Not significant.

Fig. 2. Phenograms depicting the genetic relationships among *R. leguminosarum* bv. *viciae* strains in terms of (a) chromosomal genotypes and (b) the genotypes of the symbiotic plasmids. Chromosomal genotypes were identified by MLEE at 14 loci (Gordon *et al.*, 1995). The symbiotic plasmid genotypes were identified by RFLP analysis of the *nod* gene regions. Asterisks indicate the rhizopine-producing isolates.

The phenogram depicting the genetic similarity of the *R. meliloti* symbiotic plasmid genotypes shows that there is less clustering among plasmid genotypes than among host genotypes (Fig. 1b). Those strains designated as group A2 on the basis of the MLEE data analysis are also seen to cluster in the analysis of the symbiotic plasmid RFLP data. There is, however, no obvious clustering of division B symbiotic plasmids. On average, the symbiotic plasmids of the rhizopine-producing strains are significantly more alike than the symbiotic plasmids of any other division or group of isolates (Table 1).

No pronounced clustering of the 40 *R. leguminosarum* bv. *viciae* isolates was observed based on the analysis of their chromosomal genotypes (Fig. 2a). The eight rhizopine producers were no more genetically similar than any other group of eight isolates (Table 1). There is also little structure in the phenogram depicting the genetic relationships of the symbiotic plasmids of the *R. leguminosarum* bv. *viciae* isolates (Fig. 2b). The symbiotic plasmid genotypes of the rhizopine producers were significantly more similar than those of other isolates (Table 1).

For *R. meliloti* and *R. leguminosarum* bv. *viciae*, low but significant correlations were detected in comparisons of the MLEE (chromosome) and RFLP (symbiotic plasmid) genetic distance matrices (Table 2). Similar results were

obtained in comparisons of the MLEE and RFLP cophenetic matrices for each species (Table 2).

Relationships between the *mos* and *moc* gene regions of rhizopine producers

Some homology was detected when the *R. meliloti* L5-30 *moc* and *mos* region probes were hybridized to genomic DNA from the *R. leguminosarum* bv. *viciae* rhizopine producers. However, under high stringency washing conditions, many of these hybridization bands were of low intensity and this precluded any between species comparisons in the *moc* and *mos* gene regions.

Estimates of the degree of sequence divergence between the isolates of each rhizopine-producing species are presented in Table 3. Estimated sequence divergence is greater for the *mos* gene region than for the *moc* gene region in both species. On average, there is greater sequence divergence in the regions associated with rhizopine synthesis and catabolism in *R. meliloti* rhizopine producers than in *R. leguminosarum* bv. *viciae*. *R. meliloti* strain Rm220-3 contains a large deletion within the *mos* genes (Rao *et al.*, 1995). As the method for estimating sequence divergence assumes that differences in RFLP patterns are due to single base changes and not deletions or insertions (Upholt, 1977), Rm220-3 was not included in

Table 2. Genetic distance and cophenetic matrix correlations between the different levels of analysis for *R. meliloti* and *R. leguminosarum* bv. *viciae*

Significance tests are based on those described by Rohlf (1993).

Distance matrices/phenogram correlations*				
	Chromosome	Symbiotic plasmid	<i>mos</i> region†	<i>moc</i> region†
<i>R. meliloti</i>				
Chromosome	—	0.318‡	0.402‡	0.463‡
Symbiotic plasmid	0.212‡	—	0.601‡	0.655‡
<i>mos</i> region	0.428‡	0.519‡	—	0.660‡
<i>moc</i> region	0.411‡	0.635‡	0.631‡	—
<i>R. leguminosarum</i> bv. <i>viciae</i>				
Chromosome	—	0.154‡	−0.319	−0.095
Symbiotic plasmid	0.130‡	—	0.041	0.298
<i>mos</i> region	−0.315	−0.078	—	0.179
<i>moc</i> region	−0.134	0.237	0.222	—

* The upper two rows of the matrix for each organism present the cophenetic matrix correlations and the lower half the genetic distance matrix correlations.

† These correlations are based on the data for the rhizopine-producing isolates only.

‡ Significance at the 0.01 level.

Table 3. Hybridization results and estimates of between isolate sequence divergence in the *mos* and *moc* gene regions of *R. meliloti* and *R. leguminosarum* bv. *viciae* rhizopine-producing isolates

Isolate	Probe	No. of fragments	No. of genotypes	Sequence divergence	
				Mean	Range
<i>R. meliloti</i>	5.7 kb <i>mos</i> region	61	12	4.5 %	0–7.4 %
	15.1 kb <i>moc</i> region	45	7	1.6 %	0–2.7 %
<i>R. leguminosarum</i> bv. <i>viciae</i>	5.3 kb <i>mos</i> region	10	3	1.6 %	0–4.5 %
	13.7 kb <i>moc</i> region	29	6	0.5 %	0–1.1 %

the calculation of between isolate divergence. The relatedness of the isolates based on genetic distance estimates for the *moc* and *mos* regions of both rhizobial species are depicted in Figs 3 and 4.

Comparisons of the genetic distance and cophenetic matrices of the *R. meliloti* rhizopine producers revealed significant correlations for all comparisons (Table 2). The strongest correlations were observed for the *mos* versus *moc* region comparisons and for the comparisons of the *mos* and *moc* regions versus the *nod* region of the symbiotic plasmids. For *R. leguminosarum*, no significant correlations between chromosome, symbiotic plasmid, *mos* or *moc* region genetic distance or cophenetic matrices were detected (Table 2).

DISCUSSION

The analyses presented by Eardly *et al.* (1990), Segovia *et al.* (1993), Gordon *et al.* (1995) and Rossbach *et al.* (1995)

all reveal that *R. meliloti sensu lato* consists of two major divisions of strains (A and B). In addition, two subgroups are found within the division A strains. Group A1 isolates have a cosmopolitan distribution and can be isolated from a variety of annual and perennial medicks, while group A2 strains are unusual in that they are able to initiate an effective symbiosis with *Trigonella suavissima*, an endemic Australian species. Division B strains have only been isolated from annual medicks growing in the eastern Mediterranean basin (Eardly *et al.*, 1990; Gordon *et al.*, 1995). Eardly *et al.* (1990) suggest that the differences between division A and B isolates are sufficient to warrant assigning division B strains separate specific status. Less clear cut is the taxonomic status of division A2 strains. Their status as a distinct group of strains is supported by our RFLP analysis, as the *nod* regions of their symbiotic plasmids tend to be more similar than the *nod* regions of symbiotic plasmids from group A1 or division B strains (Fig. 1b). However, there is insufficient information to

Fig. 3. Phenograms depicting the genetic relationships among *R. meliloti* rhizopine producers based on RFLP analyses of the *mos* and *moc* gene regions.

Fig. 4. Phenograms depicting the genetic relationships among *R. leguminosarum* bv. *viciae* rhizopine producers based on RFLP analyses of the *mos* and *moc* gene regions.

decide whether group A2 isolates are sufficiently distinct from A1 isolates to justify separate specific status or whether they represent two 'biovars' of *R. meliloti sensu stricto*.

At present the available data suggest that rhizopine production does not occur in division B or group A2 strains, although relatively few strains have been examined (Wexler *et al.*, 1995; Rossbach *et al.*, 1995). The observed distribution of rhizopine production cannot be explained on the basis of host plant specificity, as rhizopine-producing strains have been isolated from

Fig. 5. Consensus dendrogram of the phenograms for the symbiotic plasmids and *mos* and *moc* gene regions of the rhizopine-producing *R. meliloti* isolates.

Medicago sativum and *Medicago littoralis* (Wexler *et al.*, 1995). Furthermore, *in planta* tests have shown that the rhizopine-producing strain L5-30 can synthesize 3-O-methyl-*scyllo*-inosamine within a variety of host annual and perennial medick species and in *T. sawissima* (Wexler *et al.*, 1995).

There are no reports which demonstrate that the symbiotic mega-plasmids of *R. meliloti* are naturally self-transmissible. Given the apparent absence of plasmid transfer, it might be expected that the genetic relationships among the symbiotic plasmid isolates would reflect the genetic relationships observed at the host level. Such a result was not observed, as there is little concordance between the relationships of the strains based on the MLEE analysis and those observed for the RFLP analysis (Fig. 1). Although significant, only very low correlations were detected between either the genetic distance matrices or phenograms generated from the MLEE or RFLP data (Table 2). The presence and absence of rhizopine production in strains with identical MLEE genotypes provides an obvious illustration of the lack of concordance between the chromosomal relationships of the strains and the genetic relationships of their symbiotic plasmids.

The strains that show the most similar *mos* and *moc* gene regions also have symbiotic plasmids with very similar *nod* regions (Fig. 5). Almost half (6/13) of the rhizopine producers form a closely related group, showing a mean 1.4% *mos* and 0.1% *moc* region sequence divergence and a symbiotic plasmid genetic distance of 0.372. As these six strains were collected from four continents, geographic origin cannot explain this result.

Cluster analysis of the MLEE data for the *R. leguminosarum* bv. *viciae* isolates failed to reveal any significant isolate associations (Gordon *et al.*, 1995). This is the expected result given that no evidence for linkage disequilibrium can be detected in this data set (Maynard Smith *et al.*, 1993; Gordon *et al.*, 1995). The chromosomal genotypes of the rhizopine producers were no more similar than any other sample of strains (Table 1). The biovars of this species cannot generally be distinguished on the basis of chromosomally determined characters (Roberts *et al.*, 1980; Young, 1985; Segovia *et al.*, 1991; Dooley & Harrison, 1993; Laguette *et al.*, 1993b). This suggests

that, for the species as a whole, rhizopine producers are not a chromosomally similar subset of strains.

As was found in *R. meliloti*, the symbiotic plasmids of the rhizopine-producing *R. leguminosarum* strains were, on average, more similar than would be expected by chance. The absence of any significant associations between the *noc* and *mos* region RFLP data or between these gene regions and the RFLP data for the symbiotic plasmids is probably due to the small number of rhizopine-producing strains and the low degree of variation detected in the rhizopine genes.

The results of this analysis suggest that the distribution of rhizopine production in *R. meliloti* and *R. leguminosarum* is independent of the genotype of the host. Rhizopine synthesis and catabolism appear to be traits associated with particular symbiotic plasmid genotypes (as defined by their *nod/nif* region). This is consistent with the observation that the rhizopine genes of all isolates examined in this study are found on the symbiotic plasmid (Wexler, 1994). The absence of rhizopine production in group A2 *R. meliloti* strains may not be so much due to the uniqueness of this group of strains as determined by chromosomal genotype, but may be due to the observation that this group of strains also has similar symbiotic plasmids. The association of rhizopine traits with particular symbiotic plasmids may also explain why these traits have only been detected in the biovar *viciae* of *R. leguminosarum*. These biovars are defined on the basis of the host harbouring particular symbiotic plasmids and not on the basis of chromosomally determined characters (Laguerre *et al.*, 1993b).

The results indicate that the *noc* and *mos* genes of *R. leguminosarum* strains are less divergent than those of *R. meliloti* strains, suggesting that the *noc/mos* genes may have transferred from the symbiotic plasmid of *R. meliloti* to a biovar *viciae* symbiotic plasmid in *R. leguminosarum*. Wexler *et al.* (1995) found the ability to catabolize 3-O-methyl-*scyllo*-inosamine in one other species of *Rhizobium*, genomic species 2 (*Phaseolus*) (Laguerre *et al.*, 1993a), but we do not know whether these traits arose in the genus *Rhizobium* or were acquired through horizontal transfer from non-*Rhizobium* species, although the trait has not been detected in any other genus of bacteria (D. M. Gordon, unpublished data; Rossbach *et al.*, 1995).

We are currently unable to explain the extant distribution of rhizopine synthesis and catabolism in the genus *Rhizobium*. No real understanding of the evolutionary forces responsible for the occurrence of this trait will be gained without additional survey and sequence studies of the *mos* and *noc* loci both within and between species. Perhaps more significantly, we may be unable to interpret the distribution of this trait until we fully understand its function and potential ecological role.

ACKNOWLEDGEMENTS

We thank Natalie Cavanagh and Nicki Featherstone for excellent technical assistance, J. A. Downie for pIJ1089 and J. Batut for pGMI149. This work was supported by the Australian Wool Research and Development Corporation.

REFERENCES

- Dooley, J. H. & Harrison, S. P. (1993). Phylogenetic grouping and identification of *Rhizobium* isolates on the basis of random amplified polymorphic DNA profiles. *Can J Microbiol* **39**, 655–673.
- Downie, J. A., Hombrecher, G., Ma, Q.-S., Knight, C. D., Wells, B. & Johnston, A. W. B. (1983). Cloned nodulation genes of *Rhizobium leguminosarum* determine host range specificity. *Mol Gen Genet* **190**, 359–365.
- Eardly, B. D., Materon, L. A., Smith, N. H., Johnson, D. A., Rumbaugh, M. D. & Selander, R. K. (1990). Genetic structure of natural populations of the nitrogen-fixing bacterium *Rhizobium meliloti*. *Appl Environ Microbiol* **56**, 187–194.
- Evans, P. & Howieson, J. (1993). Better inoculants improve persistence and production of lucerne. *Aust Grain April/May*, 12–13.
- Gordon, D. M., Wexler, M., Reardon, T. B. & Murphy, P. J. (1995). The genetic structure of *Rhizobium* populations. *Soil Biol Biochem* **27**, 491–499.
- Jensen, E. S. (1987). Inoculation of pea by application of *Rhizobium* in the plant furrow. *Plant Soil* **97**, 63–70.
- Laguerre, G., Fernandez, M. P., Edel, V., Normand, P. & Amarger, N. (1993a). Genomic heterogeneity among French *Rhizobium* strains isolated from *Phaseolus vulgaris* L. *Int J Syst Bacteriol* **43**, 761–767.
- Laguerre, G., Geniaux, E., Mazurier, S. I., Casartelli, R. R. & Amarger, N. (1993b). Conformity and diversity among field isolates of *Rhizobium leguminosarum* bv. *viciae*, bv. *trifolii*, and bv. *phaseoli* revealed by DNA hybridisation using chromosome and plasmid probes. *Can J Microbiol* **39**, 412–419.
- Maynard Smith, J., Smith, N. H., O'Rourke, M. & Spratt, B. G. (1993). How clonal are bacteria? *Proc Natl Acad Sci USA* **90**, 4384–4388.
- Murphy, P. J., Heycke, N., Banfalvi, Z., Tate, M. E., de Bruijn, F. J., Kondorosi, A., Tempé, J. & Schell, J. (1987). Genes for the catabolism and synthesis of an opine-like compound in *Rhizobium meliloti* are closely linked and on the Sym plasmid. *Proc Natl Acad Sci USA* **84**, 493–497.
- Murphy, P. J., Heycke, N., Trenz, S. P., Tatet, P., de Bruijn, F. J. & Schell, J. (1988). Synthesis of an opine-like compound, a rhizopine, in alfalfa nodules is symbiotically regulated. *Proc Natl Acad Sci USA* **85**, 9133–9137.
- Murphy, P. J., Wexler, M., Grezemski, W., Rao, J. P. & Gordon, D. M. (1995). Rhizopines – their role in symbiosis and competition. *Soil Biol Biochem* **27**, 525–529.
- Nei, M. & Li, W.-H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci USA* **76**, 5269–5273.
- Rao, J. P., Grezemski, W. & Murphy, P. J. (1995). *Rhizobium meliloti* lacking *mosA* synthesizes the rhizopine *scyllo*-inosamine in place of 3-O-methyl-*scyllo*-inosamine. *Microbiology* **141**, 1683–1690.
- Roberts, G. P., Leps, W. T., Sliver, L. E. & Brill, W. J. (1980). Use of two-dimensional polyacrylamide gel electrophoresis to identify and classify *Rhizobium* strains. *Appl Environ Microbiol* **39**, 414–422.
- Rohlf, F. J. (1993). Numerical taxonomy and multivariate analysis system, version 1.80. New York: Exeter Software.
- Rossbach, S., Rasul, G., Schneider, M., Eardly, B. & de Bruijn, F. J. (1995). Structural and functional conservation of the rhizopine catabolism (*noc*) locus is limited to selected *Rhizobium meliloti* strains and unrelated to their geographical origin. *Mol Plant–Microbe Interact* **4**, 549–559.
- Saint, C. P., Wexler, M., Tate, M. & Murphy, P. J. (1993).

Characterization of genes for synthesis and catabolism of a new rhizopine induced in nodules by *Rhizobium meliloti* Rm220-3: extension of the rhizopine concept. *J Bacteriol* **175**, 5205–5215.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Segovia, L., Piñero, D., Palacios, R. & Martínez-Romero, E. (1991). Genetic structure of a soil population of non symbiotic *Rhizobium leguminosarum*. *Appl Environ Microbiol* **57**, 426–433.

Segovia, L., Young, J. P. W. & Martínez-Romero, E. (1993). Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type 1 strains as *Rhizobium etli* sp. nov. *Int J Syst Bacteriol* **43**, 372–374.

Selander, R. K., Caugant, D. A., Ochman, H., Musser, J. M., Gilmour, M. N. & Whittam, T. S. (1986). Methods of multi locus enzyme electrophoresis for bacterial genetics and systematics. *Appl Environ Microbiol* **51**, 873–884.

Sokal, R. R. & Rohlf, F. J. (1981). *Biometry*, pp. 787–795. New York: W. H. Freeman.

Tempé, J. & Petit, A. (1983). La Piste des opines. In *Molecular Genetics of the Bacteria Plant Interaction*, pp. 14–32. Edited by A. Pühler. Berlin: Springer-Verlag.

Truchet, G., Debellé, F., Vasse, J., Terzaghi, B., Garnerone, A.-M.,

Rosenberg, C., Batut, J., Maillet, F. & Denarié, J. (1985). Identification of a *Rhizobium meliloti* pSym2011 region controlling the host specificity of root hair curling and nodulation. *J Bacteriol* **164**, 1200–1210.

Upholt, W. B. (1977). Estimation of DNA sequence divergence from comparison of restriction endonuclease digests. *Nucleic Acids Res* **4**, 1257–1265.

Wexler, M. (1994). *Molecular biology and distribution of rhizopine genes in Rhizobium species*. PhD thesis, University of Adelaide, Australia.

Wexler, M., Gordon, D. M. & Murphy, P. J. (1995). The distribution of inositol rhizopine genes in *Rhizobium* populations. *Soil Biol Biochem* **27**, 531–537.

Wilson, K. (1991). Preparation of genomic DNA from bacteria. In *Current Protocols in Molecular Biology*, vol. 1, pp. 2.4.1–2.4.5. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. Brooklyn, NY: Green Publishing Associates & John Wiley.

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.

Received 16 November 1995; revised 5 February 1996; accepted 9 February 1996.