FEMS Microbiology Ecology 15 (1994) 147–160

Published by Elsevier

FEMSEC 00566

Survival and dispersion of genetically modified rhizobia in the field and genetic interactions with native strains

Penny R. Hirsch * and John D. Spokes

Soil Science Department, AFRC IACR Rothamsted Experimental Station, Harpenden, Hertfordshire AL5 2JQ, UK

(Received 20 January 1994; revision received 4 May 1994; accepted 10 June 1994)

Abstract: A genetically modified strain of the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum* biovar *viciae* was used to inoculate a typical host, pea, and a control non-host cereal crop in the field. The inoculant was monitored for survival and spread from the site of application, and for genetic interactions with the native population. It could be identified by chromosomally located antibiotic resistance markers and additional markers conferred by the transposon Tn*5* inserted on its conjugative symbiotic plasmid. These markers facilitated enumeration of the strain on selective agar, enabling survival and spread to be monitored over a six year period. Although culturable cell numbers dropped two to three orders of magnitude after the first year, subsequently they remained around 10^2 viable cells per g soil, even in subplots where only the non-host cereals had been grown. However, peas did give the inoculant a small survival advantage compared with non-hosts. Soil cultivation appeared to play a major role in inoculant dissemination from the site of application. Transfer of the Tn*5* marker to other rhizobia could be monitored by screening for isolates with Tn*5*-encoded antibiotic resistance in the absence of the inoculant chromosomal markers. Over three years, more than 4000 pea root nodules were screened for indigenous rhizobia that had acquired the Tn*5*-marked symbiotic plasmid from the inoculant. None were detected, although overall about 2% of nodules contained the inoculant strain, and transfer of the Tn*5*-marked symbiotic plasmid to three out of four *R. leguminosarum* biovar *viciae* isolates from the field site could be demonstrated under laboratory conditions.

Key words: Rhizobium leguminosarum; Tn*5*; GMO release; GMO survival; Gene transfer

Introduction

Bacterial inoculants have been applied in agriculture for over 100 years. Advances in molecular biology and microbial genetics over the past two decades have made the construction of bacteria with novel genes routine and have brought closer the exploitation of inoculants improved by genetic manipulation. These have potential as agricultural inoculants but there are reservations about the consequences and implications of their use. Despite many reports on the survival of bacteria after field application, inoculants are difficult to monitor when culturable cell numbers drop, and little is understood about the ability of introduced microorganisms to persist and the de-

* Corresponding author. Tel: 0582 763133; Fax: 0582 760981.

SSDI 0168-6496(94)00049-2

gree to which they may spread from the site of introduction. Even less is known about their impact on the native soil population, and the frequency of genetic exchanges between inoculant and indigenous bacteria, although plasmid transfer between introduced strains has been demonstrated under favourable conditions in soil, such as the rhizosphere [1,2]. This is of particular concern, as it could result in the propagation of a gene in a new genetic background, well adapted to survival in local conditions. Paradoxically, genetically modified strains also offer the means to answer some of these questions. Marker genes not normally present in the soil population can be introduced into inoculant strains to facilitate monitoring that would otherwise not be possible.

We report here such an experiment using a genetically modified strain of the symbiotic nitrogen-fixing bacterium *R. leguminosarum* biovar *viciae* released in the field at Rothamsted as an inoculant on pea, a host plant, and non-host cereals, and subjected subsequently to different cropping regimes. Rhizobia have a long history of widespread use in agriculture as inoculants for leguminous plants, and offer the advantage over some other soil bacteria in that they can be isolated from soil relatively free from contamination by other microorganisms via the root nodules which they induce on compatible host plants.

The experiment was designed to examine inoculant persistence in the presence of host and non-host legumes, and non-legume crops, and to see if transfer of the genetic marker Tn*5* to the native population could be detected. The inoculant strain RSM2004 was constructed to maximize the potential for detecting gene transfer, with the Tn*5* marker inserted in a conjugative symbiotic plasmid known to transfer to a range of other rhizobia at relatively high frequencies, up to 10% of acceptor bacteria receiving the plasmid when crossed with RSM2004 [3]. Details of the construction of RSM2004 and a preliminary description of the field experiment have been reported [4].

The Tn*5* marker was chosen because the neomycin resistance which it encodes is known to be expressed in a wide variety of Gram-negative bacteria [5] and it provides a DNA sequence that can be identified following hybridization to homologous DNA probes. Its ability to transpose was also seen as an advantage for this experiment: after transfer of genetic material to a new host, Tn*5* can move to a new site in the genome even if the DNA on which it entered the host is lost; thus it can act as a marker for transient genetic interactions.

Initially, we intended to compare the behaviour of the same genetically modified inoculant in three countries with different soil and climatic condition: Rothamsted, UK; Dijon, France, and Bayreuth, FRG. Preliminary results showed that RSM2004 could not be detected three months after application in Dijon, and did not appear to survive the first winter in Bayreuth although it was still present three years after its introduction at Rothamsted [6]. We present here the detailed results from monitoring the introduced strain at Rothamsted over a six year period.

Materials and Methods

Bacterial strains, methods and probes

General methods for working with *Rhizobium* including growth conditions, TY (Tryptone Yeast) and YM (Yeast Mannitol) media, DNA extraction, and screening plasmid profiles are described in [7]. Other molecular methods, e.g. preparation of DNA probes and hybridization to gel blots and colony lifts to identify the presence of homologous sequences, were essentially as described [8]. Antibiotics were purchased from Sigma.

The parent strain from which RSM2004 was derived, from the John Innes Institute culture collection, is *R. leguminosarum* biovar *viciae* strain 248 isolated from a *Vicia faba* root nodule from a field in Great Witchingham, Norfolk. It is thus native to the UK but not to Rothamsted soil. The construction of RSM2004 involved sequentially selecting spontaneous mutants resistant to streptomycin and rifampicin before introducing Tn*5* (an element not normally present in rhizobia or other UK soil) on a suicide vector and screening for insertion of Tn*5* on the symbiotic plasmid. From several transposon mutants, RSM2004 was

chosen as it had no apparent disruption of phenotype (apart from gaining the resistance genes carried by Tn*5*) and its symbiotic competence in plant infection tests, growth rate, and survival in laboratory culture was apparently identical to the parental strain. The absence of vector sequences was confirmed by the lack of homology to vector DNA probes [4]. The probe used to detect Tn*5* in *Rhizobium* isolates was the plasmid pKan2. This consists of the central 3.5 kb *Hin*dIII fragment of Tn*5* cloned in pBR322 [9].

The ability of RSM2004 to transfer the Tn*5* marker to other rhizobia was confirmed in standard laboratory crosses, in which 10^8–10^9 colony forming units (cfu) of each parent were mixed and incubated overnight on a nitrocellulose membrane (25 mm diameter, 0.22 μm pore, Millipore) on a TY plate before washing off and plating on selective TY agar. In crosses to 10 other *R. leguminosarum* biovar *viciae* strains, there were 10^{-1}–10^{-4} Tn*5*-carrying transconjugants per parent, depending upon the recipient strain; to other *Rhizobium* strains the frequency was lower, at 10^{-2}–10^{-7}. These transconjugants appeared to contain the Tn*5*-marked symbiotic plasmid from RSM2004. Transfer of Tn*5* to *Escherichia coli* and *Enterobacter agglomerans* could also be detected at low frequencies, but no autonomously replicating plasmid could be demonstrated and Tn*5* appeared to have transposed into the acceptor genome [3].

Although common sense suggests that the deliberate release of bacteria carrying transferable antibiotic resistance genes may be undesirable, Tn*5* has been used widely in laboratories for over 20 years in genetic studies of many different bacterial genera, often with no special precautions but there are no reports of health problems, and the antibiotics to which it confers resistance are not used as a first line of defence against human bacterial infections (unlike, for example the penicillins, or gentamicin).

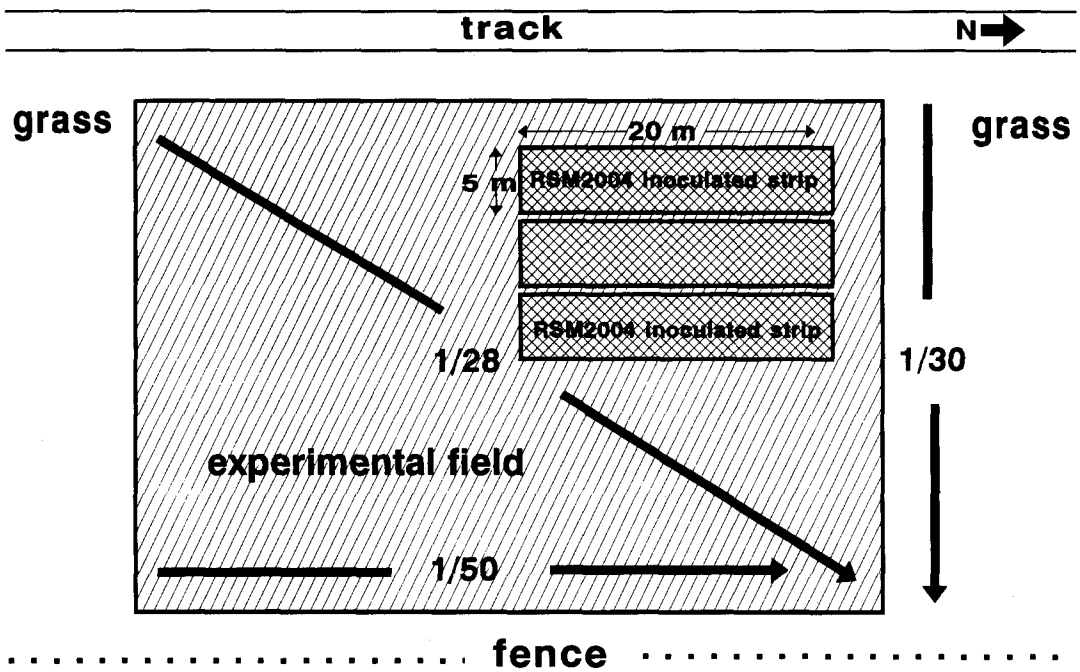

Fig. 1. Plan of field release site. The field site of the experimental release is surrounded by permanent grass extending over 20 m north and south. Other experimental fields lie beyond the track to the west and the fence to the east. The arrows denote the directions of the slopes on the site for which the gradients are given.

Inoculant preparation and application

The inoculant RSM2004 was used both as a seed coating and to form granules. It was grown in liquid YM culture before injecting into bags containing a sterile commercial formulation, HiStick® (European Patent Application No. 87306342.2-2111 "Inoculant Composition for Plants" - Agricultural Genetics Co. (AGC) Ltd.) kindly provided by the AGC. The bags were incubated for several days, then a small sample of each was resuspended and plated on selective TY agar to obtain a viable cell count (cfu) before the remainder was added to 500 ml sterile water and mixed with the seeds. To make granules, an equal weight of $CaSO_4$ (British Gypsum fine casting plaster) was blended with HiStick containing the inoculant strain, then sterile deionized water added and mixed in to give approximately 40–45% moisture, the level at which appropriately sized aggregates form: these were sieved through a 5 mm mesh. After preparation of inoculants in the laboratory the coated seeds and granules were securely packed in plastic bags and transported to the field. The inoculant granules have a soft, loose structure and rapidly loose their integrity when saturated with water, disintegrating within a few weeks of application in field soil conditions, enhancing the dispersal of the inoculant bacteria (J. Jebb, personal communication).

In a parallel experiment to look at bulk movement of soil using soil bacteria as a marker, commercial chick peas were coated with a *Rhizobium "cicer"* inoculant formulation provided by John Jebb, AGC.

Field release

Untreated seed of pea, *Pisum sativum* cv Progreta, was kindly provided by the PGRO, Peterborough, UK. Lucerne was obtained from John Chambers, Kettering, UK. The phaseolus bean (*Phaseolus vulgaris*) variety was Edmund. Cereal varieties were: spring wheat (*Triticum aestivum*) - cv Alexandria; spring barley (*Hordeum vulgare*) - cv Klaxon; and winter wheat (*T. aestivum*) - cv Mercia. Unless otherwise stated, the sowing densities and treatment of the crops were to the standard Rothamsted Farm practice, but cultivation equipment was washed with hypochlorite solution (Chloros - Hays Chemical Distribution Ltd., Leeds, UK) diluted to 1% between sub-plots and after use, and plant material was incinerated after harvesting.

The release site (Fig. 1) was situated in the 'Garden Plots' area of the Rothamsted Farm. It is known to have good natural drainage with no run-off into drainage ditches or history of bulk water or soil flow after heavy rain. The site has a Batcome series soil, loam over clay with flints overlying chalk, known agriculturally as a light clay and more specifically as a fine loamy chromic luvisol with 32% clay, pH 7.5 [10]; J. Catt, personal communication. It has been managed as a typical arable agricultural soil. In spring 1987, the field was prepared as a seed bed and drilled with spring wheat, leaving unsown an area containing three 20 m × 5 m strips separated by 0.5 m paths. The inoculated peas and chick peas were planted manually in two of these strips. In the pea plot, inoculant granules were placed in the furrows before planting the inoculated seed. A third strip was broadcast with inoculant granules over the surface before planting half the width of the strip with spring wheat, the other half with barley (neither inoculant-coated) using a mechanical seed drill. The action of the drill turned over the soil thus covering the inoculant granules. Initial results from the two halves of the strip were indistinguishable and samples were subsequently pooled: for simplicity the strip is referred to as 'cereals' throughout this paper, although the same combination of spring wheat and barley was used for the following two years. The three strips and 1 m of the surrounding uninoculated cereal were covered by a fruit net to exclude birds. This was surrounded by an electric fence to exclude small mammals, especially rabbits. Apart from removing any clods of soil from footwear and equipment before leaving the site, and sterilizing sampling and cultivation equipment, no other special precautions above those normally used for small-scale field trials were taken. No further inoculation was performed after the first year.

Approximately 10^{12} viable RSM2004 (cfu) were added to each strip. The seed coating contained 10^6 rhizobia per seed, peas were planted at 80 per m^2, resulting in a total contribution of ap-

prox. 10^8 RSM2004 per m^2. Granules were applied to give 10^{10} per m^2, approximately equivalent to the total number of indigenous *R. leguminosarum* biovar *viciae* estimated in field soil, i.e. 5×10^4 infective cells per g dry soil, assuming that the dry weight of soil to 25 cm depth under 1 m^2 of the field site is about 280 kg [11] and that the majority of aerobic soil bacteria including rhizobia are found in the top 25 cm of arable soils. This represents 10–100 × the normal commercial rate for inoculants, to overcome problems of competition for nodulation of the peas from the indigenous rhizobial population. Thus, the contribution from granules to the bulk soil was approximately 500 × that of the seed-coating inoculant (which is, however, particularly important for host nodulation as it delivers a locally high concentration of the inoculant to the emerging root). The *R. "cicer"* inoculant, intended to act as a marker for bulk soil movement, was applied at the commercial rate (i.e 10^5–10^6 rhizobia per seed, equivalent to 10^7–10^8 per m^2 or 10^2–10^3 rhizobia per g soil). Previous investigations had found no evidence of indigenous rhizobia capable of nodulating chick peas in the field site.

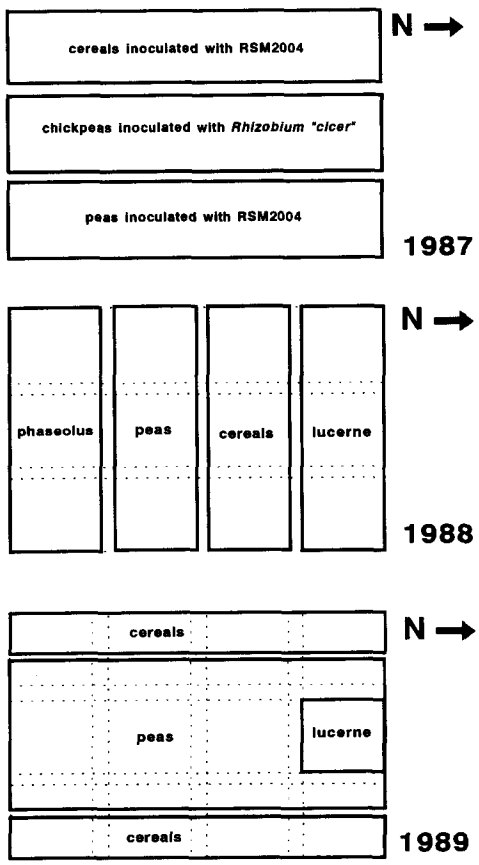

Fig. 2. Experimental field release strategy. In 1987, the three strips were inoculated with rhizobia as indicated. In 1988 four strips were planted at 90° creating 12 sub-plots. In 1989 most of the site was planted with peas and cereals with one lucerne subplot that had been left over the winter, creating 20 subplots. No inoculants were applied in 1988 and 1989. The field including the release site has been planted only with cereals since 1989.

The field plan and cropping sequence are shown in Fig. 2. After the first year the site was ploughed north-south (N-S) within the three strips and the soil was sampled to examine migration of *R. "cicer"* and RSM2004. The field surrounding the release site was planted with spring barley in 1988 and the strips inoculated in 1987 were planted in four 5 m × 16 m strips (peas, phaseolus beans, lucerne and spring barley/wheat) running east-west (E-W) across the three original treatments to give 12 sub-plots. After harvesting, the strips were ploughed E-W, leaving a sub-plot of lucerne to overwinter. In 1989, the third year, most of the sub-plots were planted with peas or spring barley/wheat, retaining the lucerne plot, and the surrounding field was again planted with spring barley. Subsequently the field has been kept in arable cultivation with spring barley in 1990, winter wheat in 1991 and 1992, and spring wheat in 1993, the original sub plots being treated the same way as the surrounding field.

Plant infection tests

Rhizobial soil population densities from field soil samples (prepared as described below) were estimated from the number of nodulated host plants when four-fold soil dilutions were added to four replicate plants at each dilution, using the density estimate tables of R.E. Fisher and F. Yates [12], as described in [13]. This method determines total numbers (infective cells per g soil) of different rhizobial biovars and species, depending upon which host plant is used. It has recently been shown to correspond with direct colony counts (cfu) made by plating soil dilutions

on semi-selective agar, then using specific DNA probes to identify colonies as *Rhizobium* biovars and species [14]. The test plant for *R. leguminosarum* biovar *viciae* was hairy tare (*Vicia hirsuta*), whereas for *R. meliloti* lucerne (*Medicago sativa*) was used (both supplied by John Chambers, Kettering, UK). White clover (*Trifolium repens* cv Aberystwyth S184) was used to enumerate *R. leguminosarum* biovar *trifolii*. These small-seeded test plants were grown on N-free Fåhraeus agar slopes in test tubes [7]. To estimate *R. leguminosarum* biovar *phaseoli* populations, surface-sterilized phaseolus beans (*Phaseolus vulgaris* cv Edmund) were grown in 10 cm pots containing sterile calcined montmorillonite clay (Terragreen - Oil Dri Corporation of America), dilutions of soil extract were applied, and the plants were watered with sterile N-free feed [7]. Rhizobia were isolated from surface-sterilized root nodules by crushing and spotting onto TY agar, transferring bacterial growth to fresh TY plates, then replicating to selective agar media (TY with neomycin or rifampicin at 100 μg ml^{-1}) to check the antibiotic resistance phenotype, and to nitrocellulose membrane filters (0.45 μm pore, Schleicher and Schüll) to perform in situ lysis for DNA hybridization following overnight incubation on TY agar.

The presence or absence of *R. "cicer"* was determined by mixing 400 g field soil (consisting of five sub-samples) mixed with an equal volume of sterile sand in a 10 cm pot with surface-sterilized pre-germinated chick peas, *Cicer arietinum*. After six weeks, roots were examined for nodules. This strategy, employing the host root system to probe much larger soil samples than is possible with the standard plant infection assays (utilizing 1–10 g soil), is more sensitive but non-quantitative.

Sampling bacteria from soil

Soil samples were taken from the field using a trowel surface-sterilized with 70% ethanol between each sub-sample. The top 10 cm of soil were removed and a sample of about 100 g taken at this depth and placed in a polythene bag. For pooled samples, ten independent 100 g sub-samples taken at evenly spaced intervals over the plot (about 1 m apart from the large strip plots in the first year, about 0.3 m apart for smaller sub-plots in the second and subsequent years) were pooled in the same bag. The samples were sieved through a 5 mm mesh to aid mixing and removal of small stones, fauna, plant roots etc. The sieve was washed thoroughly and sterilized with 70% ethanol between samples and care was taken to avoid any cross-contamination. After further mixing with a sterile spatula, 1 g of the mixed sub-samples was taken and resuspended in 10–100 ml sterile sodium hexametaphosphate (2%) by vigorous vortexing for 1–2 min. Suspensions were immediately diluted further and used to inoculate test plants or plated on selective agar (described below). The moisture content of the remaining soil sample was calculated by weighing before and after drying for 48 h at 80°C. Sampling when the ground was waterlogged (field capacity is about 30% water wt/wt) was avoided and in dry conditions the site was irrigated before sampling. Consequently, the moisture content of soil samples was always in the range 10–20% wt/wt

Direct counting of RSM2004 from soil was possible on TY agar supplemented with rifampicin and neomycin which counterselected the indigenous bacterial population, and the fungicide cycloheximide, all at 100 μg ml^{-1}. Fungal contamination was the main factor which limited the detection of antibiotic-resistant bacteria as cycloheximide slowed the spread of fungal colonies, but did not completely inhibit growth.

Results

Nodule occupancy

Although preliminary glasshouse pot experiments had demonstrated that, when 10^8 RSM2004 were applied to germinating pea seeds in 600 g of soil from the field site, 30% of the nodules contained the inoculant, the results from the field release showed a lower occupancy, averaging about 2.5% overall. However, the first-formed nodules on the main root sampled six weeks after planting did contain around 20% RSM2004 (i.e. Neo^r, Rif^r, Tn*5*-DNA probe homologous isolates). After ten weeks and in the final sampling

at 17 weeks, only the lateral roots were nodulated (the first-formed nodules having senesced) and they contained fewer RSM2004, resulting in the 6% first year average (Table 1). The following year no RSM2004 were detected in pea root nodules although the sample of 572 was only half of the 1987 total. In the third year, in response to the difficulty in detecting RSM2004, most of the plot was sown with peas but only 0.8% of nodules contained the inoculant strain. The proportion was higher in plants from sub-plots planted with cereals (rather than peas) in 1987, but all these RSM2004-containing nodules came from the sub-plot with phaseolus in 1988. In contrast, nodules from all four sub-plots where peas had been grown in 1987 contained RSM2004 in 1989. Since the actual numbers involved were small (Table 1), these differences are unlikely to be statistically significant.

The chick peas planted in 1987 were well-nodulated and appeared to contain the inoculant *R. "cicer"* strain, on the basis of plasmid profiles of ten separate nodule isolates (data not shown). Since no nodules had developed on uninoculated chick peas grown in soil from the site in the glasshouse, we concluded that there were insignificant numbers of indigenous rhizobia capable of nodulating chick peas. Thus, nodulation of chick peas was used to assay the presence of the *R. "cicer"* inoculant in subsequent investigations of soil and inoculant movement over the field site.

Plasmid transfer

There was no evidence over the three year period, with over 4000 pea nodules screened (and also several hundred phaseolus bean and lucerne nodules in the second year), that transfer of the

Table 1

Frequency of rhizobia containing Tn*5* in root nodules

Year	Plant	1987 Crop	1988 Crop		Number of nodules	Nodules containing RSM2004	Nodules containing transconjugants
1987	Peas			Total	1035	65 (6%)	0 (< 0.1%)
1988	Phaseolus	Peas			140	0	0
1988	Phaseolus	Cereals			130	0	0
1988	Phaseolus			Total	270	0 (< 0.4%)	0 (< 0.4%)
1988	Lucerne	Peas			135	0	0
1988	Lucerne	Cereals			125	0	0
1988	Lucerne			Total	260	0 (< 0.4%)	0 (< 0.4%)
1988	Peas	Cereals			236	0	0
1988	Peas	Peas			336	0	0
1988	Peas			Total	572	0 (< 0.2%)	0 (< 0.2%)
1989	Peas	Peas	Phaseolus		398	1 (0.2%)	0 (< 0.2%)
1989	Peas	Peas	Lucerne		477	4 (0.8%)	0 (< 0.2%)
1989	Peas	Peas	Cereals		342	1 (0.3%)	0 (< 0.3%)
1989	Peas	Peas	Peas		715	3 (0.4%)	0 (< 0.1%)
1989	Peas	Peas		Total	1932	9 (0.5%)	0 (< 0.05%)
1989	Peas	Cereals	Phaseolus		231	11 (4.8%)	0 (< 0.4%)
1989	Peas	Cereals	Lucerne		69	0 (< 1.4%)	0 (< 1.4%)
1989	Peas	Cereals	Cereals		86	0 (< 1.2%)	0 (< 1.2%)
1989	Peas	Cereals	Peas		182	0 (< 0.5%)	0 (< 0.5%)
1989	Peas	Cereals		Total	568	11 (2%)	0 (< 0.2%)
1989	Peas			Total	2500	20 (0.8%)	0 (< 0.04%)
	Peas	1987–1989		Total	4107	85 (2%)	0 (< 0.02%)

The number of nodules containing viable bacteria is given. Nodule isolates were identified as RSM2004 on the basis of ability to grow on neomycin and rifampicin and homology to Tn*5*-DNA probe. Putative transconjugants were isolates able to grow on neomycin and with Tn*5*-homology but lacking rifampicin resistance: all possible candidates were rechecked but were found to be RSM2004.

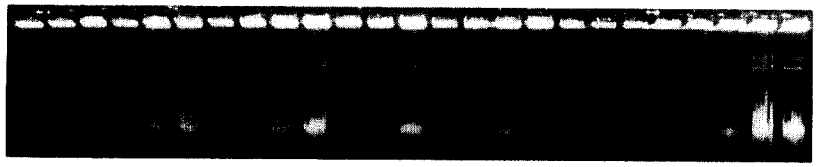

Fig. 3. Plasmid profiles of putative RSM2004 isolates from field soil. The track on the far right of the gel contains laboratory culture of RSM2004, the other tracks contain 24 separate field soil isolates from selective plates (TY agar containing rifampicin and neomycin).

Tn*5* marker to other *R. leguminosarum* strains had occurred. All nodule isolates that were neomycin resistant and had homology to the Tn*5* DNA probe had the RSM2004 phenotype: they were also rifampicin resistant, and the identity of 10–20 isolates from each batch of nodules was confirmed by examining their plasmid profiles (Fig. 3). Overall, the plasmid profiles of about 100 putative RSM2004 isolates have been examined and no differences between these and RSM2004 maintained in the laboratory have been detected.

The intrinsic antibiotic resistance levels and plasmid profiles of 240 *R. leguminosarum* biovar *viciae* isolates from the site were screened and four with distinct phenotypes representing different prevalent indigenous strains from the field soil, were chosen (Fig. 4). Spontaneous mutants to spectinomycin resistance were selected and crossed (in standard filter matings) with RSM 2004, selecting for transconjugants on TY agar supplemented with spectinomycin and neomycin. The transfer frequency to three of the isolates was 10^{-5}, but none could be detected in the fourth cross (frequency less than 10^{-8}). The transfer frequency in sterile soil was investigated in crosses between RSM2004 and two different strains (Table 2). Transfer was less efficient in soil than with the standard filter method, and could not be detected when the original cross mixtures were diluted 10-fold to give fewer than 10^6 parents per g soil.

Table 2

Transfer frequencies [a] of Tn*5* from RSM2004

Recipient strain	Cross on filters [a]	Cross in sterile soil [a]
Rhizobium meliloti RCR2001 Spc^r [b]	2×10^{-4}	8×10^{-5}
R. leguminosarum biovar *viciae* RCR1001 Spc^r [b]	1.7×10^{-3}	1.1×10^{-4}

[a] Transconjugants per parent: since number of both parents was equal, transfer frequencies apply to either parent.

[b] Spc^r = spectinomycin resistant.

A 0.2 ml aliquot of mixture of the parental strains (10^8 each) was spotted on to either 25 mm diameter nitrocellulose filter on TY agar plate (standard cross conditions), or 10 g soil in universal bottle (sterilized by autoclaving) providing surface 25 mm diameter. After overnight incubation at 28°C, the filter was washed in 1 ml and the soil was resuspended in 10 ml sterile H_2O, then diluted and plated on selective agar.

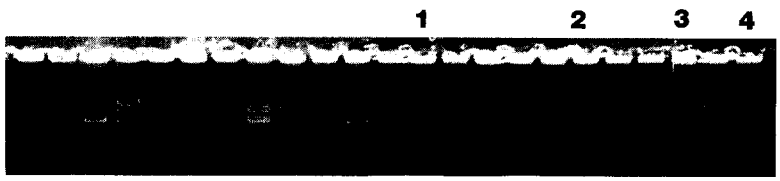

Fig. 4. Plasmid profiles of *R. leguminosarum* biovar *viciae* isolates. The gel contains 23 separate isolates from *V. hirsuta* nodules inoculated with soil from the field release site. Spectinomycin-resistant derivatives of the isolates marked 1–4 were crossed with RSM2004 using standard conditions: isolates 1, 3, and 4 received the Tn*5* marker at frequencies of 10^{-5} transconjugants per parent (donor or acceptor); transfer to isolate 2 could not be detected.

Survival in soil

The neomycin resistance encoded by Tn*5*, in conjunction with the chromosomally located rifampicin resistance, facilitated direct isolation of RSM2004 from field soil on selective agar. The first counts were done in January 1988 (four months after harvesting, but just before the plots were ploughed), one month later after ploughing, and then at monthly intervals until June 1989. For the first three samplings, five independent soil samples taken from each plot were resuspended, diluted and plated in duplicate. Resulting colony counts showed considerable variation between the five replicates from each plot, reflecting the non-homogeneous distribution of the inoculant. This was investigated further for one sub-plot where 16 independent soil samples were diluted and plated in quadruplicate (Table 3). A log-linear model was fitted to the results, showing that replicate counts for each sample were slightly more variable than a Poisson distribution would predict (significant at the 2% level). However, the variation between soil sub-samples was much greater (significant at the 0.1% level). The interpretation of these findings is that there was increased variability between replicates and in each dilution series compared with a homogeneous bacterial culture (where colony counts should have a Poisson distribution), probably due to the non-uniform nature of the soil suspensions. However, this was far less important than the great variation between samples from different sam-

Table 3

Variation in RSM2004 numbers in different samples

Sample	Replicate plate counts (cfu):			
1	6	1	0	1
2	1	8	2	12
3	16	16	9	13
4	8	7	4	12
5	3	2	2	6
6	0	0	0	0
7	6	2	6	4
8	4	3	4	6
9	16	13	11	6
10	56	51	34	32
11	3	0	1	0
12	3	2	1	3
13	13	15	18	15
14	13	5	8	6
15	3	3	6	2
16	10	9	7	8

Separate 1 g soil samples taken on 25 May 1988 from sub-plot with peas in both 1987 and 1988 were each resuspended in 10 ml sterile sodium hexametaphosphate (2%), diluted 1 in 4, then 0.1 ml was plated on selective agar. Thus, counts represent 1 in 400 dilution of the number per g soil.

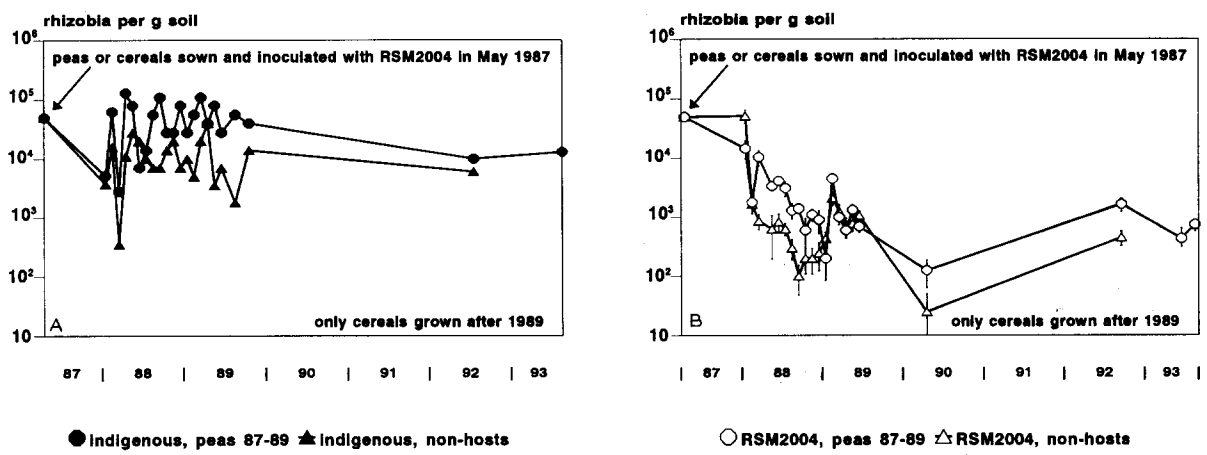

Fig. 5. Comparison of the indigenous population of *R. leguminosarum* biovar *viciae* with RSM2004 inoculated on host and non-host crops. Peas or cereals were inoculated with RSM2004 in 1987, soil samples were taken from sub-plots with peas only or non-host crops only between 1987–1989. After 1989, only cereals have been grown on all subplots. **(A)** Total populations of indigenous *R. leguminosarum* biovar *viciae*, calculated from plant infection tests; error bars are not shown but each estimate has 95% probability of varying by less than factor of 2.7 (i.e. approximate 95% fiducial limits). **(B)** Colony counts of RSM2004 made on selective agar. Means were calculated from minimum of four plates, standard errors are shown for each value.

plings within each sub-plot, presumably reflecting the heterogeneity of the field soil and the tendency of rhizobia to be clumped in microcolonies on soil or organic debris particles, peat particles released by disintegrating inoculant granules, or senesced nodules. Subsequently, ten separate soil samples taken from different parts of each sub-plot were pooled to overcome such sampling problems.

A log-linear model fitted to the data collected from 1988 and 1989 showed that the most significant effect was exerted by the previous crop, i.e. whether it was peas or cereals. From this data, the model predicted a count of 1.45×10^3 where the previous crop was pea (approximate standard error 0.16) and 5.3×10^2 (approximate standard error 0.10) where the previous crop was cereals. A more detailed statistical analysis of the effects of different crop plants on RSM2004 survival will be presented elsewhere. However, graphical representation of the data (Fig. 5) demonstrates that generally there were almost three times as many

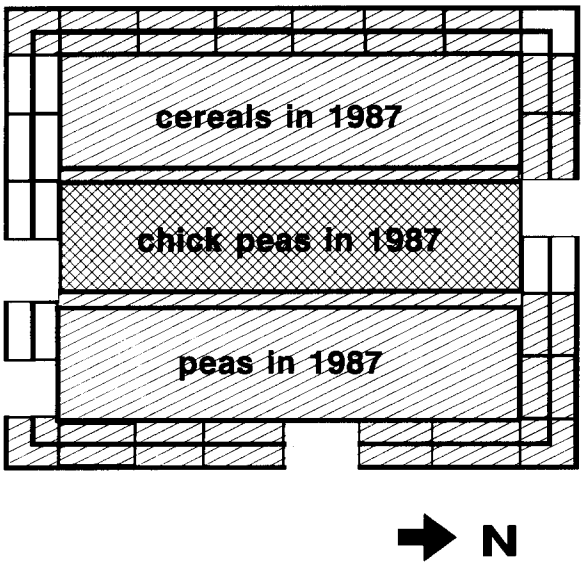

Fig. 6. Dispersion of *R. "cicer"* from the site of application. The strip inoculated with *R. "cicer"* in 1987 is cross-hatched. Samples were taken in 1989 after harvesting but before ploughing, from within blocks 0–1 m and 1–2 m from the edges of the original strips. Clear blocks indicate no nodules on chick pea plants and therefore *R. "cicer"* too low to detect, hatched areas indicate nodules, no blocks are shown where results were inconclusive due to death of test plants.

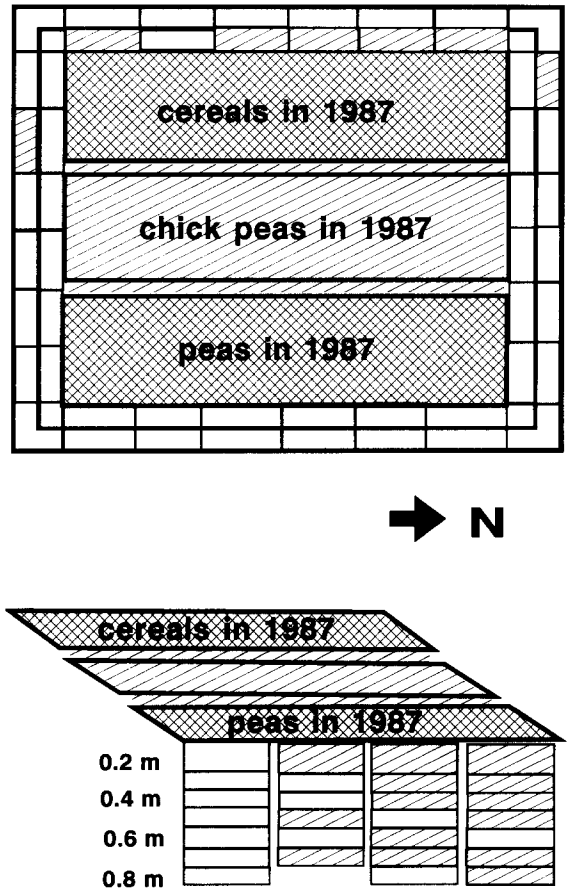

Fig. 7. Dispersion of RSM2004 from the site of application. The upper diagram shows horizontal spread of RSM2004 in November 1989 (after clearing the site but before ploughing) in blocks 0–1 m and 1–2 m from the edges of the original strips inoculated in 1987 (cross-hatched). Blocks where RSM2004 was detected are hatched; clear blocks indicate no RSM2004 was detected (i.e. fewer than 25 cfu per g soil). The lower diagram, not drawn to scale, shows vertical distribution to depth of 0.8 m (one sample was lost).

RSM2004 colonies under peas than under non-host crops and this survival advantage conferred by the host was also apparent in the total infective populations of *R. leguminosarum* biovar *viciae* estimated from plant nodulation tests. Survival of RSM2004 for over six years has been demonstrated, although no host plants have been grown since 1989 (Fig. 5).

Dispersion of inoculants

In January 1988, before ploughing, samples of soil were taken to assess spread of the *R. "cicer"*

and RSM2004 from the sites of release. Five samples from the field margin, 20 m south of the inoculated strip, did not contain *R. "cicer"*. All 20 samples taken at points 15 cm and 30 cm from the edge of the inoculated strip, 16 out of 20 at 40 cm and 1 out of 20 at 60 cm contained *R. "cicer"*. Similarly, each of five soil samples taken from within the chick pea strip, diluted and plated on selective agar contained RSM2004, although at very low numbers (fewer than 100 cfu per g soil). An unusually wet autumn with some puddles of surface water may have assisted the spread. After the strips had been ploughed N-S in January 1988 and E-W in January 1989, *R. "cicer"* was present over most of the inoculated area and the 2 m surround (Fig. 6).

A preliminary investigation of horizontal and vertical RSM2004 dispersion in 1989 detected colonies on selective agar from soil samples taken in the strip originally planted with chick peas, up to 2 m from the perimeter of the three strips inoculated in 1987, and up to 0.8 m below the surface of the strip with peas in 1987. Because plate counts were very low, these RSM2004 numbers can be estimated only roughly (Fig. 7). Nodulation tests on *V. hirsuta* indicated the presence of infective *R. leguminosarum* biovar *viciae* in all these samples.

Discussion

Nodulation by the inoculant

It is unclear why RSM2004 formed a lower proportion of pea root nodules in the field than had been predicted from preliminary experiments with pots of field soil in the glasshouse. However, the indigenous population of *R. leguminosarum* biovar *viciae* may be more competitive under the environmental conditions to which it may be presumed to be adapted, than under the more controlled environment in the glasshouse. The first-formed nodules did contain a relatively high percentage of RSM2004. This implies that the seed coating formulation gave the inoculant privileged access to nodulate the emerging root, but that the granules which introduced many more RSM2004 cells to the field soil were a less effective method for inoculating the roots in the presence of the competitive native population. Plate counts on selective agar could underestimate the number of RSM2004 cells surviving in the soil if a significant proportion loose the ability to grow in culture. However, experiments on the population dynamics of rhizobia added to soil, comparing selective plate counts and direct counts in situ using specific immunofluorescent antibodies, have shown no significant evidence of non-culturable *R. leguminosarum* cells [15].

Genetic interactions

The absence of any transconjugant rhizobia with the Tn*5* marker in the nodule isolates is not surprising since even in sterile field soil, transfer between RSM2004 and compatible acceptor strains could be detected only when more than 10^6 of each parent was present, and it has often been reported that transfer frequencies are lower in non-sterile compared with sterile soils [16,17]. The total populations in soil did not reach this level, although the non-homogeneous distribution of the inoculant could give rise to locally high densities. The developing root system in the field may provide a surface on which rhizobia coincide and grow actively, conditions required for conjugation. However, if transfer of the Tn*5*-marked plasmid occurred, the frequency was below the limit of detection. We had not anticipated any selective advantage for rhizobia carrying Tn*5* and the experiment was not designed to detect such an event. It has recently been reported that the bleomycin resistance gene which is also present on Tn*5* and is expressed in RSM2004 has DNA repair activity which has been shown to confer a survival advantage to ageing *E. coli* in the decline phase of batch culture [18]. However, comparisons of isogenic rhizobia with and without Tn*5* in Dijon have not identified any differential survival in the field (N. Amarger and S. Mazurier, personal communication).

Inoculant survival

The method of introducing the inoculant did not have a dramatic effect on the survival of RSM2004, with about three-fold fewer counts in sub-plots where granules only were supplied to

cereals compared with sub-plots where the host pea plants were inoculated by both seed-coating and granules (Fig. 5). A similar difference is apparent in the total populations of *R. leguminosarum* biovar *viciae* under peas or non-hosts (Fig. 5). Since 500-fold more inoculant was supplied in the granules than in the coated pea seeds, it is assumed that the RSM2004 applied by this route to the peas did not make a significant contribution to the overall population surviving in soil compared with that from the granules. However, seed-coating inoculation probably was important in enabling RSM2004 to nodulate peas in competition with the indigenous population and may therefore have contributed to the small survival advantage conferred by the host plant. This unexpected survival of RSM2004 in the absence of the host plant may reflect the ability of *R. leguminosarum*, unlike some other rhizobial species, to survive for long periods in soil at relatively high numbers in the absence of any host plants. The total population of *R. leguminosarum* biovar *viciae* remained at 10^4-10^5 infective cells per g soil before sowing in 1987, and after peas had been grown for three years. The population of *R. leguminosarum* biovar *trifolii* was similar, although the plot had no record of cultivation of host plants for either biovar for at least 20 years, when detailed records began. This is in contrast to another species, *R. meliloti*, which could not be detected before the host plant lucerne was cultivated. The population rose rapidly when the host lucerne was grown and declined after harvesting [19]. This indicates the importance of appraising the behaviour of individual bacterial strains and species when considering the release of genetically modified derivatives as broad generalizations at the genus level may be misleading.

Inoculant dispersion

The dispersion of microbial inoculants is difficult to assess in arable field sites. The exact boundaries of sub-plots are lost during cultivation and there is some error in their re-location from the external marker posts. It is probable that mechanical cultivation is the most important route for horizontal dispersion of the inoculant bacteria. The methods used on the site, ploughing and non-inversion tillage (harrowing), move bulk soil about 0.5 m and a dispersion parameter of 0.24 m^2 per tillage has been calculated [20] although movement of over 1 m per year has been observed for weed seeds of varying sizes in soil (G. Cussans, personal communication). The vertical movement of RSM2004 may be due to migration and movement in water films along roots and the associated channels and cracks and there may have been some horizontal dispersion in surface water after heavy rain although the site is well drained. Any inadvertent dispersion by humans (boots), vertebrates (feet) or invertebrates (on or in soil) is likely to be minor by comparison with mechanical cultivation which is a normal feature of arable agriculture.

Implications

The demonstration that RSM2004 has survived and remained viable in soil for over six years following its introduction is relevant when considering the implications of introducing other inoculants which may be altered more radically. The low level of RSM2004, estimated at 0.1–1% of the total *R. leguminosarum* biovar *viciae* population, would have been difficult to detect without the Tn*5* marker. There was no evidence of Tn*5* transfer from RSM2004 to native strains in the field, i.e. it was below the limit of detection (fewer than 0.02% of isolates could have received Tn*5*) indicating that overall, gene transfer activity did not approach the maximum level (0.1% of potential acceptors receiving Tn*5*) observed in standard laboratory crosses. In conjunction with the relatively low population of RSM2004, this implies that transfer of Tn*5* to the indigenous population is unlikely to be a major factor in its persistence in this field. Survival of RSM2004 in the absence of the host plant reflects the behaviour of *R. leguminosarum* populations in soil but not necessarily that of other rhizobial species. However the results of the field release demonstrate that it should not be assumed that introduced inoculants disappear after a few years and in arable situations, bulk movement of soil during cultivation is likely to be a major route for the spread of introduced microorganisms.

Acknowledgements

This work was supported in part by the EC Biotechnology Action Programme and by the MAFF. We thank Amanda Latham and Maureen Birdsey for skilled technical support, John Jebb and Paul Williams of AGC for supplying inoculant carriers and much useful advice, and Peter Lane for statistical analysis of the data. For guidance when planning the field experiment, we are particularly indebted to John Day, who tragically died on April 24 1994, and we would like to dedicate this paper to his memory.

References

1 Van Elsas, J.D., Nikkel, M. and Van Overbeek, L.S. (1988) Detection of plasmid RP4 transfer in soil and rhizosphere, and the occurrence of homology to RP4 in soil bacteria. Curr. Microbiol. 19, 375–381.
2 Van Elsas, J.D., Van Overbeek, L.S. and Fouchier, R. (1991) A specific marker, *pat*, for studying the fate of introduced bacteria and their DNA in soil using a combination of detection techniques. Plant Soil 138, 49–60.
3 Hirsch, P.R. (1990) Factors limiting gene transfer in bacteria. In: Bacterial Genetics in Natural Environments (Fry, J.C. and Day, M.J., Eds.), pp. 31–40. Chapman and Hall, London.
4 Hirsch, P.R. and Spokes, J.R. (1988) *Rhizobium leguminosarum* as a model for investigating gene transfer in soil. In: Risk Assessment for Deliberate Releases (Klingmüller, W., Ed.), pp. 10–17. Springer-Verlag, Berlin Heidelberg.
5 Berg, D.E. and Berg, C.M. (1983) The prokaryotic transposable element Tn*5*. Biotechnology 1, 417–435.
6 Amarger, N. Hirsch, P. and Klingmüller, W. (1990) Assessing the risks involved in the release of genetically manipulated microorganisms. In: Biotechnology R&D in the EC - Part II - Detailed Final Report of BAP Contractors in Risk Assessment (1989–1990) (Economidis, I., Ed.), pp. 55–60. CEC, Brussels.
7 Hirsch, P.R. and Skinner, F.A. (1992) The Identification and classification of *Rhizobium* and *Bradyrhizobium*. In: Identification Methods in Applied and Environmental Microbiology. (Board, R.G, Jones, D. and Skinner, F.A., Eds.), pp. 45–65. Blackwell Scientific Publications, Oxford.
8 Maniatis, T., Fritsch, E.F. and Sambrook, J. (1992) Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, NY.
9 Scott, K.F., Hughes, J.E., Gresshoff, P.M., Beringer, J.E., Rolfe, B.G. and Shine, J. (1982) Molecular cloning of *Rhizobium trifolii* genes involved in symbiotic nitrogen fixation. J. Mol. Appl. Genet. 1, 315–326.
10 Avery, B.W., Bullock, P., Catt, J.A., Newman, A.C.D., Rayner, J.H. and Weir, A.H. (1971) The soil of Barnfield. In: Rothamsted Experimental Station Report for 1971., pp. 5–37. Lawes Agricultural Trust, Harpenden.
11 Hall, A.D. (1905) The Book of the Rothamsted Experiments. John Murray, London
12 Fisher, R.A. and Yates, F. (1963) Statistical Tables for Biological, Agricultural and Medical Research. Revised 6th edition. Oliver and Boyd, Edinburgh
13 Vincent, J.M. (1970) A Manual for the Practical Study of Root Nodule Bacteria. IBP Handbook no 15, Blackwell Scientific Publications, Oxford.
14 Laguerre, G., Bardin, M. and Amarger, N. (1994) Isolation from soil of symbiotic and non-symbiotic *R. leguminosarum* by DNA hybridization. Can. J. Microbiol., in press.
15 Heijnen, C.E., Burgers, S.L.G.E. and van Veen, J.A. (1993) Metabolic activity and population dynamics of rhizobia introduced into unamended and bentonite-amended loamy sand. Appl. Environ. Microbiol. 59, 743–747.
16 Van Elsas, J.D., Trevors, J.T. and Starodub, M-E. (1989) Plasmid transfer in soil and rhizosphere. In: Risk Assessment for deliberate Releases (Klingmüller, W., Ed.), pp. 10–17. Springer-Verlag, Berlin Heidelberg.
17 Fry, J.C. and Day, M.J. (1990) Plasmid transfer and the release of genetically engineered bacteria in nature: a discussion and summary. In: Bacterial Genetics in Natural Environments (Fry, J.C. and Day, M.J., Eds.), pp.243–250. Chapman and Hall, London.
18 Blot, M., Meyer, J. and Arber, W. (1991) Bleomycin-resistance gene derived from the transposon Tn*5* confers selective advantage to *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA 88, 9112–9116.
19 Hirsch, P. (1994) Factors influencing survival of *Rhizobium*. In: AFRC Institute of Arable Crops Research Report for 1993. Rothamsted Experimental Station, Harpenden. (In the press).
20 McGrath, S.P. and Lane, P. (1989) An explanation for the apparent losses of metals in a long-term field experiment with sewage sludge. Environmental Pollution 60, 235–256.