

Transfer and loss of naturally-occurring plasmids among isolates of *Rhizobium leguminosarum* bv. *viciae* in heavy metal contaminated soils

Amir Lakzian^a, Phillip Murphy^a, Ken E. Giller^{a,b,*}

^aDepartment of Biological Sciences, Imperial College at Wye, University of London, Wye, Ashford, Kent TN25 5AH, UK

^bPlant Production Systems, Department of Plant Sciences, Wageningen University, P.O. Box 430, Postbus 430, 6700 AK Wageningen, The Netherlands

Received 9 August 2006; received in revised form 22 November 2006; accepted 1 December 2006

Available online 9 January 2007

Abstract

Plasmid transfer among isolates of *Rhizobium leguminosarum* bv. *viciae* in heavy metal contaminated soils from a long-term experiment in Braunschweig, Germany, was investigated under laboratory conditions. Three replicate samples each of four sterilized soils with total Zn contents of 54, 104, 208 and 340 mg kg⁻¹ were inoculated with an equal number (1×10^5 cells g⁻¹ soil) of seven different, well-characterized isolates of *R. leguminosarum* bv. *viciae*. Four of the isolates were from an uncontaminated control plot (total Zn 54 mg kg⁻¹) and three were from a metal-contaminated plot (total Zn 340 mg kg⁻¹).

After 1 year the population size was between 10^6 and 10^7 g⁻¹ soil, and remained at this level in all but the most contaminated soil. In the soil from the most contaminated plot no initial increase in rhizobial numbers was seen, and the population declined after 1 year to <30 cells g⁻¹ soil after 4 years. One isolate originally from uncontaminated soil that had five large plasmids (no. 2-8-27) was the most abundant type re-isolated from all of the soils. Isolates originally from the metal-contaminated soils were only recovered in the most contaminated soil. After 1 year, four isolates with plasmid profiles distinct from those inoculated into the soils were recovered. One isolate in the control soil appeared to have lost a plasmid. Three isolates from heavy metal contaminated soils (one isolate from the soil with total Zn 208 mg kg⁻¹ and two isolates from the soil with total Zn 340 mg kg⁻¹) had all acquired one plasmid. Plasmid transfer was confirmed using the distinct ITS-RFLP types of the isolates and DNA hybridization using probes specific to the transferred plasmid. The transconjugant of 2-8-27 which had gained a plasmid was found in one replicate after 2 years of the most contaminated soil but comprised more than 50% of the isolates. A similar type appeared in a separate replicate of the most contaminated soil after 3 years and persisted in both of these soils until the final sampling after 4 years. After 2 years isolates were recovered from four of the soil replicates with the chromosomal type of 2-8-27 which appeared to have lost one plasmid, but these were not recovered subsequently.

Isolate 2-8-27 was among the isolates most sensitive to Zn in laboratory assays, whereas isolate 7-13-1 showed greater zinc tolerance. Acquisition of the plasmid conferred enhanced Zn tolerance to the recipients, but transconjugant isolates were not as metal tolerant as 7-13-1, the putative donor. Laboratory matings between 2-8-27 and 7-13-1 in the presence of Zn resulted in the conjugal transfer of the same small plasmid from 7-13-1 to isolate 2-8-27 and the transconjugant had enhanced metal tolerance. Our results show that transfer of naturally-occurring plasmids among rhizobial strains is stimulated by increased metal concentrations in soil. We further demonstrate that the transfer of naturally-occurring plasmids is important in conferring enhanced tolerance to elevated zinc concentrations in rhizobia.

© 2007 Elsevier Ltd. All rights reserved.

Keywords: Conjugation; Zinc; Metal toxicity; Sewage sludge; Microbial ecology

1. Introduction

The sensitivity of *Rhizobium* to heavy metals in soil is well established (Giller et al., 1989, 1998). Toxic effects of heavy metals are a cause for concern in agricultural soils which become contaminated as heavy metals accumulate in soils which receive repeated additions of sewage sludges

*Corresponding author. Plant Production Systems, Department of Plant Sciences, Wageningen University, P.O. Box 430, 6700, Building No. 537, Haarweg 333, AK Wageningen, The Netherlands.
Tel.: +31 317 485818; fax: +31 317 484892.

E-mail address: Ken.Giller@wur.nl (K.E. Giller).

and remain there indefinitely (McGrath, 1987). Reductions in the population size of *Rhizobium leguminosarum* bv. *trifolii*, and loss of nitrogen fixation in white clover (*Trifolium repens* L.) were observed in long-term experiments in Europe (McGrath et al., 1988; Giller et al., 1989; Chaudri et al., 1993). This evidence has contributed to revisions of legislation for environmental protection of agricultural soils in the UK (MAFF/DoE, 1993). Subsequently, several studies have confirmed these results, for instance in soils from Belgium (Broos et al., 2004) and New Zealand (Horswell et al., 2003).

Lakzian et al. (2002) described the populations of *R. leguminosarum* bv. *viciae* surviving in metal-contaminated soils at Braunschweig which had received a range of sewage sludge treatments between 1980 and 1990. Across the gradient of Zn concentrations which increased from 50 to 400 mg kg⁻¹, numbers of *R. leguminosarum* bv. *viciae* decreased from 10⁵ to 10–10² cells g⁻¹ soil. Isolates were characterized according to their plasmid profiles and restriction fragment length polymorphisms (RFLPs) of the intergenic spacer (ITS) region between the 16SrRNA and 23SrRNA genes (Laguette et al., 1996). Isolates with a common ITS–RFLP profile were most abundant in all plots examined, but the number of large (100–850 kb) plasmids in isolates with this ITS–RFLP profile varied from three or four in the uncontaminated control plots to eight or nine in the most contaminated plots (Lakzian et al., 2002). The isolates with eight plasmids dominated in the most contaminated plots comprising more than 75% of the population and these were more tolerant to Zn in *in vitro* tests on plates or in buffered liquid media (Lakzian et al., 2002).

These observations led us to hypothesize that plasmid transfer had occurred in the field soils which had led to rhizobial isolates acquiring large numbers of plasmids, and that one or more of these plasmids conferred enhanced tolerance to zinc concentrations. To test this hypothesis experimentally, soils sampled from the field in Braunschweig were sterilized by γ -irradiation and inoculated with equal numbers of four isolates taken from the control soils and three isolates taken from the metal contaminated soils. Numbers of rhizobia were counted, and rhizobia were re-isolated after 1, 2, 3 and 4 years of incubation. The rhizobial isolates were characterized to investigate: (1) which types dominated in the soils with or without heavy metal stress (i.e. to test whether there was a competitive/selective advantage of having many plasmids) and (2) whether transfer of specific plasmids was found under conditions of heavy metal stress.

2. Material and methods

2.1. Soil samples and sterilization procedure

Four different treatments with different concentrations of heavy metals were selected from the Old Arable Soils field experiment at the Federal Research Centre for

Agriculture (FAL), Braunschweig, of north east Germany. The experimental site had received a range of sewage sludge treatments between 1980 and 1990 and was described in detail elsewhere (Chaudri et al., 1993; Lakzian et al., 2002). The plots sampled were a control plot which had received 180 kg N ha⁻¹ y⁻¹ (Plots 2–8), and plots that received 100 m³ sewage sludge ha⁻¹ y⁻¹ (Plots 4–23), 300 m³ sewage sludge ha⁻¹ y⁻¹ (Plots 6–2) and 300 m³ metal-amended sewage sludge ha⁻¹ y⁻¹ (Plots 7–13). Total zinc concentrations in the soils estimated by atomic absorption spectrometry after Aqua-Regia digestion were 54, 104, 208 and 340 mg kg⁻¹ soil, respectively (Lakzian et al., 2002). Soil samples (3 kg) from each treatment were placed into plastic bags, sterilized by gamma irradiation (50 k Gy) and each was subdivided into three subsamples of 1 kg. The success of the soil sterilization procedure in eliminating rhizobia was checked by inoculation of plants of hairy tare (*Vicia hirsuta* L.) with suspensions of the sterilized soil samples in test tubes containing plant growth media (Lakzian, 1998). Soil suspensions were also plated on Luria-B and tryptone-yeast (TY) (Berlinger, 1974) plates to check for survival of other microorganisms. Colonies (mainly actinomycetes) were found only in the lowest dilution. This was not considered to be a problem for the experiment as *R. leguminosarum* bv. *viciae* were shown to be absent using the plant tests. Care was taken to avoid cross-contamination between treatments during the experiment, but the bags containing the soils were not sealed which presumably allowed colonization by other microorganisms.

2.2. Soil inoculation

Seven different isolates of *R. leguminosarum* bv. *viciae* were selected for inoculation of the sterilized soil samples (four different isolates from the control plot (isolates 2-8-1, 2-8-11, 2-8-24 and 2-8-27) and three different isolates from the 300 m³ metal-amended sewage sludge ha⁻¹ y⁻¹ treatment (isolates 7-13-1, 7-13-34 and 7-13-45)). All seven isolates had different plasmid profiles (Fig. 1) and were from different ITS groups (Lakzian et al., 2002). Selected isolates of *R. leguminosarum* bv. *viciae* were grown on TY plates containing Congo Red and 10 ml aliquots of TY broth were inoculated with each isolate. Incubation was on an orbital shaker at 150 rpm min⁻¹ and 28 °C for 48 h. Ten ml aliquots of rhizobial culture were transferred to sterile universal tubes and centrifuged at 500 g for 10 min. The washing procedure was repeated twice and the washed cells were resuspended in 10 ml of sterile water. The viable cell count was estimated using serial dilutions with replicate drops on yeast-mannitol (YM) plates (Miles and Misra, 1938) and the soil samples were all inoculated with 7 × 10⁵ rhizobial cells g⁻¹ soil (10⁵ rhizobial cells of each isolate). Soil samples were incubated at 25 °C in the dark in loosely tied plastic bags to allow gas exchange but prevent excessive moisture loss. The soils were adjusted to 50% water holding capacity at the start of the experiment and at

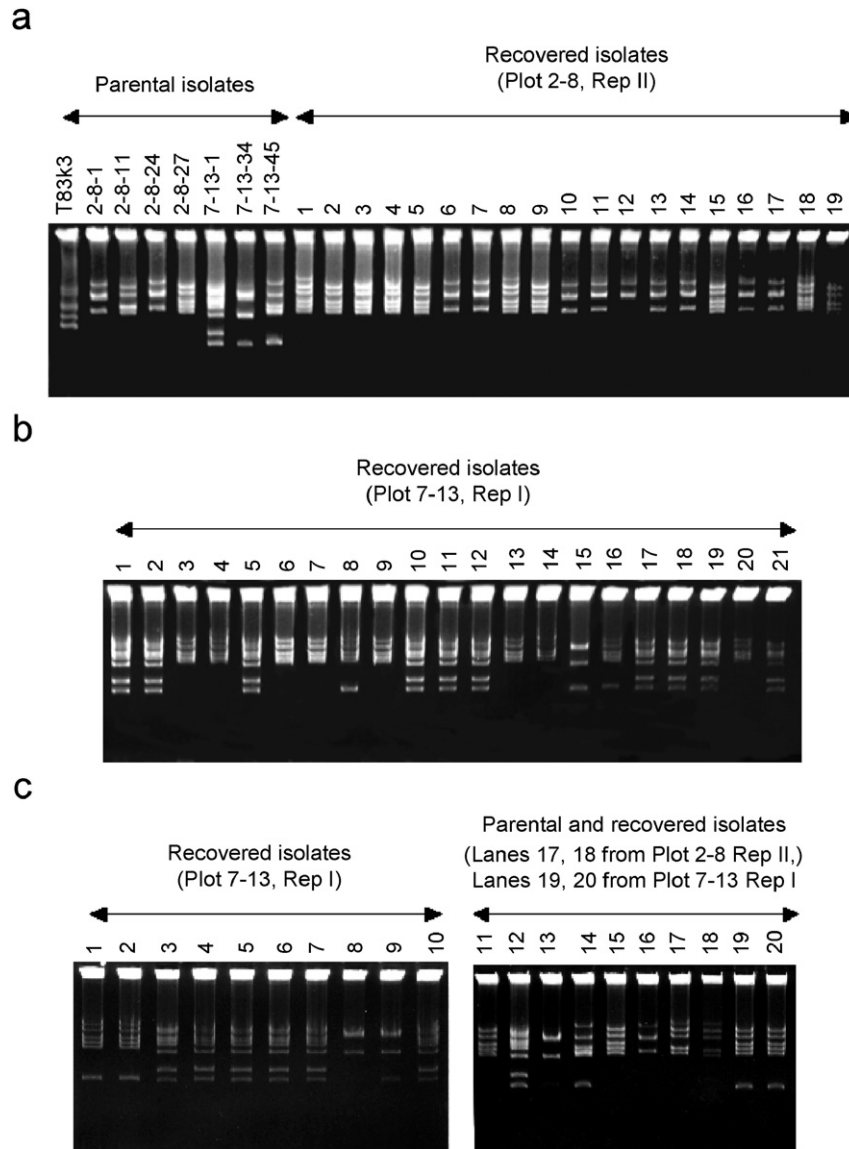


Fig. 1. Plasmid profiles of parental and recovered isolates of *R. leguminosarum* bv. *viciae* from the incubated soil samples. (a) Plasmid profiles of the seven parental isolates and 19 isolates from the control plot $180 \text{ kg N ha}^{-1} \text{ y}^{-1}$ recovered after 1 year. The isolate in lane 12 represented a new plasmid profile in the control plot which resembled parental isolate 2-8-1 with one plasmid missing (2-8-1(-1)). (b) Plasmid profiles of 21 recovered isolates from the 300 m^3 metal amended sewage sludge $\text{ha}^{-1} \text{ y}^{-1}$ after 1 year. Isolates in lanes 8 and 16 represented a new plasmid profile from the 300 m^3 metal-amended sewage sludge treatment which resembled 2-8-27 with one extra plasmid (2-8-27(+1)). (c) Plasmid profiles of isolates recovered after 2 years and parental isolates. Isolates in lanes 1, 2, 19 and 20 represent the new plasmid profile 2-8-27(+1). The isolate in lane 8 represents 7-13-34 which had lost a plasmid 7-13-34(-1). Isolates in lanes 17 and 18 represent 2-8-27 which had lost a plasmid 2-8-27(-1). The parental isolates shown are: 2-8-27, lanes 11 and 15; 7-13-1, lane 12; 7-13-34, lane 13; 7-13-45, lane 14; and 2-8-1, lane 16.

four week intervals thereafter although water loss was minimal.

2.3. Population counts and recovery of isolates from the incubated soil samples

After 1, 2, 3 and 4 years of incubation, numbers of *R. leguminosarum* bv. *viciae* were estimated by the most probable number (MPN) plant infection method (Vincent, 1970). Hairy vetch (*V. hirsuta* L.) seeds were surface sterilized with concentrated sulphuric acid for 2 min and

rapidly washed 10 times with sterile deionized water. The seeds were germinated on Petri dishes containing 1% agar. Two days later, 10–20 mm long seedlings were transferred to tubes containing 15 ml Hewitt's 'minus N' solution and agar slopes (Hewitt, 1952). Ten-fold serial dilutions of soil were prepared by shaking 10 g of soil in 90 ml sterilized distilled water, and serial dilutions up to 10^{-8} prepared (1 ml in 9 ml sterilized water). Triplicate plant infection tubes were inoculated with 1 ml aliquots of each dilution. Tubes were placed in a controlled environment cabinet with 14 h light, and day and night temperatures of 21 and

16 °C, respectively. Plants were examined for nodulation after three and five weeks. The numbers of *R. leguminosarum* bv. *viciae* were estimated using the MPNES computer program (Woomer et al., 1990). Rhizobial isolates were recovered from the 12 soil samples (approximately 20 isolates from each replicate) by isolation from root nodules of *Vicia hirsuta*. Nodules were surface-sterilized by immersing in 3% Na hypochlorite for 3 min and washing five times with sterile deionized water. The nodules were then squashed in a drop of sterilized water onto the surface of yeast-extract mannitol (YEM) agar in Petri dishes and incubated at 28 °C. Isolates were streaked out on YEM plates and a single colony picked to avoid problems of mixed isolates. The isolates were typed on the basis of their plasmid profiles to assess any changes in the population structure of *R. leguminosarum* bv. *viciae* isolates introduced to the sterilized soil samples. At the sampling after 2 years 40 additional isolates were made from Replicate I of the 300 m³ metal-amended sewage sludge ha⁻¹y⁻¹ (Plots 7–13).

2.4. Strain typing using plasmid profiles

Plasmid profiles of *Rhizobium* strains were visualized by in situ lysis on Eckhardt (1978) gels using the procedure of Hirsch et al. (1980). One lane was loaded with the *R. leguminosarum* bv. *viciae* strain T83K3 which has six plasmids of 480, 440, 300, 255, 210 and 155 kb (Wang et al., 1986).

2.5. DNA isolation, library construction and characterization

Total DNA was isolated from 5 ml of cell culture using the method described by Wilson (1987). The DNA was resuspended in TE buffer at a final concentration of approximately 200 ng µl⁻¹ and stored at 4 °C. DNA for polymerase chain reaction (PCR) amplification was prepared by diluting the stock DNA samples with sterile water to a final concentration of 10 ng µl⁻¹. The diluted stocks were then stored at -20 °C.

Total DNA (5 mg) isolated from *R. leguminosarum* bv. *viciae* strain AL7-31-1 was partially digested with *Sau3AI*. The partially digested DNA was then used to prepare a genomic DNA library in the bacteriophage vector Lambda GEM-12 (Promega) as per the manufacturers' instructions. The library was estimated to contain 12,000 pfu ml⁻¹. Restriction analysis of DNA isolated from 10 randomly selected lambda clones showed that each of the clones contained different insert sequences that ranged in size from 9 to 18 kb. The average insert size was approximately 16 kb (Lakzian, 1998).

To identify plasmid-specific probes, 35 DNA inserts isolated from randomly selected lambda clones were sequentially hybridized with plasmid DNA from isolates 2-8-1 and 7-31-1 which had been separated on Eckhardt gels as described above and transferred to Hybond N⁺

(Amersham) hybridization membrane. Efficient transfer of high molecular weight plasmid DNA to the Hybond N⁺ membrane was confirmed by hybridization of a 273-bp *Sall* internal fragment from the *nifH* gene of *Rhizobium etli* CFN-42 (Anyango et al., 1995).

2.6. PCR amplifications of intergenic spacer (ITS) regions

The PCR primers used to amplify the ITS region which separates the 16S and 23S rRNA genes were the forward primer C (5'-GGCTGGATCACCTCCTTTCT-3') corresponding to the 3' end of 16S gene, and the reverse primer D (5'-CCGGGTTTCCCCATTTCGG-3') corresponding to the 5' region of the 23S gene (Laguerre et al., 1996). The PCR reactions were carried out in 50 µl reaction volumes containing 20–30 ng of template DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 250 mM dNTP's, 0.3 mM of each primer, 1.75 mM MgCl₂ and 0.5 units of *Taq* DNA polymerase (Promega or Gibco BRL, UK) using a Perkin-Elmer thermal cycler (GeneAmp PCR System 9600) programmed as follows: initial denaturation of DNA at 94 °C for 4 min followed by 32 cycles of 30 s denaturation at 94 °C, 30 s primer annealing at 58 °C and 1 min 30 s DNA synthesis at 72 °C. The final cycle of DNA synthesis was allowed to proceed for a further 5 min before the tubes were cooled to 4 °C. The amplification products were analysed by gel electrophoresis in 1.5% agarose gels using Tris-borate-EDTA (TBE) buffer and visualized by ethidium bromide staining under UV light.

2.7. Restriction analysis and sequencing of PCR products

PCR products representing the entire ITS region were digested separately with the restriction endonuclease *HhaI* in accordance with the manufacturer's (Promega) recommended procedures. The digestion products were separated by electrophoresis on 2.5–3.5% agarose gels in TBE buffer and visualized by ethidium bromide staining under UV light.

2.8. Conjugal matings in liquid media

The donor (7-13-1) and recipient (2-8-27) strains were grown overnight in TY broth, diluted 1/10, then incubated for a further 2–3 h. The cells were then mixed and pelleted together, washed in 10 mM phosphate buffer and resuspended to a cell density of approximately 5 × 10⁵ cfu ml⁻¹ in 25 ml of PA medium containing 0 or 20 mg l⁻¹ of added Zn (as ZnSO₄) in 250 ml Erlenmeyer flasks. The cells were then incubated at 28 °C for 8 h. Each mating was carried out in triplicate and controls of donor and recipient alone were used to check the relative growth rates. Total cell counts of the control incubations and each mating were determined by plating serial dilutions from each mating on TY agar plates. Donor and putative transconjugant selection was carried out by duplicate streaking of randomly picked colonies onto freshly made TY plates

with and without Zn (60 mg l^{-1} added as ZnSO_4). Colonies which grew on both plates were then incubated overnight in TY broth and used for ITS analysis to determine the chromosomal type. The putative transconjugants (i.e. Zn-tolerant colonies with strain 2-8-27 ITS-RFLPs) were colony blotted and hybridized with a probe specific to the small plasmid of strain 7-13-1. Selected transconjugants were used for total DNA blotting and Eckhardt gel analysis.

2.9. Metal tolerance tests

The degree of zinc tolerance of isolates was assessed on TY plates to which varying concentrations of analytical grade ZnSO_4 were added. Isolates were grown in TY broth at 28°C for two days, washed twice in sterile deionized water, and three replicate drops were inoculated onto three replicate TY plates at each concentration.

3. Results

3.1. Changes in population size of *R. leguminosarum* bv. *viciae* in soil samples

The soil sterilization procedure proved successful as no nodules were found on any hairy tare plants grown on any of the soil samples, whereas the population of *R. leguminosarum* bv. *viciae* in the control plots before sterilization ranged from 1.4×10^4 to $8 \times 10^4 \text{ cells g}^{-1}$ soil (Lakzian et al., 2002). Other studies had shown that numbers of inoculated rhizobia decrease rapidly if soil samples are inoculated with high cell numbers, above commonly found rhizobial population sizes of 10^3 – 10^5 (Giller et al., 1993). For this reason, the cell numbers used for inoculation were calculated to be similar to the original size of the populations found in the soils, and all the soil samples were initially inoculated with $7 \times 10^5 \text{ cells g}^{-1}$ soil. The number of *R. leguminosarum* bv. *viciae* cells had increased after 1 year incubation to between 1.4×10^6 and $4 \times 10^7 \text{ cells g}^{-1}$ soil in all of the treatments except the most contaminated soil (300 m^3 metal-amended sewage sludge $\text{ha}^{-1} \text{ y}^{-1}$; Fig. 2). After 2 years incubation the numbers remained around 6×10^6 to $1 \times 10^7 \text{ cells g}^{-1}$ in all but the most contaminated soil in which the rhizobial numbers were significantly decreased to $1.2 \times 10^5 \text{ cells g}^{-1}$. Subsequently, rhizobial numbers stabilized around $2.3 \times 10^5 \text{ cells g}^{-1}$ in the three less contaminated soils, but progressively decreased in the most contaminated soil to $<30 \text{ cells g}^{-1}$ after 4 years.

3.2. Recovery of introduced isolates from soil samples after 1 year

In total, 216 isolates of *R. leguminosarum* bv. *viciae* were recovered from incubated soil samples after 1 year of incubation (Table 1a). Their plasmid profiles were compared with the plasmid profiles of the seven parental

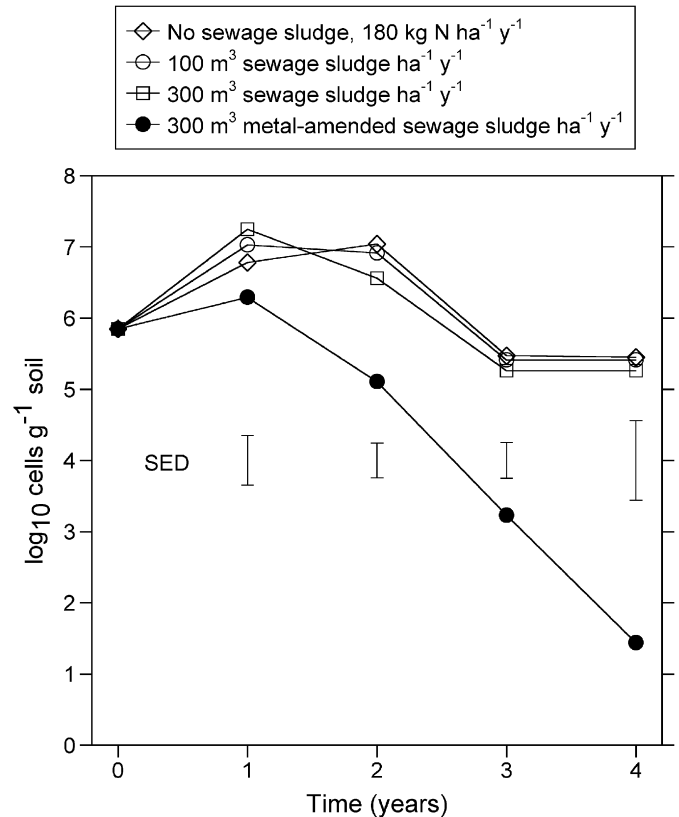


Fig. 2. Survival of *R. leguminosarum* bv. *viciae* in the soils over 4 years of incubation. The vertical bars are the pooled standard error of the difference (SED) of the means at each sampling time.

isolates. In the control soil samples from the $180 \text{ kg N ha}^{-1} \text{ y}^{-1}$ treatment (Zn concentration 50 mg kg^{-1}), three of the original plasmid profile types, 2-8-1, 2-8-24 and 2-8-27, were recovered. Isolate 2-8-27 was dominant in all three replicates, isolate 2-8-1 was found in only one of the replicate plots and isolate 2-8-24 was found in the other two replicates. One new plasmid profile type was found in one of the replicates which was similar to isolate 2-8-1, but had only two instead of three plasmid bands and this was named 2-8-1(-1) (Fig. 1a). In the 100 m^3 sewage sludge $\text{ha}^{-1} \text{ y}^{-1}$ treatment (Zn concentration 100 mg kg^{-1}) and the 300 m^3 sewage sludge $\text{ha}^{-1} \text{ y}^{-1}$ treatment (Zn concentration 200 mg kg^{-1}), the same three isolates were recovered and again isolate 2-8-27 was the dominant type in most of the plots. In one replicate of the treatment containing 200 mg kg^{-1} Zn, one new plasmid profile type (named 2-8-27(+1)) was found which had five plasmids similar in size with those in isolate 2-8-27 and one extra plasmid which was similar in size to the smallest plasmid in isolates 7-13-1, 7-13-34 and 7-13-45. In the 300 m^3 metal-amended sewage sludge $\text{ha}^{-1} \text{ y}^{-1}$ treatment (Zn concentration 340 mg kg^{-1}), five of the seven parental isolates were recovered. In two of the replicates, types originally isolated from the metal-contaminated plot (Plots 7–13) were re-isolated, but 2-8-27 was still the

Table 1

Recovery of parental isolates of *Rhizobium leguminosarum* bv. *viciae* and isolates with new plasmid profiles from different treatments of incubated soil samples after incubation for (a) 1 year, (b) 2 years, (c) 3 years and (d) 4 years

Number of isolates recovered from each soil												
Treatment	Control Plot:180 kg N ha ⁻¹ y ⁻¹			100 m ³ sewage sludge ha ⁻¹ y ⁻¹			300 m ³ sewage sludge ha ⁻¹ y ⁻¹			300 m ³ metal amended sewage sludge ha ⁻¹ y ⁻¹		
Replicate	I	II	III	I	II	III	I	II	III	I	II	III
(a) 1 year												
2-8-1	3	—	—	1	—	—	—	1	2	—	—	2
2-8-11	—	—	—	—	—	—	—	—	—	—	—	—
2-8-24	—	8	1	4	10	—	3	—	—	—	6	—
2-8-27	17	10	20	15	6	—	17	17	15	8	14	19
7-13-1	—	—	—	—	—	—	—	—	—	10	1	—
7-13-34	—	—	—	—	—	—	—	—	—	2	—	—
7-13-45	—	—	—	—	—	—	—	—	—	—	—	—
New isolates	—	1 ^a	—	—	—	—	—	1 ^b	—	2 ^b	—	—
Total isolates	20	19	21	20	16	^c	20	19	17	22	21	21
(b) 2 years												
2-8-1	2	—	1	14	12	6	—	—	—	—	—	—
2-8-11	—	—	—	—	—	—	—	—	—	—	—	—
2-8-24	—	—	—	—	—	—	—	—	—	—	—	—
2-8-27	18	18	19	6	7	14	20	20	19	1	18	19
7-13-1	—	—	—	—	—	—	—	—	—	25	1	1
7-13-34	—	—	—	—	—	—	—	—	—	3	—	—
7-13-45	—	—	—	—	—	—	—	—	—	—	—	—
New isolates	—	2 ^d	—	—	1 ^d	—	—	—	—	30 ^{b1e}	1 ^d	—
Total isolates	20	20	20	20	20	20	20	20	19	60 ^f	20	20
(c) 3 years												
2-8-1	9	1	3	9	6	6	1	—	—	—	—	—
2-8-11	—	—	—	—	—	—	—	—	—	—	—	—
2-8-24	—	3	1	—	—	—	—	—	—	—	—	—
2-8-27	13	17	16	13	11	13	14	9	16	4	9	8
7-13-1	—	—	—	—	—	3	6	8	3	9	11	13
7-13-34	—	—	—	—	—	—	—	—	—	—	—	—
7-13-45	—	—	—	—	—	—	—	3	—	3	—	—
New isolates	—	—	—	—	—	—	—	—	—	7 ^b	2 ^b	—
Total isolates	22	21	20	22	17	22	21	21	19	23	22	22
(d) 4 years												
2-8-1	5	1	3	9	2	1	1	1	—	—	—	1
2-8-11	—	—	—	—	—	—	—	—	—	—	—	—
2-8-24	—	3	3	2	1	4	—	—	—	—	—	—
2-8-27	15	16	16	8	17	14	11	10	11	2	6	8
7-13-1	—	—	—	—	—	—	7	9	7	12	11	9
7-13-34	—	—	—	—	—	—	—	—	—	—	—	—
7-13-45	—	—	—	—	—	—	2	—	—	1	—	3
New isolates	—	—	—	—	—	—	—	—	—	9 ^b	5 ^b	—
Total isolates	20	20	23	19	20	19	20	20	19	24	22	21

^a2-8-1(-1).

^b2-8-27(+1).

^cSamples lost during processing.

^d2-8-27(-1).

^e7-13-34(-1).

^fAn extra 40 isolates were characterized from this plot to check whether other types could be isolated.

most dominant type found in this treatment considering all replicates. In one replication of this treatment two isolates with new plasmid profiles were recovered with identical plasmid profiles to 2-8-27(+1) (Table 1a; Fig. 1b). By comparison of plasmid profiles it appeared that the smallest plasmid of isolates 7-13-1, 7-13-34 or 7-13-45 had been transferred to 2-8-27 to form the plasmid profile of the new isolates.

3.3. Recovery of introduced isolates from soil samples after 2 years

After a further year of incubation 2-8-27 was still the most abundant type isolated from most of the samples. In the control soil and the 100 m³ sewage sludge ha⁻¹ y⁻¹ treatment isolate 2-8-1 was also recovered, but this isolate was not recovered from the more contaminated plots. New

plasmid profiles were identified among isolates from the control and 100 m³ sewage sludge ha⁻¹y⁻¹ treatments; these were different from those found after 1 year and resembled parental isolate 2-8-27 with one plasmid missing (Table 1b; Fig. 1c). All of the isolates from the three replicates of the 300 m³ sewage sludge ha⁻¹y⁻¹ treatment had the plasmid profile of 2-8-27 (Table 1b). In the 300 m³ metal-amended sewage sludge ha⁻¹y⁻¹ treatment isolates with the plasmid profile of the parental isolate 7-13-1 were found in all replicates and were very abundant in Replicate I. Three isolates from this replicate had the plasmid profile of the parental isolate 7-13-34, and one isolate had a similar profile but lacking one plasmid (Fig. 1c, lane 8). The plasmid profile found in greatest abundance in this plot was the same as the new isolate with an extra plasmid which had been found after 1 year, plasmid profile 2-8-27(+1) (Table 1b, Fig. 1c).

3.4. Recovery of introduced isolates from soil samples after 3 and 4 years

After 3 and 4 years of incubation, 2-28-7 remained the dominant isolate from most of the treatments (Table 1c and d). Isolates 2-8-1 and 2-8-24 were both recovered from several soils, though most commonly in the least contaminated soils. As in the first 2 years, isolate 7-13-1 was isolated from all replicates of the most contaminated soil, but additionally from all replicates of the 300 m³ sewage sludge ha⁻¹y⁻¹ treatment. Isolate 7-13-5 was also recovered at low frequency from some replicates of the more contaminated soils. The new plasmid profile 2-8-27(+1) persisted in the third and fourth years in the two replicates of the most contaminated soil where it has previously been found, comprising more than a third of the recovered isolates in one replicate.

3.5. ITS-RFLP analysis of the isolates

The 2-8-27(+1) isolates which had new plasmid profile types were compared with the parental isolates using RFLP analysis of the ITS region between the 16S and 23S rRNA genes using two different restriction enzymes, *Hha*I and *Dde*I (Fig. 3). All of the isolates originally inoculated into the soil had distinct ITS-RFLPs and the ITS-RFLPs of the new isolates 2-8-27(+1) were confirmed to be the same as isolate 2-8-27 and clearly different from isolate 7-13-1.

3.6. Screening of the library for plasmid-specific probes

A DNA library was made from isolate 7-31-1 (Lakzian et al., 2002). Isolates 7-31-1 and 7-13-1 were very similar in that they had the same plasmid profile and belonged to the same ITS group although they were isolated from field plot replications of the 300 m³ metal-amended sewage sludge ha⁻¹y⁻¹. It seems likely that they represent separate isolations of the same 'strain' which was dominant in the metal-contaminated soils. Cloned Lambda DNA

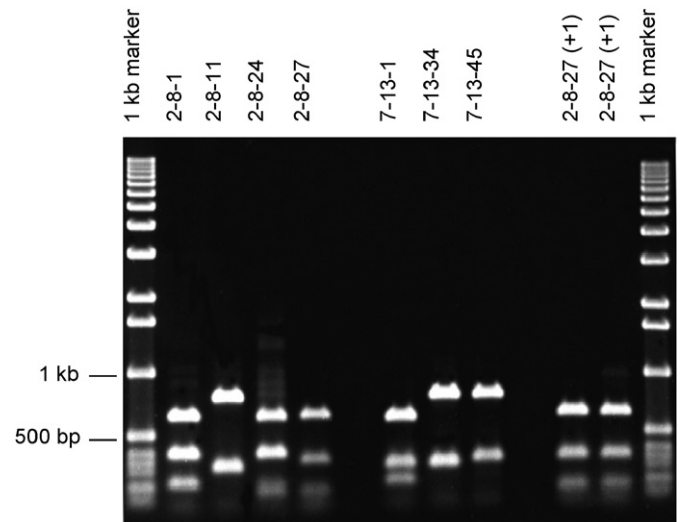


Fig. 3. RFLPs of the ITS region digested with *Hha*I of parental isolates and isolates of *R. leguminosarum* bv. *viciae* recovered from the incubated soil samples after 1 year.

fragments from the 7-31-1 library were used to probe Southern blots of plasmid DNA separated on Eckhardt gels from strains 7-31-1 and 2-8-1, a *R. leguminosarum* bv. *viciae* isolate from the control soil which had a very different plasmid profile. A large number of the cloned Lambda DNA's hybridized solely to chromosomal DNA. From 20 randomly selected lambda clones, two probes (λ AL3 and λ AL38) specifically hybridized with the smallest plasmid of isolate 7-31-1. Probes λ AL3 and λ AL38 did not hybridize to DNA of isolate 2-8-1 but λ AL38 also hybridized to DNA in the well of isolate 7-31-1 (Fig. 4). This hybridization to DNA in the well is most likely due to plasmid DNA not moving into the gel. Therefore, it seemed likely that Probes λ AL3 and λ AL38 were plasmid-specific probes. Other probes were identified which hybridized to chromosomal DNA of both isolates 7-31-1 and 2-8-1 but not to plasmid DNA, or which hybridized to plasmid and chromosomal DNA of isolate 7-31-1 but only hybridized to chromosomal DNA of isolate 2-8-1. The results of RFLP analysis of the plasmid specific probes λ AL3 and λ AL38 (using *Sac*I and *Sac*I+*Not*I) and sequence analysis revealed that they were overlapping DNA fragments (15 and 18 kb, respectively) of the same plasmid (data not shown). The two plasmid-specific probes hybridized to the smallest plasmid (named pAL7311 h) in isolate 7-31-1 (Lakzian, 1998). Both probes hybridized to the smallest plasmid of isolate 7-13-1 and to the new plasmid of each of the three new isolates 2-8-27(+1) (data not shown).

3.7. Characterization of the putative transconjugants

The two DNA sequences specific to the smallest plasmid of 7-13-1 were hybridized to total DNA digested with *Eco*RI, *Hind*III and *Sac*I from the three transconjugants and all seven isolates originally inoculated into the soils. Probe λ AL3 (Fig. 5) and probe λ AL38 (data not shown)

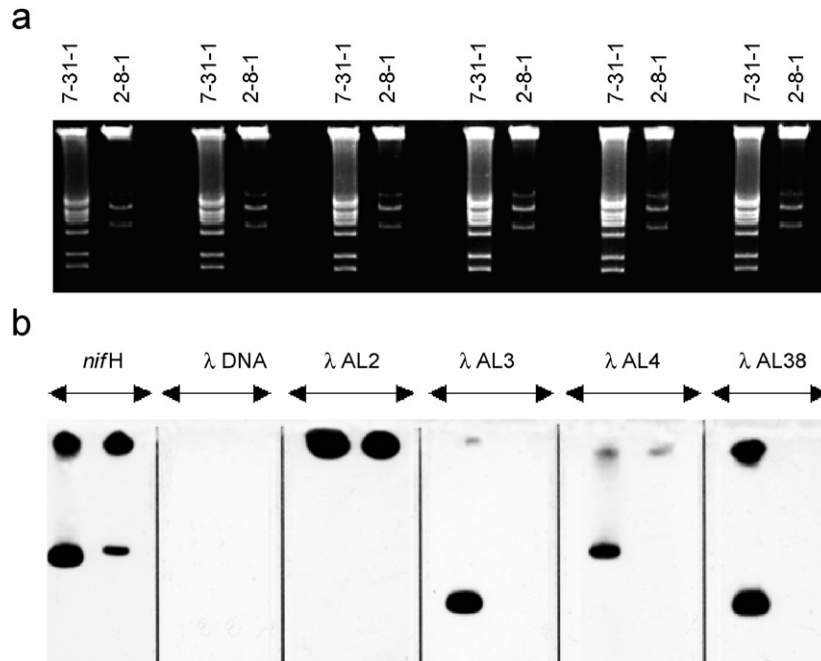


Fig. 4. Hybridization of cloned DNA fragments of *R. leguminosarum* bv. *viciae* to plasmid DNAs of two isolates 7-31-1 and 2-8-1. (a) Plasmid profiles of two isolates 7-31-1 and 2-8-1. (b) Hybridization of the above plasmid profiles with *nifH*, λ DNA and *R. leguminosarum* bv. *viciae* DNA probes λ AL2, λ AL3, λ AL4 and λ AL38.

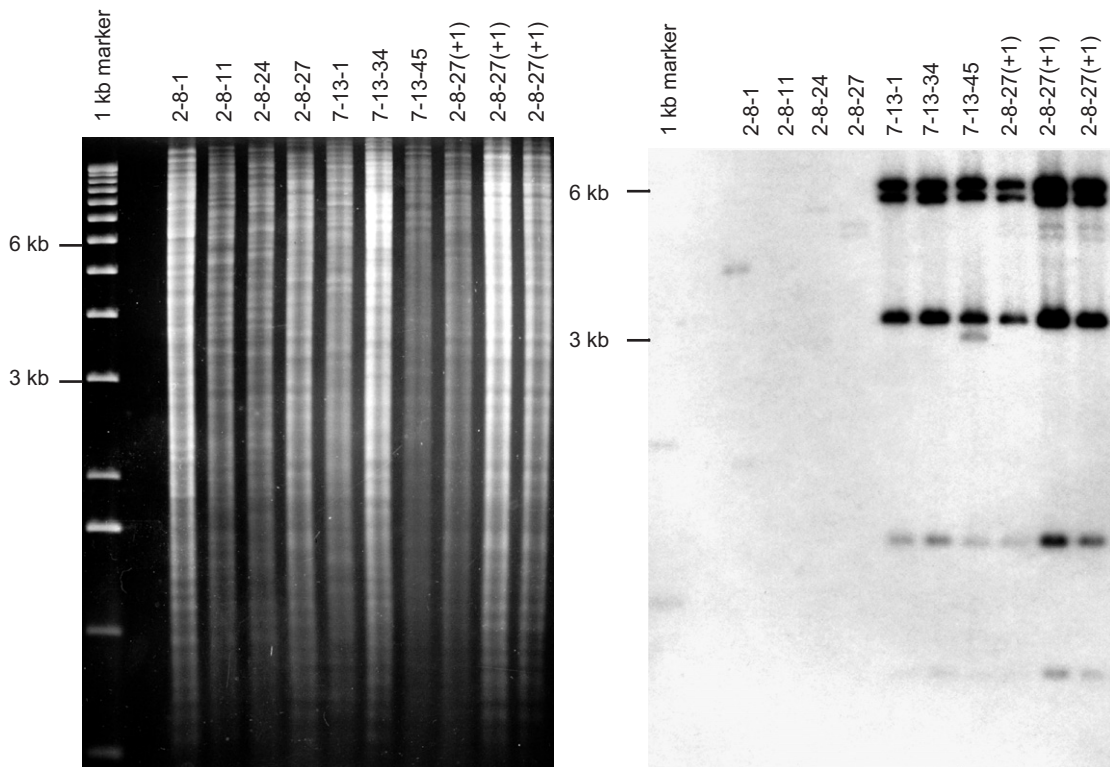


Fig. 5. (a) Total genomic DNAs digested with *EcoRI*, *HindIII* and *SacI* from the seven isolates originally inoculated into the soil (isolates 2-8-1, 2-8-11, 2-8-24, 2-8-27, 7-13-1, 7-13-34, 7-13-45) and three separate isolations of the isolates recovered after 1 year 2-8-27(+1). (b) Hybridization of plasmid-specific Probe λ AL3 to the total DNAs of the above isolates. Five strong bands can be seen which are presumably hybridizing to the digested DNA of the common plasmid. Weakly hybridizing bands in other lanes are presumably due to cross-hybridization. The common weak double bands of \sim 5 kb in the lanes of 2-8-27 and the three 2-8-27(+1) isolates confirm their relatedness.

Table 2

(a) Zn tolerance of parental and recovered isolates of *R. leguminosarum* bv. *viciae* from the incubated soils; (b) Zn tolerance of parental isolates 2-8-27 and 7-13-1 and a transconjugant 2-8-27:: pAL7311h produced by laboratory mating

Isolate	Growth on plates containing Zn (mg l ⁻¹) ^a					
	35	40	45	50	60	70
(a)						
<i>Parental isolates</i>						
2-8-1	+++	+++	+++	++-	+--	---
2-8-11	+++	+++	+++	++/--	---	---
2-8-24	+++	+++	+++	+--	---	---
2-8-27	+++	+++	+++	++/--	---	---
7-13-1	+++	+++	+++	+++	+++	++-
7-13-34	+++	+++	+++	+++	+++	++-
7-13-45	+++	+++	+++	+++	+++	++-
<i>Recovered isolates</i> ^b						
2-8-1-I	+++	+++	+++	++/--	+--	---
2-8-1-II	+++	+++	+++	++/--	+--	---
2-8-1-III	+++	+++	+++	++/--	+--	---
7-13-1-I	+++	+++	+++	+++	++/--	---
7-13-1-II	+++	+++	+++	+++	++/--	---
7-13-1-III	+++	+++	+++	+++	++/--	---
2-8-27(+1)	+++	+++	+++	+++	+++/-	---
2-8-27(+1)	+++	+++	+++	+++	+++/-	---
2-8-1(-1)	+++	+++/-	+--	---	---	---
	30	40	50	60	70	100
(b)						
2-8-27	++-	+--	---	---	---	---
2-8-27:: pAL7311h	+++	+++	+++	++/--	+--	---
7-13-1	+++	+++	+++	+++	+++	++-

^aThree replicate plates were used at each dilution: +, growth; +/-, poor growth; -, no growth on each plate.

^bIsolates of 2-8-1 recovered from Replicates I–III of the control soil and isolates of 7-13-1 recovered from I to III of the most contaminated soil.

showed five hybridizing bands to the digested DNA of three of the inoculant strains (7-13-1, 7-13-34 and 7-13-45) and the three transconjugant isolates. The RFLP patterns were identical for each hybridizing isolate indicating that all carried the same plasmid. No hybridization was initially found to isolate 2-8-27. However, by increasing the autoradiograph exposure time to 2–3 days, some common bands (weak hybridization to fragments of ~5 kb) between isolates 2-8-27 and 2-8-27(+1) were found, confirming their relatedness.

Initial toxicity tests of a range of Braunschweig isolates showed that 7-13-1 was the most Zn-tolerant isolate from these soils, whilst 2-8-27 was one of the least Zn-tolerant (Lakzian et al., 2002). Further toxicity tests were carried out to compare the Zn-tolerance of one of the transconjugants isolated from the soil inoculation experiment, 2-8-27(+1) with the recipient strain 2-8-27, and the donor strain, 7-13-1. These data confirmed the original findings of Lakzian et al. (2002) and showed that the transconjugant 2-8-27(+1) was less tolerant than 7-13-1 but more tolerant to Zn than the parental type 2-8-27 (Table 2). The recovered isolates of strain 7-13-1 (denoted as 7-13-1 I, II and III in Table 2a) did not show quite the same degree of tolerance as the original strain, though the differences in tolerance were small.

In light of the above results, we decided to test whether plasmids could readily be mobilized from 7-13-1 to 2-8-27 in suspension culture. The matings were carried out with or without Zn to test whether this had an observable effect on the rate of conjugation. The growth medium and Zn concentrations used were determined by a set of preliminary experiments which had shown that the growth rates of the donor and recipient were not significantly different from each other under these growth conditions. The use of richer media such as TY or yeast mannitol (YM) broth resulted in much faster growth rates for strain 2-8-27 whilst higher concentrations of added Zn significantly reduced the growth rate of strain 2-8-27 to below that of strain 7-13-1 (data not shown). Zinc concentrations of 60 mg l⁻¹ effectively stopped the growth of 2-8-27 for 5–7 days.

Fifty individual colonies from the lowest dilution where single colonies could be separated (10⁻²) of each replicate of each experimental mating (300 in all) were streaked in duplicate onto TY plates containing either no added zinc or 60 mg l⁻¹ of added zinc to select for the donor strain and any transconjugants. RFLP analysis of PCR-amplified ITS regions and subsequent colony blot hybridization analysis using λ AL38, identified 19 putative transconjugants. All of these were isolated from the matings

containing Zn in the growth medium. Eckhardt gels of the putative transconjugants confirmed that they had plasmid profiles identical with the transconjugants found in the soil inoculation experiment. Subsequent hybridization analysis of total DNA from three randomly selected transconjugants probed with λ AL3 and λ AL38 supported our contention that the smallest plasmid from 7-13-1 had been conjugally transferred to 2-8-27 (data not shown).

4. Discussion

4.1. Survival of inoculated rhizobia

The increase in the population sizes of *R. leguminosarum* bv. *viciae* over the year incubation period from 7×10^5 cells g^{-1} soil to between 1.4×10^6 and 4.2×10^7 cells g^{-1} soil seen in all but the most contaminated soil (Fig. 2) was probably due to the lack of competition from other soil bacteria in the sterilized soil. The slow rate of decrease in numbers of rhizobia in the most contaminated soil may be partly due compensation for the loss of rhizobia sensitive to zinc by increases in numbers of rhizobial types more tolerant to Zn (Table 1). Other studies have found that the rate of decline in rhizobial numbers in metal contaminated soils can be remarkably slow, with periods of 1–2 years required for significant decreases in numbers to occur (Chaudri et al., 1992b). Broos et al. (2005) also showed sensitivity of rhizobia to zinc in the absence of the host legume. While rhizobial numbers stabilized at large numbers around 2.3×10^5 cells g^{-1} soil after 3 years of incubation in the three least contaminated soils (Fig. 2), the population continued to decline in the most contaminated soil to less than 30 cells g^{-1} soil after 4 years.

The dominance of plasmid profile type 2-8-27 in all of the treatments after 1 and 2 years of incubation was surprising. This type was represented by only a single isolation among 50 isolates in the control plot (Plots 2–8) and was included in this study due to its distinctive plasmid profile. In fact, 2-8-27 was the least tolerant to Zn in culture of all of the isolates tested (Lakzian et al., 2002), but it grew more rapidly in culture than the other isolates used to inoculate the soils. As rhizobial numbers increased substantially over the first year, the fast growth rate of 2-8-27 may have enabled it to colonize the soil more rapidly than the other isolates, but it is surprising that this type remained abundant in all of the treatments after even 4 years. Isolates 2-8-1 and 2-8-24 both carried only three plasmids and each represented roughly 10% of the isolates originally made from this plot (Lakzian et al., 2002). Both plasmid profile types were found sporadically across the treatments after 1 year, but plasmid profile type 2-8-1 was found mainly in the two least-contaminated soils after 2 years of incubation. Isolate 2-8-11 was not recovered from any of the incubated soils.

Isolates which originally came from the metal-contaminated soil were only re-isolated from the

most-contaminated soil after the first two years (Table 1a and b). Isolate 7-13-1 comprised 90% of the population in this treatment before soil sterilization but was the most abundant type isolated in only one of the replicates, and only one isolate of this type was found among the 42 isolates from the other two replicates. After 3 and 4 years isolate 7-13-1 was also found in soils from the 300 m³ sewage sludge ha⁻¹ y⁻¹ treatment (Table 1c and d) and isolate 7-13-45 was also recovered from some of the more contaminated soils. Simple plating on media containing zinc (Table 2) supported the conclusion that survival of isolates originally from the metal-contaminated soils is favoured by their greater metal tolerance. Presumably, these isolates from heavy metal contaminated soils (7-13-1 and 7-13-45) are poor competitors compared with isolates from the uncontaminated soils in the absence of heavy metal stress, as they were not re-isolated from the control soils (Table 1).

4.2. Plasmid loss

One isolate was recovered which appeared to have lost a single plasmid, although it is possible that the plasmid had integrated into the chromosome. It has been argued that carrying a large plasmid complement places greater energy demands on bacterial cells, so plasmids will tend to be lost unless there is an active selection pressure to maintain them (Shaw, 1987). The parental type 2-8-1 of the isolate which had lost a plasmid after 1 year itself carried only three plasmids and comprised about 10% of the *R. leguminosarum* bv. *viciae* population in the field plot from which it was isolated (Lakzian, 1998). This type (2-8-1) with a deleted plasmid was not re-isolated after 2 years, but two isolates of 2-8-27 which had lost a plasmid were identified, both in a replicate of the control plot and in the most contaminated soil, and one isolate of 7-13-34 which had lost a plasmid was found in the most contaminated soil. In the original populations in the field soils these two plasmid profile types both formed <2% of the field population in the soils from which they had been isolated (Lakzian, 1998). Under the incubation conditions where the inoculated rhizobia multiplied, opportunities for rapid growth may have been more important for survival than maintenance of this particular plasmid. Presumably only functions which were not essential to the survival or competitiveness of the rhizobia under these conditions were carried on the plasmids. Other studies in our laboratory have shown that loss of plasmids from strains of *R. leguminosarum* can result from repeated broth culture over 9 or 10 cycles (M. Berendsen; unpublished results).

4.3. Plasmid transfer

We have clear evidence for two separate occurrences of transfer of the same plasmid (pAL7311 h) from an isolate from metal-contaminated soil to an isolate with five plasmids from the uncontaminated soil. The identity of a

putative donor (Isolate 7-13-1) and recipient (Isolate 2-8-27) of the plasmid was indicated by their plasmid profiles and ITS–RFLPs (Fig. 1). The identity of isolate 2-8-27 as the recipient was confirmed as the 2-8-27(+1) isolates had the same ITS–RFLP pattern (Fig. 3) and shared weakly hybridizing bands in RFLP analysis of total DNA digests (Fig. 5).

The precise identity of the donor is less clear as Eckhardt gel hybridization and total DNA hybridization studies using the two plasmid-specific probes indicated that the smallest plasmid (which is the one presumed to have been transferred) is the same or very similar in 7-13-1, 7-13-34 and 7-13-45 (Fig. 5). Transfer of this plasmid was only detected under conditions of heavy metal stress, which suggested there was selection pressure for this plasmid, raising the question as to whether the plasmid conferred tolerance to heavy metals. As Zn is the metal thought to cause the toxic effects to rhizobia in the Braunschweig soils (Giller et al., 1998) we tested the tolerance of parental isolates and some of the recovered isolates. This indicated that the transconjugants which had gained a small plasmid had enhanced metal tolerance, but they were not as metal tolerant as the isolates which had originally come from the metal-contaminated soils (Table 2). Further support for the role of the small plasmid was gained from matings in liquid broth between the two parental types thought likely to be the recipient and donor of the small plasmid. Transfer of the plasmid was only detected when Zn was added to the liquid growth medium whereas De Rore et al. (1994) found no positive effect of Zn on frequency of conjugation over 4 h of a small plasmid encoding metal resistance genes. The positive effect of Zn observed here in matings conducted over 8 h could have been due to selection for the transconjugants in the matings conducted in the presence of Zn, rather than an increase in the frequency of conjugation.

Eckhardt gel analysis and total DNA hybridization with λ AL3 and λ AL38 suggested that the transconjugants identified were all identical to those found in the soil incubation experiment (data not shown). The transconjugants had a greater metal tolerance than the recipient type, but not as strong a metal tolerance as the donor (Table 2b), again similar in ranking to the results found in the soil incubation experiment. Results from such assays of metal tolerance on agar plates are hard to reproduce exactly as the results depend on the initial inoculum density and on the 'available' concentration of Zn in the media which might vary slightly as metals are progressively precipitated by slow reactions in the agar (Bird et al., 1985).

Kinkle and Schmidt (1991) found that the frequency of transfer of the symbiotic plasmid pJB5JI between strains of rhizobia was higher in sterile than non-sterile soils, and peak transconjugant frequencies occurred with an inoculum density of 10^7 cells g^{-1} soil. Conditions in this experiment were similar to the conditions which they found to

promote plasmid transfer. Addition of nutrients and heavy metals to soil samples significantly increased the frequency of conjugative transfer of plasmids carrying metal resistance genes in soil microcosms (Top et al., 1995; Bruins et al., 2000). It is possible that liberation of nutrients due to soil sterilization might have stimulated plasmid transfer in our study, though this effect is likely to be relatively short-lived over the first few weeks of this 2-year incubation study.

4.4. Survival of rhizobia in heavy metal contaminated soil

The most remarkable result of this study is the survival of rhizobia in the heavy metal contaminated soil which appear to be sensitive to heavy metals. The dominance of isolate 2-8-27 in all soils is hard to explain, although its fast rate of growth may have allowed it to colonize the soils rapidly. Soil sterilization results in liberation of substrates for microbial growth due to killing of the microbial biomass so that results may not be representative of what might occur in non-sterile soil. In preliminary competition experiments isolate 2-8-1 dominated when isolates were inoculated together in plant tests in equal numbers and appeared to be the most competitive for nodulation of *V. hirsuta* (data not presented). The toxicity of the metals in soil is demonstrated by the decline in rhizobial numbers which was observed in the most contaminated soil after 4 years. This decline in rhizobial numbers occurred despite the increase in dominance of isolates which originally came from the metal contaminated soil, and the transconjugants, which appear to have slightly more metal tolerance than the other isolates. Such slow rates of death of rhizobia in soils polluted with heavy metals at similar loadings to the soils studied here is a consistent feature across a number of studies (Giller et al., 1998), though the reasons for this are unclear. In laboratory assays Zn is clearly bacteriocidal, though the effects depend on the concentration and length of exposure (Chaudri et al., 1992a; Knight and McGrath, 1995; Broos et al., 2005). Soil is a highly oligotrophic environment (Williams, 1985) and it is possible that the rhizobia must actively grow and divide to be affected by the metal toxicity. Another possibility is that bacteria find refuge from heavy metal exposure within microsites of bacterial colonies or soil microaggregates.

It seems likely that the most metal contaminated soil will eventually become dominated by the strains originally isolated from the metal contaminated soils, or by the transconjugants. Even if this were to occur, the numbers of the metal-tolerant strains surviving after 4 years (<30 cells g^{-1} soil) were below the critical threshold (50 cells g^{-1} soil) normally considered to be necessary to ensure good nodulation in legumes without inoculation (Singleton and Tavares, 1986). It is thus likely that symbiotic nitrogen fixation by legumes would be impaired in soils with this moderate degree of heavy metal contamination even if the zinc tolerant rhizobia survived in low numbers.

Acknowledgements

This research was conducted with financial support from the EU-Environment Programme of DGXII. Amir Lakzian is grateful to the Government of Iran for a scholarship. We dedicate this paper to Phillip Murphy, an inspirational scientist who died unexpectedly and suddenly in 2005.

References

- Anyango, B., Wilson, K.J., Beynon, J.L., Giller, K.E., 1995. Diversity of rhizobia nodulating *Phaseolus vulgaris* L. in two Kenyan soils of contrasting pHs. *Applied and Environmental Microbiology* 61, 4016–4021.
- Beringer, J.E., 1974. R-factor transfer in *Rhizobium leguminosarum*. *Soil Biology & Biochemistry* 84, 188–198.
- Bird, N.P., Chambers, J.G., Leech, R.W., Cummins, D., 1985. A note on the use of metal species in microbiological tests involving growth media. *Journal of Applied Bacteriology* 59, 353–355.
- Broos, K., Uyttendaele, M., Mertens, J., Smolders, E., 2004. A survey of symbiotic nitrogen fixation by white clover grown on metal contaminated soils. *Soil Biology & Biochemistry* 36, 633–640.
- Broos, K., Beyens, H., Smolders, E., 2005. Survival of rhizobia in soil is sensitive to elevated zinc in the absence of the host plant. *Soil Biology & Biochemistry* 37, 573–579.
- Bruins, M.R., Kapil, S., Oehme, F.W., 2000. Microbial resistance to metals in the environment. *Ecotoxicology and Environmental Safety* 45, 198–207.
- Chaudri, A.M., McGrath, S.P., Giller, K.E., 1992a. Metal tolerance of isolates of *Rhizobium leguminosarum* biovar *trifolii* from soil contaminated by past applications of sewage sludge. *Soil Biology & Biochemistry* 24, 83–88.
- Chaudri, A.M., McGrath, S.P., Giller, K.E., 1992b. Survival of the indigenous population of *Rhizobium leguminosarum* biovar *trifolii* in soil spiked with Cd, Zn, Cu and Ni salts. *Soil Biology & Biochemistry* 24, 625–632.
- Chaudri, A.M., McGrath, S.P., Giller, K.E., Rietz, E., Sauerbeck, D., 1993. Enumeration of indigenous *Rhizobium leguminosarum* biovar *trifolii* in soils previously treated with metal-contaminated sewage sludge. *Soil Biology & Biochemistry* 25, 301–309.
- De Rore, H., Top, E., Houwen, F., Mergeay, M., Verstraete, W., 1994. Evolution of heavy metal resistant transconjugants in a soil environment with a concomitant selective pressure. *FEMS Microbiology Ecology* 14, 263–274.
- Eckhardt, T., 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. *Plasmid* 1, 584–588.
- Giller, K.E., McGrath, S.P., Hirsch, P.R., 1989. Absence of nitrogen fixation in clover grown on soil subject to long term contamination with heavy metals is due to survival of only ineffective *Rhizobium*. *Soil Biology & Biochemistry* 21, 841–848.
- Giller, K.E., Nussbaum, R., Chaudri, A.M., McGrath, S.P., 1993. *Rhizobium meliloti* is less sensitive to heavy-metal contamination in soil than *R. leguminosarum* bv. *trifolii* or *R. loti*. *Soil Biology & Biochemistry* 25, 273–278.
- Giller, K.E., Witter, E., McGrath, S.P., 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils—a review. *Soil Biology & Biochemistry* 30, 1389–1414.
- Hewitt, E.J., 1952. *Sand and Water Culture Methods used in the Study of Plant Nutrition*. Commonwealth Agricultural Bureaux, Farnham Royal Bucks.
- Hirsch, P.R., Van Montagu, M., Johnston, A.W.B., Brewin, N.J., Schell, J., 1980. Physical identification of bacteriocinogenic, nodulation and other plasmids in strains of *Rhizobium leguminosarum*. *Journal of General Microbiology* 120, 403–412.
- Horswell, J., Speir, T.W., van Schaik, A.P., 2003. Bio-indicators to assess impacts of heavy metals in land-applied sewage sludge. *Soil Biology & Biochemistry* 35, 1501–1505.
- Kinkle, B.K., Schmidt, E.L., 1991. Transfer of the pea symbiotic plasmid pJB5JI in nonsterile soil. *Applied and Environmental Microbiology* 57, 3264–3269.
- Knight, B., McGrath, S.P., 1995. A method to buffer the concentrations of free Zn and Cd ions using a cation exchange resin in bacterial toxicity studies. *Environmental Toxicology and Chemistry* 14, 2033–2039.
- Laguerre, G., Mavingui, P., Allard, M.-R., Charnay, M.-P., Louvrier, P., Mazurier, S.-I., Rigottier-Gois, L., Amarger, N., 1996. Typing 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. *Applied and Environmental Microbiology* 62, 2029–2036.
- Lakzian, A., 1998. Diversity and Metal Tolerance of *Rhizobium leguminosarum* bv. *viciae* in Soils Contaminated with Heavy Metals. Wye College, University of London, p. 169.
- Lakzian, A., Murphy, P., Turner, A., Beynon, J.L., Giller, K.E., 2002. *Rhizobium leguminosarum* bv. *viciae* populations in soils with increasing heavy metal contamination: abundance, plasmid profiles, diversity and metal tolerance. *Soil Biology & Biochemistry* 34, 519–529.
- MAFF/DoE, 1993. Review of the rules for sewage sludge application to agricultural land: soil fertility aspects of potentially toxic elements. Report of the Independent Scientific Committee, PB 1561. MAFF Publications, London.
- McGrath, S.P., 1987. Long-term metal transfers following applications of sewage sludge. In: Coughtrey, P.J., Martin, M.H., Unsworth, M.H. (Eds.), *Pollutant Transport and Fate in Ecosystems*. Blackwell Scientific Publications, Oxford, pp. 301–317.
- McGrath, S.P., Brookes, P.C., Giller, K.E., 1988. Effects of potentially toxic metals in soil derived from past applications of sewage sludge on nitrogen fixation by *Trifolium repens* L. *Soil Biology & Biochemistry* 20, 415–424.
- Miles, A.A., Misra, S.S., 1938. The estimation of the bactericidal power of blood. *Journal of Hygiene, Cambridge* 38, 732–748.
- Shaw, P.D., 1987. Plasmid ecology. In: Kosuge, T., Nester, E. (Eds.), *Plant–Microbe Interactions*. Macmillan, New York, pp. 3–39.
- Singleton, P.W., Tavares, J.S., 1986. Inoculation response of legumes in relation to the number and ineffectiveness of indigenous *Rhizobium* populations. *Applied and Environmental Microbiology* 51, 1013–1018.
- Top, E.M., De Rore, H., Collard, J.M., Gellens, V., Slobodkina, G., Verstraete, W., Mergeay, M., 1995. Retromobilization of heavy metal resistance genes in unpolluted and heavy metal polluted soil. *FEMS Microbiology Ecology* 18, 191–203.
- Vincent, J.M., 1970. *A Manual for the Practical Study of Root-Nodule Bacteria*. Blackwell, Oxford.
- Wang, C.L., Beringer, J.E., Hirsch, P.R., 1986. Host plant effects on hybrids of *Rhizobium leguminosarum* biovars *viciae* and *trifolii*. *Journal of General Microbiology* 132, 2063–2070.
- Williams, S.T., 1985. Oligotrophy in soil: fact or fiction? In: Fletcher, M., Floodgate, M. (Eds.), *Bacteria in their Natural Environments*. Special Publication of the Society for General Microbiology. Academic Press, New York, pp. 81–110.
- Wilson, K.J., 1987. Preparation of genomic DNA from bacteria. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (Eds.), *Current Protocols in Molecular Biology*. Greene Publishing and Wiley/Interscience, New York, pp. 241–245.
- Woomer, P., Bennet, J., Yost, R., 1990. Overcoming the inflexibility of most-probable number procedures. *Agronomy Journal* 82, 349–353.