***In situ* transfer of an exogenously isolated plasmid between *Pseudomonas* spp. in sugar beet rhizosphere**

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A method for studying plasmid transfer in the rhizosphere is described. This work demonstrates plasmid transfer in an unenclosed rhizosphere under field conditions. The donor (*Pseudomonas marginalis* 376N) and recipient (*Pseudomonas aureofaciens* 381R) bacteria and the conjugative mercury resistance plasmid (pQBR11) studied were all isolated from the bacterial community indigenous to sugar beet rhizosphere. Spontaneous nalidixic acid and rifampicin resistant mutants of these bacteria were used as donors and recipients of pQBR11 for *in situ* matings. Fresh field soil was mixed with donors and recipients to give a soil mating mix (SMM) which was placed underground on the surface of a sugar beet root storage organ. Plasmid transfer in the SMM was determined after 24 h at frequencies between 5.1×10^{-5} and 1.3×10^{-8} transconjugants per recipient. Higher transfer frequencies (1.3×10^{-2} to 1.7×10^{-6}) were recorded on the peel adjacent to the SMM. No transfer of mercury resistance was detected in SMM controls incubated at 20 °C *in vitro* or placed in soil at distances of more than 5 cm from plants.

Keywords: rhizosphere, plasmid transfer, soil bacteria, *Pseudomonas*

INTRODUCTION

The prospect of releasing genetically manipulated micro-organisms (GMMOs) into the environment has highlighted the need to evaluate the mechanisms and frequencies of natural gene transfer. Conjugative plasmids are an important means of gene transfer in soil and rhizosphere habitats (Trevors *et al.*, 1987), where the study of the behaviour of indigenous plasmids is of particular interest. As a result of the difficulties normally associated with working in the natural environment, many researchers have used simplified microcosms, laboratory strains or non-indigenous plasmids to model transfer. In aquatic systems, however, plasmid transfer has been studied in unenclosed natural systems using indigenous plasmids (Bale *et al.*, 1988; Fry & Day, 1990). Similar experiments are needed in terrestrial systems, to assess directly the natural activity of plasmids.

Other work in model or simulated systems typically describes low plasmid transfer frequencies in a range of soils. The transfer frequency can, however, be increased in the presence of roots, by nutrient amendment, or by

soil sterilization (van Elsas *et al.*, 1990; Top *et al.*, 1990; Stotzky, 1989; Klingmüller, 1991). These modifications increase the availability of nutrients or reduce competition from other microbiota. In this study we describe the use of the rhizosphere associated with sugar beet storage organs (the beet) to investigate natural plasmid transfer. These root structures can grow to over 3 kg and support substantially higher bacterial densities than the surrounding soil.

To enable realistic studies of plasmid transfer in the terrestrial environment it was considered essential that only indigenous plasmids and bacteria be used. Consequently, after isolation and characterization, isolates were reintroduced into the sugar beet rhizosphere at environmentally relevant densities to demonstrate and measure the frequency of *in situ* plasmid transfer.

METHODS

Bacterial strains, plasmids, culture media and enumeration.

The bacterial strains and plasmids used in this study are given in Table 1. All strains were stored at –70 °C and cultured on plate count agar (PCA; Oxoid CM463) at 28 °C amended with the appropriate antibiotics. Selective agents were included in media at the following concentrations unless otherwise stated; rifam-

Abbreviation: SMM, soil mating mix.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics*	Source or reference
<i>Pseudomonas putida</i> UWC1	Rif ^r	McClure <i>et al.</i> (1989)
<i>Pseudomonas aureofaciens</i> 381		This study
381R	Rif ^r	This study
373		This study
373N	Nal ^r	This study
373R	Rif ^r	This study
<i>Pseudomonas marginalis</i> 376		This study
376N	Nal ^r	This study
<i>Pseudomonas fluorescens</i> 366		This study
366R	Rif ^r	This study
391		This study
391N	Nal ^r	This study
391R	Rif ^r	This study
pQBR11†	Tra ⁺ , Hg ^r , 294 kb	This study

* Abbreviations used: Rif^r, rifampicin resistance; Nal^r, nalidixic acid resistance; Tra⁺, transfer-proficient conjugative plasmid; Hg^r, resistance to mercuric ions. Some other antibiotic resistant mutants of these strains also used (see Results and Discussion).

† Twenty-two other mercury resistance plasmids were also isolated and investigated in this study (see Results and Discussion).

picin (Sigma; 100 µg ml⁻¹), nalidixic acid (Sigma; 300 µg ml⁻¹) and HgCl₂ (BDH; 0.1 mM). Broth cultures were grown in nutrient broth (NB; Oxoid CM1) overnight in an orbital shaker (190 r.p.m.) at 28 °C. Tryptone Soya Broth Agar (TSBA; Tryptone Soya Broth, Oxoid CM129, and 1.5%, w/v, Oxoid Agar no. 1 L11) was used for filter plate matings and amended with cycloheximide (Sigma; 50 µg ml⁻¹) for the isolation of strains from the rhizosphere. Donors for *in situ* and soil matings were grown in NB amended with HgCl₂ (0.05 mM).

Bacteria were enumerated by dilution and spread plating for low dilutions or drop plated, three drops at 20 µl, for higher dilutions, on suitably amended PCA plates. All counts, expressed per gram, were corrected to dry weights (soil at 105 °C, peel at 85 °C). Plasmid transfer frequencies were calculated as the final transconjugant count divided by the final recipient count.

Crop management and definitions. The sugar beet were grown at the Oxford University Farm, Wytham, Oxford, UK, in a 10 m × 10 m plot (map reference SP473088). On 28 April 1992 sugar beet (*Beta vulgaris* var. Amethyst) pelleted seeds (Germains UK) were sown at 15 cm intervals. The pellets included the fungicides Thiram, Hymexazol and Methiocarb. An additional row was sown on 2 July 1992. The plot was fertilized at 0.15 kg m⁻² (N:P:K 13:13:20, Hydrofertilisers) and treated with a triazinone beet crop, contact-residual herbicide (Goltex; Bayer) and a contact-ingestion organochlorine insecticide (Gammacol; ICI) 5 d before sowing. The

soil at the site is described as an Evesham Series heavy clay (clay 53.5%, sand 24.9%, silt 21.6%, pH 7.7, with organic content 8.5%). Potted plants were prepared by digging up single field-grown plants with the surrounding root soil intact and placing them in 20 cm pots. These plants were kept outside and grown on for 2–3 weeks before use, and watered from below to maintain field capacity.

Fine peel, taken to represent the rhizoplane, was removed with a scalpel (approximately 40 mg cm⁻²) from the beet after clinging soil had been knocked away. Rhizosphere soil was defined as the soil adhering to and within 5 mm of the beet. This was sampled by paring soil back to within 5 mm of the beet and prising or gently scraping off the remaining soil. Fresh field soil was collected at a depth of 7–10 cm and at least 10 cm from the nearest plant.

Isolation of bacterial strains from the rhizosphere. Fine peelings of the beet were resuspended at 5% (w/v) in quarter-strength Ringer's solution (Oxoid BR52) and shaken (Luckham R100 shaker, 250 r.p.m., room temperature, 20 min) or homogenized (Waring Blender, 3 min on ice). Bacterial suspensions were diluted, spread onto TSBA and incubated for 96 h at 15 °C. Colonies were picked, purified and identified by analysis of methylated fatty acid profiles using the Microbial Identification System (MIS-MIDI, Newark, DE, USA) essentially as described by Thompson *et al.* (1993). Briefly, 50 mg cells from TSBA plates (incubated 24 h, 28 °C) were saponified using NaOH in methanol and then methylated with methanol and HCl. Fatty acid methyl ester derivatives (FAMES) were extracted with methyl *tert*-butyl ether and the base washed organic phase stored at -20 °C. FAMES were analysed on a Hewlett Packard series II gas chromatograph (5890) using a phenyl methyl silicone capillary column (25 m × 0.2 mm × 0.33 µm). A pattern recognition algorithm compared FAME profiles with the MIDI-MIS TSBA aerobic data base (version 3.2), naming species and sub-species most like the sample and giving similarity indices. All identifications given had similarity indexes of 0.7 or greater.

Exogenous isolation of conjugative plasmids from the rhizosphere. Conjugative plasmids conferring resistance to Hg²⁺ were isolated from sugar beet rhizosphere bacteria using the exogenous isolation method (Fry & Day, 1990) adapted for the rhizosphere. A beet peel homogenate was mixed with an overnight broth culture of *Pseudomonas putida* UWC1 (1 ml of each) and incubated on a dry TSBA plate at 20 °C for 24 h. This mating mix was resuspended in 6 ml 0.1 × NB and serially diluted onto PCA plates amended with rifampicin and HgCl₂ to select for *P. putida* UWC1 transconjugants which had gained plasmids conferring resistance to Hg²⁺.

Filter plate matings. Overnight cultures (1 ml) in NB of donor and recipient were mixed and deposited onto 25 mm diameter cellulose nitrate membrane filters (Whatman) and incubated, bacteria side up, on TSBA plates for 24 h at 20 °C. The filters were vortex-mixed in 10 ml 0.1 × NB, diluted and enumerated on appropriately amended PCA plates to count donors, recipients and transconjugants.

In situ mating. Overnight cultures of *Pseudomonas aureofaciens* 381R in NB (without antibiotics) and *Pseudomonas marginalis* 376N(pQBR11) in NB amended with 0.05 mM HgCl₂ were harvested (Eppendorf microcentrifuge 5415C, 14000 r.p.m., 1 min), washed twice and resuspended in 0.85% saline. Fresh field soil (29–33% moisture) was mixed with a spatula and divided into 1 g quantities in sterile universal bottles. Mean initial bacterial concentration in the soil was determined at 9.4 × 10⁵ c.f.u. g⁻¹ (*n* = 6, *cv* = 114%). Saline suspensions of donor and recipient cells (100 µl total) were added to the soil

(donor first) and mixed with a spatula (final moisture 35–39%). Each soil mating mix (SMM) was placed, with a spatula, onto a sterile 25 mm diameter 0.45 µm pore size cellulose nitrate membrane filter (Whatman) in a sterile plate and introduced to the rhizosphere of potted field plants or to plants at the field site (transport time 40 min). The soil was eased away from the side of the beet using a trowel and a sterile spatula; the inoculated mixture supported on the filter was then pressed down onto the root 7–10 cm below the surface and the surrounding soil replaced. A replicate SMM filter was similarly placed on the opposite side of the root. After 24 h the SMMs were recovered. The soil was collected from each filter into a sterile universal bottle, transported to the laboratory and vortex-mixed in 10 ml 0.1 × NB to resuspend bacteria. The peel from the root surface where the SMM had been applied was carefully removed with a scalpel into a microcentrifuge tube. Peelings from the two mating sites on each plant were pooled and vortex-mixed in 1 ml 0.1 × NB. Peel and SMM suspensions were diluted and the bacteria enumerated on PCA plates amended with (i) nalidixic acid and HgCl₂ for donors, (ii) rifampicin for recipients, or (iii) rifampicin and HgCl₂ for transconjugants. The identity of a subsample of transconjugants (60–70 colonies) was initially confirmed by growth on fresh PCA amended with rifampicin and HgCl₂ and lack of growth on PCA amended with nalidixic acid and HgCl₂.

Control experiments were as follows: (i) SMMs placed on filters in universal bottles and incubated at 20 °C for 24 h, (ii) SMMs placed on filters in Petri dishes and incubated at room temperature for 1.5 h, and (iii) SMMs held between pairs of filters placed in the soil 5–10 cm from the experimental plant. Other controls determined the rate of mutation of donors and recipients to transconjugant phenotype.

Soil matings. Donor and recipient cells from overnight broth cultures were washed twice, resuspended in 0.85% saline and mixed (100 µl g⁻¹) with fresh field soil in universal bottles. Nutrient-amended soil matings were included, using overnight cultures washed and resuspended in NB and mixed with the soil. The soil matings were incubated for 24 h at 20 °C, vortex-mixed 10% (w/v) in 0.1 × NB, diluted and plated onto appropriately amended PCA plates to count donors, recipients and transconjugants.

Plasmid isolation and restriction endonuclease digestion. Plasmid presence was confirmed using the method of Rochelle *et al.* (1986) as modified from Kado & Liu (1981) for plasmid extraction and visualization on agarose gels. Plasmid DNA for restriction endonuclease digestion was prepared by the sucrose gradient method of Wheatcroft & Williams (1981) and digested using enzymes supplied by Pharmacia in accordance with the manufacturer's instructions. Electrophoresis was performed through 0.6% (w/v) agarose gels in 1 × TBE buffer (0.089 M Tris/borate, 0.002 M EDTA) at 5 V cm⁻¹.

Isolation of proteins and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Recipient and donor cellular protein patterns were compared by SDS-PAGE on 6% or 10% (w/v) polyacrylamide gels (Laemmli, 1970). Cellular proteins were isolated from overnight broth cultures using the method of Xu & Gross (1988). Briefly, 250 µl overnight broth culture was centrifuged and the pellet resuspended in 200 µl water and 250 µl sample buffer (0.125 M Tris/HCl, pH 6.8; 4% (w/v) SDS; 20% (v/v) glycerol; 1.4 M 2-mercaptoethanol). After 1 h incubation at 37 °C the mixture was centrifuged (Eppendorf microcentrifuge 5415C, 14000 r.p.m., 1 min) and 20 µl supernatant containing soluble protein loaded per well. Protein bands were visualized by staining with Coomassie Brilliant Blue.

Statistical analysis. Coefficients of variation (cv), one-way analysis of variance (ANOVA), Bartlett's Box *F* test, Cochran's *C* test, Hartley's *F*_{max} test and other statistical tests were calculated using Unistat version 4.56. For one-way ANOVA, count data were transformed by log₁₀ *x* and frequency data by log₁₀ (*x* + 1 × 10⁻⁹) to ensure homogeneity of variances.

RESULTS AND DISCUSSION

Isolation of strains and plasmids

From a collection of bacteria isolated from the sugar beet rhizoplane on TSBA and identified by fatty acid analysis, 11 pseudomonads were chosen as potential donors or recipients for *in situ* matings. These were three strains of *P. aureofaciens*, two of *Pseudomonas fluorescens*, two of *P. marginalis*, two of *Pseudomonas putida*, and two of *Pseudomonas syringae*. Spontaneous rifampicin and nalidixic acid resistant strains were selected where possible, grown overnight in broth and quantitatively plated to confirm stable resistance. One isolate gave only a nalidixic acid resistant strain (376N), two gave only rifampicin resistant strains (381R, 366R), and two gave strains of both resistances (373N, 373R, 391N, 391R) (Table 1). Stable nalidixic acid and rifampicin resistant strains were evaluated as potential donors and recipients respectively.

Twenty-three mercury resistance plasmids were exogenously isolated from peel homogenates in three independent experiments using *P. putida* UWC1 as a recipient. Twelve of these plasmids (chosen to include plasmids from each experiment) were transferred by conjugation from *P. putida* UWC1 into the potential donor (nalidixic acid resistant) strain 376N (transfer frequencies 1.5 × 10⁻³–1.0) by filter plate matings. Similarly, transfer of seven of these plasmids was attempted from *P. putida* UWC1 to 373N; five transferred (1.6 × 10⁻⁶–4.0 × 10⁻⁴) and two did not (limits of detection 4.0 × 10⁻⁹ and 1.7 × 10⁻⁹). Transfer was attempted for only seven of the 12 plasmids as it became apparent that 373N was a poor recipient for some of the plasmids and that transfer from it to the chosen recipients was poor. No transfer to 391N by five of the 12 plasmids could be detected despite low limits of detection in three cases (3.4 × 10⁻¹⁰–8.1 × 10⁻¹⁰).

Those plasmids in 376N and 373N were transferred in filter matings to the four available rifampicin resistant strains to identify a high transfer frequency combination of donor, recipient and plasmid. No plasmid transfer could be detected to 366R (three plasmids from 373N and four from 376N) or to 391R (five plasmids from 373N and four from 376N). Plasmid transfer frequencies to 381R and 373R are given in Table 2. Isogenic transfer from 373N to 373R was not considered because of the potential difficulties of differentiating between donor and recipient cells from *in situ* matings.

From the combinations listed in Table 2, the donor *P. marginalis* 376N containing pQBR11 and the recipient *P. aureofaciens* 381R were chosen for *in situ* mating experiments because they gave a high transfer frequency of 4.4 × 10⁻³ (*n* = 2) in these preliminary experiments and because the bacteria could be readily distinguished.

Table 2. Comparison of filter plate mating frequencies for 12 exogenously isolated mercury resistance plasmids in three possible pairings of donor and recipient pseudomonad strains

Plasmid	Plasmid transfer frequency* (T/R) for different donor and recipient combinations		
	376N × 381R	376N × 373R	373N × 381R
pQBR2	6.0×10^{-5}	1.6×10^{-5}	5.4×10^{-6}
pQBR3	3.2×10^{-4}	ND	9.5×10^{-6}
pQBR4	1.2×10^{-4}	7.6×10^{-5}	ND
pQBR5	2.0×10^{-4}	ND	ND
pQBR6	9.3×10^{-5}	1.1×10^{-5}	3.0×10^{-6}
pQBR7	1.5×10^{-3}	4.7×10^{-5}	ND
pQBR10	1.2×10^{-4}	ND	4.5×10^{-5}
pQBR11	4.4×10^{-3}	7.4×10^{-6}	4.3×10^{-5}
pQBR14	1.6×10^{-3}	ND	ND
pQBR19	2.8×10^{-5}	4.9×10^{-7}	ND
pQBR23	5.3×10^{-5}	3.2×10^{-5}	2.3×10^{-7}
pQBR24	5.4×10^{-5}	ND	1.6×10^{-7}

* All frequencies are the mean of two independent experiments expressed as transconjugants (T) per recipient (R) between combinations of *P. marginalis* 376N and *P. aureofaciens* 381R or 373R. ND, Not determined.

The mean transfer frequency of pQBR11 between 376N and 381R, estimated from further filter plate matings, was 3.3×10^{-4} transconjugants per recipient ($n = 13$, $cv = 235\%$).

Rifampicin and nalidixic acid resistance markers were chosen because they allowed clean selection from rhizosphere bacteria, as resistance to these antibiotics was rare in indigenous bacteria ($< 1 \times 10^{-9}$). Rifampicin selection was preferred for recipients as this antibiotic performed better when spreading greater amounts of homogenate on plates to select transconjugants at low densities. Stable rifampicin resistant strains could not be generated from the nalidixic acid resistant donor 376N ($< 1 \times 10^{-10}$), further improving the sensitivity of transconjugant selection.

Mercury resistance plasmids were chosen for this study as they are common in terrestrial environments (Kelly & Reaney, 1984) and gave unambiguous selection against environmental backgrounds. Typically 1.9% ($n = 8$, $cv = 92\%$) of cultured beet rhizoplane bacteria were able to grow in the presence of 0.1 mM $HgCl_2$. However, no sugar beet rhizosphere bacteria with resistance to both mercury and rifampicin were isolated ($< 1 \times 10^{-10}$). The reason for the prevalence of mercury resistance is uncertain because the experimental site has no known history of mercury pollution.

In situ mating experiments

In situ mating experiments between 376N(pQBR11) and 381R on three potted and three field plants demonstrated plasmid transfer in 10 out of 12 occasions at frequencies

Table 3. Transfer frequencies (transconjugants per recipient) of pQBR11 between *P. marginalis* 376N and *P. aureofaciens* 381R from *in situ* matings in the rhizosphere of sugar beet

Experiments 1–3 were on potted plants outside at soil temperatures of 19 or 20 °C and experiment 4 was on field plants at 15 °C. All matings were for 24 h with two replicates per plant. Peel mating transfer frequencies are for peel pooled from two matings on the same plant. The mean coefficient of variation within replicate transfer frequencies was 129%.

Expt no.	Plant no.	Source*	Transfer frequency	Total count of donors and recipients (c.f.u. g ⁻¹)†	
				Initial	Final
1	1	SMM	$< 4.5 \times 10^{-7}$	1.5×10^8	2.0×10^7
	1	SMM	2.4×10^{-6}	1.5×10^9	3.9×10^8
2	2	SMM	2.1×10^{-7}	2.1×10^9	2.7×10^9
	2	SMM	2.7×10^{-6}	2.1×10^9	2.8×10^9
	2	Peel	1.3×10^{-2}	NA	2.4×10^7
3	3	SMM	1.3×10^{-8}	2.4×10^8	9.6×10^8
	3	SMM	4.4×10^{-7}	2.4×10^8	6.1×10^8
	3	Peel	1.7×10^{-6}	NA	8.8×10^9
4	4	SMM	2.2×10^{-7}	3.8×10^8	1.3×10^9
	4	SMM	1.6×10^{-8}	3.8×10^8	5.9×10^9
	4	Peel	4.2×10^{-5}	NA	3.5×10^8
4	5	SMM	4.8×10^{-6}	3.8×10^7	2.3×10^8
	5	SMM	5.1×10^{-5}	3.8×10^7	1.5×10^8
	5	Peel	3.8×10^{-4}	NA	3.5×10^8
4	6	SMM	3.2×10^{-6}	3.8×10^6	4.3×10^7
	6	SMM	$< 4.8 \times 10^{-7}$	3.8×10^6	4.3×10^7
	6	Peel	5.0×10^{-4}	NA	7.7×10^7

* SMM, soil mating mixes, fresh field soil mixed with donor and recipient bacteria applied directly to the root surface; Peel, removed from the root under the SMM.

† NA, Not applicable, as initial counts could not be made on peel at time of SMM application.

ranging from 5.1×10^{-5} to 1.3×10^{-8} (Table 3). Transfer frequencies on the peel (1.3×10^{-2} to 1.7×10^{-6}) were higher than those from the corresponding SMM (Table 3). These elevated transfer frequencies may result from greater transfer between bacteria colonizing or growing on the rhizoplane or from faster growth of transconjugants on the peel.

The large variation ($cv = 129\%$) between replicate mating experiments prevented the detection of significant differences ($P = 0.58$) between plants, such as between those sampled at different stages of development or at different soil temperatures. Jacobs *et al.* (1985) have shown that the beet rhizoplane is not a uniform habitat but has increased bacterial activity associated with a

Fig. 1. Soluble cellular protein profiles of transconjugant bacteria isolated from *in situ* mating, experiment 4. Lanes: 1–10, *P. aureofaciens* 381R transconjugants; 11, 381R culture used as recipient; 12, *P. marginalis* 376N(pQBR11) culture used as donor. Comparison of band patterns in lanes 1–10 with lane 11 confirms the identity of these transconjugants as 381R. Cellular extracts were separated on 10% SDS-PAGE and stained with Coomassie Brilliant Blue.

vertical zone of secondary root emergence. Of necessity the SMMs were positioned close to this zone and an unequal association with it may have contributed to the 10- to 35-fold differences found between the transfer frequencies of replicates.

Confirmation of selection markers

Since the work described here relied on isolating transconjugants from the environment it was essential to confirm that the selection markers functioned correctly. Ten putative transconjugants per experiment were chosen at random and examined by SDS-PAGE analysis of soluble cellular proteins to confirm that they were the recipient strain 381R (Fig. 1). Plasmids were extracted from at least five transconjugants for each mating experiment and analysed by restriction endonuclease digestion to confirm their identity as pQBR11 (Fig. 2). SDS-PAGE and plasmid profiling were reliable methods for confirming the identity of transconjugants isolated on selection plates. Plasmid analysis also confirmed that the plasmid transferred was pQBR11 and not another indigenous mercury resistance plasmid.

Control experiments and the influence of the rhizosphere

Plasmid transfer was not detected in any of the SMM control experiments *in vitro* ($< 1.2 \times 10^{-8}$), *in situ* more than 5 cm from the plants ($< 1.6 \times 10^{-8}$), or after 1.5 h mating at room temperature ($< 8.0 \times 10^{-9}$), used to simulate transport conditions. The transfer of pQBR11 between 376N and 381R could not be detected after 24 h at 20 °C ($< 2.26 \times 10^{-9}$) in normal soils *in vitro*. However, after soil amendment with nutrient broth (0.1 ml g^{-1}) transfer frequencies of 1.1×10^{-6} ($n = 3$, $\text{cv} = 63\%$) were observed.

The failure to detect transconjugants in all the control soil mating experiments clearly demonstrated the positive

Fig. 2. Restriction digests of pQBR11 DNA isolated from 376N (donor) and 381R transconjugants derived from *in situ* rhizosphere matings. Lanes: 1, *EcoRI/HindIII* digest of λ bacteriophage DNA; 2, *XbaI* digest of pQBR11 isolated from the donor in experiment 4; 3–7, *XbaI* digest of pQBR11 isolated from five transconjugants in experiment 4; 8–13, as for lanes 2 to 7, but *XhoI* digests; 14, *XhoI* digest of pQBR11 from the original plasmid isolation.

influence of the rhizosphere in promoting plasmid transfer. This effect has also been demonstrated in microcosm experiments using wheat rhizospheres (van Elsas *et al.*, 1988) for investigating transfer of the clinically isolated plasmid RP4 (Datta *et al.*, 1971) between introduced bacteria. Increased plasmid transfer in nutrient-enriched soils has been observed previously (Stotzky, 1989; Top *et al.*, 1990; Wellington *et al.*, 1990; Klingmüller, 1991). Although the mechanisms by which the rhizosphere promotes plasmid transfer have not been resolved, it is likely that nutrients play a key role. Plant root exudates can account for up to 40% of carbon fixed by photosynthesis (Whipps, 1990), which results in a rhizosphere of increased bacterial density and in more metabolically active populations (Zagallo & Katznelson, 1957). These nutrient-enhanced sites can also attract motile bacteria (Bashan, 1986).

The fall in plasmid transfer frequencies observed between the peel and rhizosphere soil may be related to a declining concentration of nutrient exudates away from the root. Transfer frequencies on the peel were higher than those in nutrient-amended soil. This may also relate to nutrient availability, as the bacteria in amended soil were growing on a declining nutrient base. Rochelle *et al.* (1989) have shown that mercury resistance plasmid transfer frequencies between pseudomonads were dependent on the nutrient concentration in the mating plate medium up to

3.5 g C l⁻¹. Nutrient availability affects the growth rate and metabolic condition of bacteria, which are considered to be significant factors in plasmid transfer. Nonetheless, no correlation between increase in bacterial numbers and transfer frequency was found and no simple relationship between nutrients, growth and transfer frequency could be determined. Other factors which may also affect transfer frequencies include the influence of soil and root surfaces on conjugation, or the plasmid itself may improve the competence of recipients on the peel. Clearly additional studies are needed to resolve the mechanisms of rhizosphere influence on plasmid transfer.

Experimental methods

To achieve realistic mating conditions the SMM inocula were applied at densities typical of rhizosphere soils. Fresh field soil, with lower bacterial counts (9.4×10^5 c.f.u. g⁻¹, $n = 6$, cv = 114 %) than rhizosphere soil, was used for SMMs to avoid increasing bacterial densities above those normally found in the rhizosphere.

In situ transfer frequencies in the SMMs were recorded at final densities for the introduced bacteria ranging from 4.3×10^7 to 5.9×10^9 c.f.u. g⁻¹ (Table 3). This range was similar to that in the natural sugar beet rhizosphere soil (1.2×10^7 to 2.3×10^8 c.f.u. g⁻¹; mean = 1.1×10^8 c.f.u. g⁻¹, $n = 6$, cv = 40 %). *In situ* transfer was also observed on the beet surface, where final bacterial densities of 2.4×10^7 to 8.8×10^9 c.f.u. g⁻¹ were recorded (Table 3). Normal bacterial densities on the peel averaged 7.7×10^8 c.f.u. g⁻¹ ($n = 11$, cv = 82 %) on other field plants sampled during the period of the experiments. Further experiments are planned where the numbers of donors in the SMM are reduced to the densities of bacteria expressing mercury resistance (2×10^6 c.f.u. g⁻¹).

Bacterial counts were similar (no significant difference, $P = 0.32$) in field plant rhizosphere soil (1.1×10^8 c.f.u. g⁻¹, $n = 6$, cv = 40 %) and potted plant rhizosphere soil (1.35×10^8 c.f.u. g⁻¹, $n = 6$, cv = 29 %). As described above, no significant differences in plasmid transfer frequency on field plants and potted plants were detected. This indicates that potted field plants are suitable experimental models for studying plasmid transfer in the rhizosphere.

Although this *in situ* method does not exactly simulate natural conditions it does allow the precise control of inoculum density and its placement in an otherwise natural rhizosphere. This provides an effective model with which to realistically study factors that influence conjugation in the rhizosphere. Factors which can be directly evaluated with this method include plant age, plant condition, and position on the root. Prior acclimatization of donor and recipient on separate roots should also be considered. The experimental approach described here provides insights into the behaviour of a natural system and allows microcosm results to be tested in the field. Although untested, by the production of smaller SMMs and careful attention to their placement, this method could be applicable to other plants with substantial roots.

Experimental variables

Experimental variables likely to have an influence on plasmid transfer in natural environments (Rochelle *et al.*, 1989) were examined during *in situ* experiments. No trends could be detected between transfer frequency and temperature (15–20 °C), soil moisture (29–33 %), increase in mating populations, beet weight (62–360 g) or plant age (54–92 d). Initial donor to recipient (D:R) ratios in the SMMs ranged from 0.16 to 1.4 and at the end of the matings from 0.4 to 1.6. No correlations could be found between final D:R ratio or initial SMM mating densities and transfer frequency.

Conclusions

The results presented here describe *in situ*, conjugative plasmid transfer using both natural isolates and an indigenous plasmid in the rhizosphere of sugar beet. The observed frequencies indicate that environmental plasmid transfer may not be an uncommon event provided bacteria are present in appropriate habitats. The method described allows for the realistic study of the factors that influence *in situ* plasmid transfer. Such studies should lead to a better understanding of plasmid ecology and permit the development of models for predicting likely frequencies of gene transfer in the terrestrial environment.

ACKNOWLEDGEMENT

A. K. L. was supported by a NERC-CASE studentship award.

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Received 17 March 1993; revised 15 June 1993; accepted 8 July 1993.