Persistence of a *xylE* marker gene in *Pseudomonas putida* introduced into soils of differing texture

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The growth and survival of two genetically engineered micro-organisms (GEMs), *Pseudomonas putida* strain PaW8 containing either marker plasmid pLV1013 (IncQ) or pLV1016 (IncP), both of which contain the *xylE* gene, were investigated in soils ranging in texture from a sandy loam to a peat. Viable GEMs were enumerated over a period of 89 d. Analysis of the decay kinetics indicated that GEM survival, as monitored by the persistence of the *xylE* gene, was biphasic in the sandy and clay loams. Both organisms showed evidence of initial growth in the peat soil prior to a rapid death phase. The results indicated that soil texture has an effect on the survival of genetically engineered *P. putida*, death decreasing with increasing clay content.

Introduction

Given current concerns about the risks associated with the use of genetically engineered micro-organisms (GEMs) in open ecosystems it is important to be able to determine reliably the survival of such organisms (Tiedje *et al.*, 1989; Domsch *et al.*, 1988) and how this survival is influenced by primary environmental factors. The situation is complicated by the highly variable abiotic and biological conditions offered within a soil and by soils of different texture. It is known that *Rhizobium* sp. and *Escherichia coli* survive for longer in heavier-textured soils and in sandy soils amended with bentonite clay (Heynen *et al.*, 1988; Richaume *et al.*, 1989; van Elsas *et al.*, 1990). Such survival studies are a necessary part of release experiments, with much of the current work carried out on a 'case-by-case' basis. Although these studies yield valuable data, an alternative approach to predicting the persistence and survival of GEMs is necessary. The development of appropriate survival models would provide a useful alternative to separate microcosm studies but requires the identification of quantifiable parameters.

In this paper we compare the survival of two *Pseudomonas putida* constructs, one containing an IncQ and the other an IncP plasmid marker, in a range of soils and investigate the influence of soil texture on the

survival and death kinetics of each inoculant. Preliminary attempts to identify suitable parameters with which to compare and possibly model survival are also made.

Methods

Bacterial strains and plasmids. The recombinant *Pseudomonas putida* PaW8 strain containing the plasmids pLV1013 or pLV1016 has been described previously (Morgan *et al.*, 1989; Winstanley *et al.*, 1989, 1991). pLV1013 is a broad-host-range nonconjugative IncQ plasmid derived from the RSF1010 vector, containing a *xylE* gene insert coding for catechol 2,3-dioxygenase. This is under the control of λ P_R and its temperature-sensitive repressor cI₈₅₇. pLV1016 is a stable cointegrate between the IncP conjugative plasmid R68.45 and pLV1012, a nonconjugative plasmid carrying the *xylE* gene under P_R/cI₈₅₇ regulation (Winstanley *et al.*, 1991). The copy numbers of the two plasmids are 15–30 and 2–3 respectively (Morgan *et al.*, 1989; Winstanley *et al.*, 1991). Both plasmids carry kanamycin-resistance genes. Organisms carrying these recombinant plasmids were obtained from Dr J. R. Saunders, Department of Genetics and Microbiology, University of Liverpool, UK.

P. putida PaW8 containing either pLV1013 or pLV1016 was grown for 24 h at 30 °C in nutrient broth containing kanamycin (50 µg ml⁻¹). Prior to inoculation into soil the cells were harvested by centrifugation at 5000 g for 10 min at 4 °C (Sorvall RC5C), washed twice in sterile saline (0.8% w/v), and resuspended in sterile distilled water at a cell density of approximately 1 × 10⁸ c.f.u. ml⁻¹.

Soils. Three soils were used in this study: a sandy loam (Rivington series); a clay loam (Hallsworth series); and a peat soil (Wilcocks series). Particle size distribution and pH are shown in Table 1 (Crompton, 1966). The field capacities of the three soils were determined, by standard procedures (Black, 1965), as 25%, 45.5% and 442% moisture content (w/w) for the sand, clay and peat soils respectively. Soils were collected from 0–10 cm depth, sieved through a

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Abbreviation: GEM, genetically engineered micro-organism.

Table 1. Some properties of the soils used

Soil series	Particle size (%)			Total C (%) (mg g ⁻¹)	pH (H ₂ O)	Sampling site and grid ref.
	Sand (20–2000)	Silt (2–20 µm)	Clay (<2 µm)			
Hallsworth	45	19	36	11.9	5.0	Cockle Park Farm, Northumberland, UK NZ 202912
Rivington	60	16	19	6.25	5.5	Cockle Farm Park, Northumberland, UK NZ 202940
Wilcocks	58	17	14	20.0	4.0	Redesdale Experimental Husbandry Farm, Northumberland, UK NY 828924

4 mm screen and stored at 4 °C prior to use. The moisture contents (w/w) of the sand and clay soils were below 50% of field capacity when they were collected from the field. The peat was air-dried to a moisture content below 50% of field capacity. The sandy loam (268.5 g at 11%, w/w, moisture content), the clay loam (262.5 g at 26%, w/w, moisture content) and the peat soil (291 g at 425%, w/w, moisture content) were weighed into pots and remoistened to 50% of field capacity with distilled water containing either *P. putida* PaW8(pLV1013) or *P. putida* PaW8(pLV1016) so that each soil contained GEMs at approximately 1×10^7 c.f.u. (g dry wt soil)⁻¹. Control pots were remoistened with sterile distilled water. The experiments were conducted in microcosms, with three replicates of each combination of soil and micro-organism.

Microcosm sampling. Microcosms were sampled on 11 occasions, at 0, 2, 5, 8, 11, 14, 19, 24, 29, 59 and 89 days. Micro-organisms were extracted using a dispersion/differential centrifugation technique (Hopkins *et al.*, 1991), in which the soils (5 g) are dispersed in 0.1% (w/v) sodium cholate and centrifuged. The supernatants are collected and the pellet then resuspended and shaken in Tris buffer (0.05 M, pH 7.4), ultrasonicated in a low-energy ultrasonic cleaning bath for 1 min in sodium cholate, then shaken in sterile distilled water. The pooled supernatants are then centrifuged at 5000 g for 10 min and the pellet resuspended in sterile saline (0.8%, w/v) to give a final volume of 5 ml. All extractions and centrifugations were carried out at 4 °C.

Serial dilutions of the soil extracts were plated, in triplicate, on to nutrient agar containing cycloheximide (50 µg ml⁻¹) for the uninoculated soils, and cycloheximide and kanamycin (50 µg ml⁻¹) for the inoculated soils. The plates were incubated for 48 h at 28 °C, then heated to 42 °C for 2 h before being sprayed with catechol (1%, w/v). Colonies turning yellow, indicating the presence of a functional *xylE* gene, and presumably therefore the recombinant plasmid, were counted.

Results

The data in Figs 1(a) and (b) suggest that the model recombinant bacteria behaved differently in the different soils: in peat, *P. putida* PaW8(pLV1013) and PaW8(pLV1016) showed single first-order kinetics of mortality, whereas in the sandy and clay loams they exhibited dual first-order kinetics of mortality – the

Fig. 1. Survival of (a) *P. putida* PaW8(pLV1013) and (b) *P. putida* PaW8(pLV1016) in clay (■---■), sand (▲---▲) and peat (●---●) soil over time. $n = 9$ (three plates from each of three microcosms). The vertical bars represent the standard error of the mean. (In this and other figures, where error bars are not shown, these were smaller than the symbols.)

decay curve is biphasic, with each phase having a constant but different death rate. This is expressed mathematically below.

Single first-order kinetics

At a given time the population can be represented as:

$$c = c_0 \exp(-k_1 t) \quad (1)$$

where c = total population density (c.f.u. g⁻¹), c_0 = initial population density (at $t = 0$) (c.f.u. g⁻¹), k_1 = first-order rate constant (d⁻¹) and t = time (d). The half-life of the population, $t_{1/2}$, is given by:

$$t_{1/2} = 0.693/k_1 \quad (2)$$

Dual first-order kinetics

At a given time the population can be represented as:

$$c_t = c_f + c_s \quad (3)$$

where c_t = total population density (c.f.u. g⁻¹), c_f = density of the short-lived population, exhibiting fast decay (c.f.u. g⁻¹) and c_s = density of the longer-lived population, exhibiting slow decay (c.f.u. g⁻¹).

From equation (1),

$$c_t = c_{f0} \exp(-k_{1f} t) + c_{s0} \exp(-k_{1s} t) \quad (4)$$

where c_{f0} = initial population density of organisms showing fast decay (c.f.u. g⁻¹), c_{s0} = initial population density of organisms showing slow decay (c.f.u. g⁻¹), k_{1f} = first-order rate constant for fast decay (d⁻¹) and k_{1s} = first-order rate constant for slow decay (d⁻¹).

P. putida PaW8(pLV1013) and PaW8(pLV1016) in the sand and clay soils exhibit dual first-order kinetics of mortality. Although the majority (97–98%) of the initial inoculum is short-lived (half-lives of 1–2 d), a small proportion (2–3%) of the initial inoculum survives longer (half-lives of 7–17 d).

The decrease of the total population, c_t , in Figs 2 and 3 resembled the simultaneous decay of two radioisotopes with differing half-lives; c_t was resolved into two components, c_f for fast decay and c_s for slow decay, in the following way. A line was fitted to the last four or five points at large times, for which $c_s \gg c_f$, to obtain k_{1s} , the first-order rate constant for slow decay (Table 2), which was then used to predict c_{s0} , the initial fraction of the inoculum likely to survive over extended periods, and the values of c_s at the various sampling times. The values of c_f at these times were found by subtracting these predicted c_s from the obtained c_t , and fitting a line to the first four or five values of c_f to obtain k_{1f} , the rate constant for the

Fig. 2. Survival of *P. putida* PaW8(pLV1013) in sand (a) and clay (b) soils over 89 d. The graphs show dual first-order kinetics of mortality. $n = 9$. The points are experimental observations $\pm$ standard errors of the total population. The dashed and dotted lines are the resolution of the total population into short-lived and long-lived phases, respectively, and add to give the continuous line fitting the total observed population.

fast decay phase, and c_{f0} (Table 2). Each line was fitted by linear regression of the natural logarithm of population density (c_t , c_f or c) versus time, with the number of data points selected to give the lowest value of P , not the highest value of R^2 , i.e. the most significant result (Rose, 1991). From Figs 2 and 3 it can be seen that the balance between the phases showing fast and slow decay changes constantly, with the 'populations' becoming equal at the cross-over points [10.5 d for PaW8(pLV1013) in sand; 11.6 d for PaW8(pLV1016) in sand; 15.3 d for PaW8(pLV1013) in clay; 13.7 d for PaW8(pLV1016) in clay].

Both *P. putida* PaW8(pLV1013) and PaW8(pLV1016) in peat showed single first-order kinetics of mortality (Fig. 4). There was evidence of an initial fall in the populations of recombinants containing pLV1013 and

Fig. 3. Survival of *P. putida* PaW8(pLV1016) in sand (a) and clay (b) soils over 89 d. The graphs show dual first-order kinetics of mortality. $n = 9$. See Fig. 2 for details.

Fig. 4. Survival of (a) *P. putida* PaW8(pLV1013) and (b) *P. putida* PaW8(pLV1016) in peat soil. The graphs show single first-order kinetics of mortality over 8–59 d (a) and 8–29 d (b). $n = 9$. The vertical bars represent the standard error of the mean.

Table 2. Dynamics of microbial populations; dual first-order kinetics

Plasmid in <i>P. putida</i> PaW8	Soil	c_{t0}	c_{r0}	c_{r0}/c_{t0}	k_{1f}	t_{1f}	c_{s0}	c_{s0}/c_{t0}	k_{1s}	t_{1s}	R^2
pLV1013	Sand	5.66×10^6	5.55×10^6	0.981	0.55	1.3 ± 0.1	1.05×10^5	0.019	0.099	7.0 ± 0.5	0.986***
pLV1013	Clay	1.36×10^5	1.32×10^5	0.969	0.30	2.3 ± 0.2	4.27×10^3	0.031	0.041	16.7 ± 0.9	0.995***
pLV1016	Sand	8.08×10^5	7.92×10^5	0.981	0.44	1.6 ± 0.2	1.57×10^4	0.019	0.075	9.3 ± 1.0	0.981***
pLV1016	Clay	5.01×10^5	4.86×10^5	0.971	0.33	2.1 ± 0.1	1.44×10^4	0.029	0.039	17.7 ± 0.8	0.987***

Symbols: c_{t0} , total initial population at time 0 (c.f.u. g⁻¹); c_{r0} , initial population density of organisms showing fast decay (c.f.u. g⁻¹); k_{1f} , first-order rate constant for fast decay (d⁻¹); t_{1f} , half-life of fast-decay population (d), mean $\pm$ standard error of estimate; c_{s0} , initial population density of organisms showing slow decay (c.f.u. g⁻¹); k_{1s} , first-order rate constant for slow decay (d⁻¹); t_{1s} , half-life of slow decay population (d), mean $\pm$ standard error of estimate; R^2 , proportion of the variance in c_t accounted for by equation (4), calculated by the method of Rose (1991). *** Significant at $P < 0.001$.

pLV1016, followed by an increase. However, after the peak, there was a fast first-order decay, with short half-lives of 2–3 days (Table 3).

For all soils, the indigenous population declined slowly over the 89 d, decreasing by, at most, one order of magnitude. The decay phase in these, uninoculated, pots

Table 3. Dynamics of microbial populations; single first-order kinetics

Organisms	Soil	c_0	k_1	$t_{1/2}$	R^2
Indigenous	Sand	3.2×10^7	0.018	38 ± 5	0.850***
	Clay	3.4×10^7	0.0066	106 ± 50	0.364*
	Peat	1.1×10^8	0.035	20 ± 2	0.906***
PaW8(pLV1013)†	Peat	3.5×10^6	0.25	2.8 ± 0.2	0.957***
PaW8(pLV1016)‡	Peat	1.3×10^5	0.28	2.4 ± 0.3	0.929**

Symbols: c_0 , initial population density (c.f.u. g⁻¹); k_1 , first-order rate constant (d⁻¹); $t_{1/2}$, half-life of population (d), mean $\pm$ standard error of estimate; R^2 , proportion of the variance in c accounted for by equation (1). * Significant at $P < 0.05$; ** significant at $P < 0.01$; *** significant at $P < 0.001$.

† For the period 8–59 d inclusive.

‡ For the period 8–29 d inclusive.

followed single first-order kinetics (Fig. 5). No background levels of catechol 2,3-dioxygenase-positive colonies were detected in the control microcosms.

Discussion

The data presented demonstrate the influence of different soil types on the survival over time of *P. putida* containing either the conjugative plasmid pLV1013 or the non-conjugative plasmid pLV1016. The two plasmids were chosen to determine whether there were differences in their survival in the three soils. However, no significant differences were observed between constructs as judged by their half-lives in the different systems (Tables 2 and 3). This is in agreement with previous studies using these organisms in which *P. putida* PaW8(pLV1013) and PaW8(pLV1016) showed similar survival patterns in lake water (Winstanley *et al.*, 1991).

Although both plasmids have been shown to be >99% stable in *P. putida*, with colony-forming ability and direct detection, using ELISA, of the 2,3-dioxygenase shown to be in good agreement (Morgan *et al.*, 1989; Winstanley *et al.*, 1991), it remains possible that the inoculant persists but without a functional plasmid. This may explain in part the difference in the decay kinetics found in the peat since in this soil the organisms experienced a period of rapid growth between 9 and 12 d and data presented by Winstanley *et al.* (1991) have suggested that under these circumstances the low copy number of the IncP replicon pLV1016 may lead to a loss of the plasmid. This does not, however, account for the fact that pLV1013, which has the larger copy number of the IncQ plasmid, behaves similarly in this soil, and this suggests that the differences may in fact be due to the interaction of the inoculum with its physicochemical and biological environment. Work by Richaume *et al.* (1989) has shown that

Fig. 5. The indigenous population of sand (a), clay (b) and peat (c) soils over 90 d. The graphs show single first-order kinetics of mortality over 90 d. $n = 9$. The vertical bars represent the standard error of the mean.

abiotic factors such as pH or temperature can result in the rapid decline of microbial inocula.

It is important that GEMs released into soil must compete successfully with the indigenous microflora, including the parent strain (van Elsas *et al.*, 1991). Here, we have not compared the survival of the GEM with that of the wild-type since the presence of *xyIE* was

considered essential for detection and identification in a mixed microbial system such as non-sterile soil (Winstanley *et al.*, 1991). Although the expression of the *xylE* gene is temperature controlled, there is some, albeit limited, expression in *P. putida* PaW8(pLV1013) and PaW8(pLV1016) when grown at low temperatures (Winstanley *et al.*, 1991). Carrying the gene therefore places only a small metabolic burden on the host, whereas expression of the gene, following isolation and growth at elevated temperatures, increases this burden. However, this only occurs when the organism has been transferred to a medium rich enough to compensate for the additional metabolic load on the cells and should have little or no effect on the ability of the organisms to survive in soils. The latter is important since studies with genetically modified strains of *Pseudomonas fluorescens* have shown that where the genetic modification places a metabolic load on the GEM its competitive ability relative to the parent strain is reduced (van Elsas *et al.*, 1991). Other studies, however, suggest that there are no significant differences in the growth of genetically engineered *P. putida* and its parent strain in freshly moistened soil (Yeung *et al.*, 1989) or in the survival of genetically engineered *Erwinia carotovora* and its wild-type in non-sterile soil (Orvos *et al.*, 1990).

The survival of the GEMs was shown to be affected by soil type. Figs 2 and 3 show the two strains of *P. putida* exhibiting dual first-order decay kinetics in the clay and sandy soils. Whether these phases represent distinct populations in the inoculum, as suggested by Winstanley *et al.* (1991), which are randomly or genetically determined remains unknown, although several workers (Alexander, 1981; van Elsas *et al.*, 1991) have suggested that the initial rapid decay may be due to enhanced protozoal grazing at high population densities of the inoculum. Danso *et al.* (1975) and Habte *et al.* (1978) have shown that following the addition of rhizobia to soil in high cell numbers (1×10^8 c.f.u. g⁻¹), the decline in population density corresponded with a parallel increase in the numbers of indigenous protozoa. It has also been demonstrated that the survival of *Rhizobium phaseoli* increased in soils previously treated with anti-protozoal agents (Chao *et al.*, 1981).

The addition of bentonite clay to soil has been shown to enhance bacterial survival (Marshall & Roberts, 1963; Stotzky, 1980, 1986) and to reduce protozoal grazing on rhizobia (Heynen *et al.*, 1988). This enhanced survival in the presence of clay minerals has also been noted with *P. fluorescens* added to coarse-textured soils and in sandy soils amended with bentonite (van Elsas *et al.*, 1986, 1990). In our study, the clay soil (Hallsworth series) extended the survival of the inocula, with half-lives during the second phase of 16.7 and 17.7 d for *P. putida* carrying pLV1013 and pLV1016, respectively, compared

to 7 and 9.3 d in the sandy soil (Rivington series). This is in agreement with the *Rhizobium* studies (Heynen *et al.*, 1988). In finer-textured soils it is likely that there are a greater number of suitable micro-niches than in a sandy soil where the introduced bacteria can become established and thereby protected from protozoal grazing (Heynen *et al.*, 1988).

It is likely that a complex interaction of biological and abiological factors regulates the survival of the inoculum. Microbial growth in soil usually occurs at surfaces and it is possible that the cross-over points shown in Figs 2 and 3 represent a change in the balance between growth and cell death with growth increasing during the second phase perhaps as a result of successful establishment within the soil matrix.

The decline of both *P. putida* PaW8(pLV1013) and PaW8(pLV1016) in peat was faster and more complete than that in either the sandy or the clay soil. This followed an initial increase in population over the first 9–12 d of the study. These results are in contrast to previous studies in which inocula have been shown to survive longer in organic soils (Alexander, 1981), and in upland soils (K. Senoo and others, reported at the IUMS Congress, Osaka, 1990), where it was suggested that water-saturated micropores gave protection to GEMs from predators. However, organic soils have also been shown to harbour large populations of protozoa (Clarholm, 1981). It might be reasonable to suggest that the low pH of the peat (Table 1) was responsible for the rapid loss of inoculum were it not for the fact that only in this soil was there any obvious growth of the inoculum.

The indigenous microflora, as measured by viable counting, following extraction using dispersion and differential centrifugation (Hopkins *et al.*, 1991) was seen to decline between 0.5 and 1.0 order of magnitude over the 89 d of the study. The kinetics for this decay were single first-order and distinct from those of either inoculant. This supports previous studies on the changes found in viable counts from soil microcosms (Orvos *et al.*, 1990) and indicates that the microcosm design was not responsible for the observed decline in the inocula.

By interpreting our survival curves using first-order rate equations, we have tried to identify quantifiable parameters of potential value in the modelling of inoculant survival. In doing so we have been able to describe survival in terms of the half-life of the population. This has made it relatively simple to show that there are no differences between constructs in each of the soils tested but that there are differences between soils. The cross-over point is another potentially useful and quantifiable parameter which, as suggested, may indicate the time taken for the population density to decline to a point at which protozoan grazing is drastically reduced or the time taken for colonization and

subsequent growth of the inoculum. As such it may be valuable in estimating the time required for establishment of the inoculum. Finally, if the inoculum consists of two distinct 'populations', one short-lived, the other long-lived, then, by calculating the intercepts of the y-axis, it is possible to estimate the proportion of the initial inoculum likely to survive over extended periods; this would be particularly useful in risk assessment.

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