APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1990, p. 401–408
0099-2240/90/020401-08$02.00/0
Copyright © 1990, American Society for Microbiology
Vol. 56, No. 2

Transport of a Genetically Engineered *Pseudomonas fluorescens* Strain through a Soil Microcosm

JACK T. TREVORS,[1]* JAN D. VAN ELSAS,[2]* LEO S. VAN OVERBEEK,[2] AND MARY-ELLEN STARODUB[1]

Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1,[1] and Research Institute Ital, 6700 AA Wageningen, The Netherlands[2]

Received 7 August 1989/Accepted 27 October 1989

Vertical soil microcosms flushed with groundwater were used to study the influence of water movement on survival and transport of a genetically engineered *Pseudomonas fluorescens* C5t strain through a loamy sand and a loam soil. Transport of cells introduced into the top 1 cm of the vertical soil microcosms was dependent on the flow rate of water and the number of times microcosms were flushed with groundwater. The presence of wheat roots growing downward in the microcosms contributed only slightly to the movement of *P. fluorescens* C5t cells to lower soil regions of the loamy sand microcosms, but enhanced downward transport in the loam microcosms. Furthermore, the introduced *P. fluorescens* C5t cells were detected in the effluent water samples even after three flushes of groundwater and 10 days of incubation. As evidenced by a comparison of counts from immunofluorescence and selective plating, nonculturable C5t cells occurred in day 10 soil and percolated water samples, primarily of the loamy sand microcosms. Vertical soil microcosms that use water movement may be useful in studying the survival and transport of genetically engineered bacteria in soil under a variety of conditions prior to field testing.

In view of the numerous possible applications of genetically engineered microorganisms (GEMs) in the environment (4, 7, 28, 32, 34a, 36, 40), research into the fate of these organisms and their genetic material after release has become increasingly important (29, 30, 34, 35, 37, 38a; J. D. van Elsas and J. T. Trevors, *in* M. Day and J. Fry, ed., *Bacterial Genetics and Natural Environments*, in press). To date, research has focused on the introduction and recovery of microorganisms in environmental samples (34a, 35), bacterial survival (3, 8, 10, 31, 35, 36, 38b), genetic interactions (37–38a; van Elsas and Trevors, in press), and analyses by gene probing (8, 13, 17, 30, 34a, 36a); on total-community DNA extraction, purification, and quantitation (13, 17, 29, 34a); and on DNA amplification using the polymerase chain reaction (26, 34a). However, less is known about environmental transport and dispersal of GEMs, even though transport has been mentioned as a factor important for environmental risk assessment (28). Transport of bacteria in soil has been mostly studied from an environmental engineering point of view, often using conditions of saturated water flow (9, 11, 22, 24). Viable bacteria were shown to be removed from percolated wastewater by both physical retention and cell death (11). However, in water-saturated soil profiles, bacteria were readily transported due to transport through soil macropores or cracks, providing continuous water films (24, 27). Fewer studies have addressed the fate (transport) of bacteria after agronomical applications (15, 18, 25, 36). It was suggested that *Rhizobium* and *Pseudomonas putida* cells were only transported in soil microcosms under the influence of percolating water or a burrowing earthworm (15), whereas in field microplots transport of *P. fluorescens* from inoculated top soil to deeper layers was reported (36). Other studies showed that inoculated bacterial populations were able to develop along (wheat) roots (39) and that soil water flow affected the rhizosphere distribution of introduced bacterial cells (18). Although none of these studies used GEMs, some of the methods used and conclusions drawn are useful for predicting the fate of introduced GEMs.

Since soil water flow apparently regulates bacterial survival and movement (15, 18, 24), from a risk assessment perspective it will be necessary to study this aspect by using soil microcosms which mimic the environment. Therefore, in this study a microcosm was used in which the effect of percolating water on the bacterial distribution could be assessed in soil with or without growing wheat plants.

MATERIALS AND METHODS

Organisms and growth conditions. The recombinant *P. fluorescens* R2f strain, denoted C5t, was constructed as described elsewhere (J. D. van Elsas, L. S. van Overbeek, A. M. Feldmann, and O. de Leeuw, manuscript in preparation). Briefly, a 4.3-kilobase *Bam*HI-*Bam*HI fragment containing the unexpressed *Bacillus thuringiensis* subsp. *israelensis* δ-endotoxin gene was inserted into the unique *Bam*HI site of transposon Tn*5* (5.7 kilobases; contains *nptII*, a kanamycin resistance gene) in suicide vector pSUP101 (23), resulting in Tn*5*::*tox*. Tn*5*::*tox* was introduced into the *P. fluorescens* R2f chromosome by conjugating the mobilizing *Escherichia coli* S17.1 donor strain (23) with a rifampin (50 μg/ml)-resistant derivative of *P. fluorescens* R2f. The presence of one copy of the 10-kb Tn*5*::*tox* element in the R2f genome and the absence of vector sequences were confirmed by Southern hybridization analysis, using appropriate probes and standard techniques (16). The transposon Tn*5*::*tox* insert was completely stable for 16 generations in dilute tryptone soya broth (Oxoid) without selective pressure.

Strain C5t was grown for 16 h at 28°C in 50 ml of TY broth (tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g; deionized water, 1 liter; pH 7.2), supplemented with 50 μg of rifampin and 50 μg of kanamycin per ml. It was stored at −80°C in the presence of 20% glycerol.

For introduction into soil, cells were aseptically harvested by centrifugation at 7,500 × *g* (10 min at 20°C), washed in

* Corresponding authors.

sterile saline (0.85% NaCl), and suspended in sterile distilled water to give a cell density of about 1.5×10^9 CFU/ml.

Soils. Two soils, Ede loamy sand and Guelph loam, were used throughout this study. The characteristics of both soils have been described before (36–38a). Briefly, Ede loamy sand is a "beekeerd" type, slightly acid (pH 6.2) soil which contains 3.5% organic matter. Its water-holding capacity is about 35%. Guelph loam is near neutral (pH 6.5), contains 3.4% organic matter, and has a water-holding capacity of 56%.

Fresh portions of both soils were mixed, sieved (4-mm mesh), and immediately used for inoculation, after air drying to the appropriate moisture content (10% for Ede loamy sand and 20% for Guelph loam). Soils were fertilized with Sporumix NPK fertilizer at a rate of 1 g/kg of soil.

Soil inoculation. The washed bacterial cell suspensions were immediately used to inoculate soil portions, which were then layered on top of vertical soil microcosms. Cells were mixed into the soil for 10 min with a sterile spatula.

Seed inoculation. In one set of microcosms, transport of seed-inoculated *P. fluorescens* C5t was studied. Surface-sterilized wheat seeds (6) were covered with C5t cells in carboxymethyl-cellulose suspension, using the method of Iswandi et al. (14). Inoculated seeds were air dried for 3 h before being placed in the top 1-cm layer of small soil microcosms (four seeds per column). The average number of viable C5t cells per seed, determined as described in reference 14, was about log 8.7.

Soil microcosms. Portions (70 g) of Ede loamy sand or Guelph loam soil, remoistened to, respectively, 18 and 30% (vol/wt) moisture (respectively, 52 and 54% of water-holding capacity) with sterile distilled water, were packed to a height of about 9 cm in sterile 60-ml syringe tubes 13.4 cm long and 2.5 cm in diameter (Fisher Scientific Co., Toronto, Ontario, Canada) to give a soil bulk density of 1.4 g (wet weight)/cm^3 (small microcosms). In addition, 750-g portions of Ede loamy sand were also packed to bulk density 1.4 g (wet weight)/cm^3 and 26-cm height in ethanol-sterilized plastic graduated cylinders, 34 cm long and 5 cm in diameter, with a 2-mm hole pierced in the bottom (large microcosms). A small plug of sterile glass wool in the bottom of the tubes prevented the soil from escaping.

Portions (7 g for small microcosms and 75 g for large microcosms) of soil inoculated with about 10^8 (log 8) viable *P. fluorescens* C5t cells per g were packed onto the top of the soil columns to depths of about 1 cm in small microcosms and 2.6 cm in large microcosms. Care was taken to establish the same moisture content and bulk density as in the remainder of the soil columns. Control soil columns contained uninoculated soil. Inoculated microcosms either were left fallow or received six germinated (3 days) wheat (*Triticum aestivum* cv. *Sicco*) seeds which were placed on the top. All microcosms received a thin (5 g) layer of sterile gravel 2 to 3 mm in diameter to prevent channeling of water during percolation and to cover the germinated seeds. The microcosms were incubated vertically in a growth chamber under a 16/8-h light/dark cycle (20/16°C).

Soil percolation. After 4, 7, and 10 days of incubation, small microcosms were subjected to various water flow treatments as outlined below (no flow, low flow, and high flow). Large microcosms were only flushed at low flow rate. Groundwater was used for percolation since during dry periods in the growing season agricultural fields may be irrigated with groundwater. For percolation of the Ede loamy sand columns, slightly acid (pH 6.0) groundwater obtained from the Wageningen pumping station was used, whereas Guelph loam columns were flushed with neutral groundwater (pH 7.0) obtained from a well located at Greensville, Ontario, Canada. Neither of the two water samples contained bacteria able to grow (28°C, 7 days) on C5t-selective King B agar (proteose peptone, 20 g; K_2HPO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 1.5 g; glycerol, 10 g; agar 15 g; water, 1 liter; pH 7.2). In addition, plating on TY agar (tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g; agar, 15 g; water, 1 liter; pH 7.2) revealed that Wageningen water was virtually free of bacteria (<log 1.0 CFU/ml), whereas Greensville water contained about log 3.0 CFU/ml. Groundwater stocks were kept at 4°C until use.

One set of small microcosms with inoculated soil received no water throughout the 10-day incubation period (no flow, control treatment). A second set was brought to saturation on days 4, 7, and 10 by adding groundwater at a flow rate of 3 ml/h, followed by further slow percolation and collection of about 2.5 ml of percolation water in sterile tubes attached to the bottom of the microcosms (low flow, 3-ml/h treatment). On days 4, 7, and 10, a third set was also brought to saturation at 3 ml/h and then flushed at a flow rate of 9 ml/h, until about 9 ml of water was collected (high flow, 9-ml/h treatment). Large microcosms were slowly brought to saturation at 7.5 ml/h and then flushed at the same rate until about 50 ml of percolation water was collected (about 16 h). Small microcosms containing inoculated seeds were either left unpercolated (control of migration along roots) or percolated at low flow rate on day 10.

The groundwater was manually delivered to the center of the soil surface, using gravitational flow and polypropylene microbore tubing (small microcosms) or using a Bio-Rad Econo Column peristaltic pump (large microcosms). Soil microcosms that were not sacrificed (see below) were subjected to additional percolations on subsequent sampling days. In addition, some microcosms were only percolated once on day 10.

Earlier data on the translocation of *P. fluorescens* C5t cells from inoculated Ede loamy sand top layers containing C5t cells either throughout the whole layer or only in the central core (30 to 50% of the volume) through small and large microcosms (10 cm high) suggested that the effect of the cylinder wall on bacterial transport (preferential transport along the soil-wall interphase) was negligible.

Sampling of soil microcosms. About 2 h after each percolation treatment, duplicate small microcosms were dissected in three sections, using a small ethanol-sterilized saw; the top 1 cm (0- to 1-cm layer) in which the C5t strain was originally introduced represented the first sample. The remaining soil column was sliced into two equal sections (1- to 5.5- and 5.5- to 10-cm layers). The samples obtained from all soil layers were homogenized by thoroughly mixing in sterile petri dishes. In samples of planted columns, rhizosphere soil, defined as the soil tightly adhering to roots, was carefully separated from non-rhizosphere soil and also analyzed.

Subsamples, 1 g, of all homogenized samples were shaken vigorously in 9 ml of 0.1% sodium pyrophosphate, using a Vortex mixer (45 s, two times, full speed). Serial dilutions of these soil suspensions were then plated in duplicate on C5t-selective agar (King B supplemented with 50 μg of kanamycin, 50 μg of rifampin, and 100 μg of cycloheximide per ml) and TY agar (supplemented with 100 μg of cycloheximide per ml) for total bacterial counts. Serially diluted percolated water was also plated on these media (small and large microcosms). To detect low cell numbers, 5- and 25-ml volumes of percolation water of large microcosms were also

TABLE 1. Survival and transport of *P. fluorescens* C5t in small Ede loamy sand and Guelph loam microcosms after one percolation with groundwater 4 days after organism introduction[a]

Soil	Depth (cm) of sample or water sample	Log CFU per g of dry soil or ml of percolated water[b]				
		No percolation		Percolation (3 ml/h)		Percolation (9 ml/h) (no plants)
		No plants	Plants	No plants	Plants	
Ede loamy sand						
NR[c]	0–1	6.64	6.57	6.61	6.54	5.99
NR	1–5.5	5.15	4.93	4.88	5.12	4.69
NR	5.5–10	[0][d]	[0]	4.24	4.08	4.35
R[c]	0–1	ND[e]	6.48	ND	6.71	ND
R	1–5.5	ND	5.64	ND	5.13	ND
R	5.5–10	ND	[0]	ND	4.37	ND
	Percolated water	ND	ND	4.67	4.38	4.86
Guelph loam						
NR	0–1	5.58	5.75	5.60	5.74	5.31
NR	1–5.5	[0]	[0]	2.54	3.37*[f]	2.40
NR	5.5–10	[0]	[0]	[0]	2.62*	[0]
R	0–1	ND	6.39	ND	6.00	ND
R	1–5.5	ND	[0]	ND	3.67	ND
R	5.5–10	ND	[0]	ND	2.91	ND
	Percolated water	ND	ND	1.77	3.08*	2.09

[a] Initial (3-h) survival (CFU per gram of dry soil) was log 7.63 in Ede loamy sand and log 6.51 in Guelph loam. All data represent geometric means of duplicate experiments.
[b] No percolation and percolation at 3 and 9 ml/h: no-flow, low-flow, and high-flow treatments, respectively.
[c] NR, Nonrhizosphere soil; R, rhizosphere soil.
[d] [0] = arithmetic zero; counts below the limit of detection (about log 1.6 per g of dry soil).
[e] ND, Not determined, as no rhizosphere soil or percolated water was present.
[f] *Significantly higher than counts in corresponding samples without plants ($P < 0.05$).

filtered over a 0.45-μm nitrocellulose filter, and filters were placed on C5t-selective plates. Petri plates were incubated inverted at 28°C for 24 (total counts) or 48 (selective C5t counts) h. All counts from soil were calculated on a soil dry weight basis. Rhizosphere soil counts were corrected for the weight of the roots present.

Immunofluorescence (IF) counting of C5t. A specific anti-R2f antiserum was raised in a rabbit as described previously (20). After purification, it was conjugated with fluorescein isothiocyanate as described previously (1). Antiserum dilutions were made in phosphate-buffered saline supplemented with 0.05% sodium azide. A 1:25 antiserum dilution was used for studying soil specimens. The conjugated antiserum only cross-reacted with two *P. putida* strains from our collection; however, no staining of other pseudomonads, *Escherichia coli*, *Enterobacter cloacae*, *Bacillus* spp., or *Rhizobium* spp. was observed. Cross-reacting (background) bacteria in control (uninoculated) soils were at or slightly above the limit of detection (about 10^3 to 10^4 cells per g of dry soil).

Soil sampling, flocculation, IF sample preparation, and enumeration were done as described by Postma et al. (20) for sand and clay soil. Results were corrected for background (control) counts.

Analysis of colonies by hybridization. On day 10, colonies grown on C5t-selective agar were screened for the presence of the *tox* fragment, using colony filter hybridization (16). The 4.3-kilobase *tox* fragment used as a probe was obtained by *Bam*HI digestion of plasmid pSUP101::Tn*5*::*tox*, isolated and purified by standard procedures (16), followed by electroelution with an electrophoretic sample concentrator (model M50; ISCO, Lincoln, Neb.). After phenol-chloroform extraction, ethanol precipitation, and dissolution, the purified fragment was radioactively labeled by nick translation, using [^{32}P]ATP (16).

Colony lifts, using nitrocellulose filters, and subsequent hybridizations were performed by a modification of the method of Grunstein and Hogness (16, 36a). After both low- and high-stringency washes (16), autoradiograms were obtained by exposing Kodak XAR film at −80°C, using an intensifying screen.

Statistical treatment of data. Analysis of variance was used on subsets of data to check for significant differences due to the experimental factors used (percolation regimen and presence or absence of plants).

RESULTS

Organism survival in Ede loamy sand and Guelph loam. Only a fraction of the added C5t cells was recoverable as culturable cells after introduction into both soils, since the numbers of CFU after 3 h (denoted as zero counts) were 3 (Ede loamy sand)- to 30 (Guelph loam)-fold lower than the added cell densities. In both soils, the C5t cell densities per gram of dry topsoil then showed a gradual decline over the 10-day incubation period: in Ede loamy sand, from log 7.63 to log 4.06; and in Guelph loam, from log 6.51 to log 5.32 (Tables 1 to 3).

Wheat plants grew well in both soils in the small microcosms; after 10 days, plants in Ede loamy sand were 10 to 15 cm in height and plants in Guelph loam were 15 to 20 cm. As from day 4, roots could be obtained from all soil layers in both soils. The presence of wheat roots in the top layers led to a 2.5- to 4.4-fold increase in C5t cell densities in Guelph loam after 4, 7, and 10 days, whereas in Ede loamy sand a 12-fold increase was only noted at day 10 (Tables 1 to 3).

Determination of the C5t cell densities in the top layers of both soils by IF at day 10 revealed that population sizes in the Ede loamy sand were significantly ($P < 0.05$) greater (up to 100-fold) than those determined by selective plating (Table 3); in Guelph loam, only slight (insignificant) differences were noted between IF counts and selective plate counts (Table 3).

TABLE 2. Survival and transport of *P. fluorescens* C5t in small Ede loamy sand and Guelph loam microcosms after two percolations with groundwater 7 days after organism introduction

Soil	Depth (cm) of sample or water sample	Log CFU per g of dry soil or ml of percolated water[a]				
		No percolation		Percolation (3 ml/h)		Percolation (9 ml/h) (no plants)
		No plants	Plants	No plants	Plants	
Ede loamy sand						
NR[b]	0–1	4.86	5.38	5.74	5.82	5.76
NR	1–5.5	[0][c]	1.90	3.85	4.44	4.40
NR	5.5–10	[0]	[0]	3.52	3.66	4.24
R[b]	0–1	ND[d]	5.48	ND	5.81	ND
R	1–5.5	ND	[0]	ND	4.66	ND
R	5.5–10	ND	[0]	ND	3.77	ND
	Percolated water	ND	ND	4.06	3.76	4.49
Guelph loam						
NR	0–1	5.49	5.88	5.34	5.58	5.50
NR	1–5.5	[0]	[0]	[0]	3.36*[e]	1.60
NR	5.5–10	[0]	[0]	1.60	2.55*	[0]
R	0–1	ND	6.23	ND	5.74	ND
R	1–5.5	ND	1.60	ND	3.60	ND
R	5.5–10	ND	[0]	ND	2.81	ND
	Percolated water	ND	ND	0.70	1.83*	1.00

[a] All data represent geometric means of duplicate experiments. No percolation and percolation at 3 and 9 ml/h: no-flow, low-flow, and high-flow treatments, respectively.
[b] NR, Nonrhizosphere soil; R, rhizosphere soil.
[c] [0] = arithmetic zero; counts below the limit of detection (about log 1.6 per g of dry soil).
[d] ND = not determined, as no rhizosphere soil or percolated water was present.
[e] * Significantly higher than counts in corresponding samples without plants ($P < 0.05$).

Transport of introduced cells by plant roots or percolation. Data obtained in unpercolated soil columns after 7 and 10 days (Tables 2 and 3) revealed that the introduced bacterial cells had hardly moved down the soil column in both unplanted soils, whereas transport and survival of low numbers of introduced bacteria were observed in the 1- to 5.5-cm layers of planted columns of both soils. However, at day 4, introduced C5t cells were recoverable from the unplanted Ede loamy sand 1- to 5.5-cm layer, at cell densities of about 3% of the inoculated top layers.

Upon percolation, cells were readily transported to the 1- to 5.5- and 5.5- to 10-cm layers and to the percolation water

TABLE 3. Survival and transport of *P. fluorescens* C5t in small Ede loamy sand and Guelph loam microcosms after three percolations with groundwater 10 days after organism introduction

Soil	Depth (cm) of sample or water sample	Log CFU or cells per g of dry soil or ml of percolated water[a]					
		No percolation		Percolation (3 ml/h)			Percolation (9 ml/h) (no plants)
		No plants	Plants	No plants	Plants	Plants[b]	
Ede loamy sand							
NR[c]	0–1	4.06 (6.06)	4.04	5.44 (6.30)	5.26	3.81	5.04 (6.29)
NR	1–5.5	[0] ([0])[d]	1.60	4.08 (5.23)	3.67	2.28	4.00 (5.52)
NR	5.5–10	[0] ([0])	[0]	3.25 (4.61)	3.37	1.84	3.10 (4.93)
R	0–1	ND[e]	5.12	ND	5.72	4.33	ND
R	1–5.5	ND	2.11	ND	3.83	2.66	ND
R	5.5–10	ND	[0]	ND	3.69	1.60	ND
	Percolated water	ND	ND	3.93 (3.43)	3.99	3.06	3.80 (3.35)
Guelph loam							
NR	0–1	5.32 (5.57)	4.99	5.40 (5.58)	5.45	5.49	5.46
NR	1–5.5	1.82 ([0])	[0]	2.09 ([0])	3.96*[f]	3.83	[0]
NR	5.5–10	[0] ([0])	[0]	[0] ([0])	3.00*	3.22	[0]
R	0–1	ND	5.34	ND	5.34	5.89	ND
R	1–5.5	ND	2.02	ND	3.96	3.84	ND
R	5.5–10	ND	[0]	ND	3.56	3.43	ND
	Percolated water	ND	ND	[0] (2.76)	2.62* (3.06)	3.28	1.48

[a] All data represent geometric means of duplicate experiments. IF counts are given in parentheses. No percolation and percolation at 3 and 9 ml/h: no-flow, low-flow, and high-flow treatments, respectively.
[b] Percolated for the first time on day 10.
[c] NR, Nonrhizosphere soil; R, rhizosphere soil.
[d] [0] = arithmetic zero; counts below the limit of detection (about log 1.6 CFU or log 4.5 cells [IF] per g of dry soil and log 1.0 CFU or log 2.0 cells per ml of percolated water).
[e] ND, Not determined, as no percolated water or rhizosphere was present.
[f] *Significantly higher than counts in corresponding samples without plants ($P < 0.05$).

TABLE 4. C5t cells detected in percolation water obtained from small microcosms as a percentage of the culturable C5t populations present in the inoculated top layers

Water flush no. (day)	Ede loamy sand			Guelph loam		
	Percolation (3 ml/h)		Percolation (9 ml/h) (no plants)	Percolation (3 ml/h)		Percolation (9 ml/h) (no plants)
	No plants	Plants		No plants	Plants	
1 (4)	0.4	0.2	8.0	0.01	0.13	0.07
2 (7)	1.5	0.3	5.0	0.001	0.02	0.005
3 (10)	1.1	1.9	6.2	0	0.08	0.013
1 (10)	5.0	7.1	ND	ND	0.21	ND

[a] Percolation at 3 and 9 ml/h: low- and high-flow treatments, respectively. ND, Not determined.

in Ede loamy sand, regardless of the presence or absence of wheat plants, the percolation regimen, or the number of water flushes (Tables 1 to 3). There was no significant difference ($P < 0.05$) in the C5t cell densities in these samples between planted and unplanted Ede loamy sand columns. Percolation of Ede loamy sand at a high flow rate resulted in the elution of 2.6- to 8-fold more C5t cells than percolation at a low flow rate. In percolated day 10 Ede loamy sand samples, total C5t cell populations determined by IF exceeded those determined by selective plating by 7- to 68-fold, suggesting the presence of nonculturable cells (2). The presence of excess water due to percolation apparently stimulated bacterial survival in the Ede loamy sand top layers after 7 and 10 days (Tables 2 and 3), since C5t cell densities were significantly higher ($P < 0.05$) in these samples than in corresponding unpercolated soil samples.

Percolation also induced transport of low numbers of introduced cells to lower soil layers and into the percolation water in Guelph loam columns; the presence of wheat roots significantly ($P < 0.05$) enhanced the numbers of C5t cells encountered in the rhizosphere and nonrhizosphere samples from the 1- to 5.5- and 5.5- to 10-cm layers and in the percolated water, as compared with the unplanted columns (Tables 1 to 3). In the percolated Guelph loam samples (day 10), there was no pronounced difference between the total (IF) and the culturable C5t cell populations, except for the percolated water of the unplanted columns (Table 3).

The C5t cell densities observed in the lower Guelph loam layers after low-flow percolation either were below the limit of detection (about log 1.6 per g of dry soil) or ranged from 0.001 to 0.2% of those in the inoculated top layers (Tables 1 to 3); in contrast, the cell densities in the Ede loamy sand lower layers ranged from 0.2 to 7.1% of those in the top layers. In addition, the culturable C5t cells in the percolation water as a percentage of the culturable C5t population in the top layers were consistently lower in the Guelph loam than in Ede loamy sand (Table 4) in all treatments. The presence of plant roots again increased bacterial translocation in Guelph loam, but not in Ede loamy sand, whereas increased flow rate resulted in the elution of higher percentages in both soils.

For both soils, the patterns of distribution of the introduced C5t cells throughout the columns were largely determined after the first groundwater flush, since subsequent percolations did not substantially modify these patterns (Tables 1 to 3); the differences in cell numbers noted in the subsequent samples may be due to progressive loss of viability of cells. However, C5t cells were generally detectable in the percolation water even after two and three percolations (Tables 2 and 3).

TABLE 5. Transport of *P. fluorescens* C5t through large Ede loamy sand soil microcosms

Water flush no. (day)	Log CFU per 50 ml of percolated water[a]	
	C5t	Total CFU
1 (4)	5.07 (0.04)	6.20
2 (7)	<2.40 (ND)	ND
3 (10)	1.04 (0.0014)	7.37

[a] All data represent geometric means of duplicate experiments. C5t numbers as percentages of cells in the inoculated top layers are given in parentheses. ND, Not determined.

Transport in large soil microcosms. After percolation of the large Ede loamy sand columns, low numbers of C5t cells were also detectable in the percolation water. After one percolation, log 5.07 CFU, or about 0.04% of C5t cells in the top layers, were detected in the percolation water (Table 5). No C5t cells were found in the percolation water of the second flush. Filtration of percolation water obtained after the third flush at day 10 permitted the detection of log. 1.04 CFU of C5t or 0.0014% of the cells in the soil top layers.

Transport of seed-inoculated cells. Without percolation, there was transport of the seed-inoculated C5t strain only to the layer immediately below the top 1 cm carrying the inoculated seeds in Ede loamy sand and Guelph loam (Table 6). The C5t cell numbers observed in these layers were higher in Ede loamy sand than in Guelph loam, even though the numbers in the top layers were higher in the Guelph loam. After percolation of the Guelph loam soil (low flow), there was a significant increase in C5t cell numbers in the lower soil layers, in both the rhizosphere and nonrhizosphere soil samples ($P < 0.05$). In addition, C5t cells were detectable in the percolation water of this soil at a density of log 4.51 per ml (Table 6).

Total counts. All counts, including counts obtained in the uninoculated (control) soil columns, of samples of the two soils were roughly in the range log 7.0 to log 9.0 per g of dry soil (data not shown). There was no significant ($P < 0.05$) difference in the total counts in the various soil layers irrespective of the percolation regimen, as variations in these

TABLE 6. Transport of *P. fluorescens* C5t in small Ede loamy sand and Guelph loam microcosms after seed inoculation

Soil	Depth (cm) of sample or water sample	Log CFU per g of dry soil or ml of percolated water[a]			
		No percolation		Percolation (3 ml/h)	
		NR	R	NR	R
Ede loamy sand	0–1	5.54	6.31	ND[b]	ND
	1–5.5	4.53	3.71	ND	ND
	5.5–10	[0][c]	[0]	ND	ND
Guelph loam	0–1	6.64	6.90	6.52	6.77
	1–5.5	2.07	1.77	4.48	4.85
	5.5–10	[0]	[0]	4.15	4.15
	Percolated water (Guelph loam)	ND		4.51	

[a] All data represent geometric means of duplicate experiments. NR, Nonrhizosphere soil; R, rhizosphere soil.

[b] ND, Not determined.

[c] [0] = arithmetic zero; below the limit of detection (about log 1.6 per g of dry soil).

counts were high. In the percolation water of the two soils, about log 4.0 to log 5.0 CFU/ml were detected in both percolation regimens, i.e., low and high flow.

Stability of *tox* marker in introduced cells. All of >500 fluorescent colonies grown on selective KB agar obtained from day 10 samples from no-flow, low-flow, and high-flow treatments showed a hybridization signal to the 4.3-kilobase *tox* fragment used as a probe, suggesting the stable presence of Tn*5*::*tox* in the cells introduced into both soils in the soil columns under the conditions used. Several white slimy colonies which also appeared on selective King B agar did not react with the probe, suggesting that these were indigenous soil microorganisms resistant to rifampin and kanamycin.

DISCUSSION

This study assessed the usefulness of vertical soil microcosms for studying the survival and transport of a genetically engineered *P. fluorescens* strain through two soils of different textures in the presence and absence of wheat roots and groundwater flow. The conditions of water flow (3 ml, or 4 mm/h) and the use of groundwater were chosen so as to mimic agricultural practices.

The observed slow decline of C5t cell densities in both the unpercolated Ede loamy sand and Guelph loam is in agreement with previous findings on the survival of introduced pseudomonads in soil (3, 36, 38b; van Elsas and Trevors, in press). Adverse biological factors such as competition, antagonism, predation, and niche exclusion and abiotic factors, which are both variable in time and space, likely caused the differential survival in the Ede loamy sand and Guelph loam. The enhanced survival in the percolated Ede loamy sand may have been due to colonization of previously unoccupied niches followed by growth. The observation in Ede loamy sand of IF-stainable cell populations 100-fold larger than the populations culturable on C5t-selective agar suggested the occurrence in this soil of nonculturable cells, like those described by Colwell et al. (2) for bacteria introduced into aquatic systems. It is unknown whether these populations represent viable, dying, or already dead cells. Death of cells in soil is difficult to measure and is often a complex process (19).

Movement of the introduced bacterial cells in the absence of groundwater flow was only detected in the presence of plant roots, but was virtually absent in unplanted soil; the detection of C5t cells on day 4 in the 1- to 5.5-cm layer (Table 1) of Ede loamy sand may have been due to sample cross-contamination. The lack of transport observed was in line with previous findings, which have indicated a general lack of movement of introduced bacteria in unplanted soils kept at constant moisture content (15, 21, 38a). Physical barriers in soil probably restrict bacterial movement unless conditions are extremely moist or water flow is present.

The presence of growing wheat roots influenced the distribution of the introduced cells in unpercolated columns of both soils, enhancing downward transport. The low cell densities found in the 1- to 5.5-cm soil layers were indicative of transport and survival of low numbers of introduced cells with the roots. Similar observations have been made before (39). The observation that plant roots can mediate cell movement may be of value in soil or seed inoculation strategies when introducing beneficial organisms into uncontained environments, such as agricultural soils.

The detected translocation of the introduced C5t cells by percolating groundwater in both soils is in agreement with previous data on bacterial transport by percolation (11, 15, 22, 24). The observation that the patterns of distribution of the introduced C5t cells, after the first percolation, did not change substantially following subsequent percolations but that nevertheless C5t cells were detected in the water samples after these flushes (Tables 1 to 3) suggested that after one flush the bulk of the introduced population may have adhered to soil particles or entered relatively protected soil pores; a minor part of the population present in more open soil spaces may have been eluted by the later percolations. The observed differences between the two soils in the cell numbers eluted into the percolated water may have been caused by either a higher retention of bacterial cells in a loam matrix as opposed to a sand matrix or by a difference in the dynamics (survival, growth, and death) of C5t populations in both soil and water. The ready elution of similar numbers of C5t cells from Ede loamy sand, irrespective of the presence of roots, and the clear influence of plant roots on the distribution patterns in the Guelph loam seemed to indicate that soil texture was an important factor. The stimulation of transport in planted Guelph loam might then be attributed to a facilitation of transport of cells with water along root channels.

The observation that, in both soils, water flow significantly affected the rhizosphere distribution of the introduced strain supports the observation of Parke et al. (18); they suggested that vertical water flow may be necessary for optimal establishment of an inoculum on a developing root system. Further, the detection of C5t cells in the percolation water of the large (26 cm) Ede loamy sand columns suggested the usefulness and predictive value of this type of simple soil microcosms. The use of filtration in conjunction with selective plating permitted the screening of large volumes of percolation water, lowering the limit of detection 50- to 250-fold. Contamination of groundwater (often used as drinking water for humans and domestic animals) with organisms introduced into field soil is undesirable, especially when the effect of the introduced organism in the food chain is unknown. However, this study also showed that both soils had a good filtering capacity, retaining in a 9-cm pathway >90% of surviving introduced cells.

It has been argued that undisturbed soil cores have better predictive value for field situations than repacked ones (24). Water flow through soil cracks and macropores probably is an important factor present in the field (24, 27), which is included in such cores. However, repacked cores such as the ones used in this study provide reproducibility and homogeneity, permitting the study of survival of bacterial cells and transport through the soil matrix. The basic information obtained should then be compared with data on bacterial movement in undisturbed soil columns. The soil microcosms used in this study could easily be adapted to accommodate such cores.

Soil microcosms similar to those used in this study have also been used by Madsen and Alexander (15). However, columns were treated with one simulated rainfall and GEMs were not used. Soil microcosms will become increasingly important for rapid, accurate, and reliable testing of survival and transport of, and genetic transfer by, GEMs which are being considered for release into uncontained environments. The use of different kinds of soil microcosms in which plant roots were physically separated from soil for studies on organism survival and genetic transfer has been described earlier (5, 12, 37, 38).

The soil microcosms used in this study may be modified by increasing the column length and diameter, to accommo-

date more soil and provide a longer soil profile. Parameters such as soil type, the type of water used (i.e., rainwater, groundwater, irrigation water, or chemical spray water), the rate of water flow and the number of applications and volumes of water applied, the type of plant cover, as well as the type of GEM and its cell density, and the application method can all be tested depending on the type of information required.

ACKNOWLEDGMENTS

This research was supported by a NATO Collaborative Research Grant awarded to J.D.v.E. and J.T.T. Research by J.T.T. is also supported by a Natural Sciences and Engineering Research Council (Canada) operating grant.

We thank A. M. Feldmann and O. de Leeuw for technical assistance with the construction of strain C5t.

LITERATURE CITED

1. **Allan, E., and A. Kelman.** 1977. Immunofluorescent stain procedures for detection and identification of *Erwinia carotovora* var *atroseptica*. Phytopathology **67**:1305–1312.
2. **Colwell, R. R., P. R. Brayton, D. J. Grimes, D. B. Roszak, S. A. Huq, and L. M. Palmer.** 1985. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. Biotechnology **3**:817–820.
3. **Compeau, G., B. J. Al-Achi, E. Platsouka, and S. B. Levy.** 1988. Survival of rifampin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. Appl. Environ. Microbiol. **54**:2432–2438.
4. **Davison, J.** 1988. Plant beneficial bacteria. Biotechnology **6**: 282–286.
5. **Dijkstra, A. F., J. M. Govaert, G. H. N. Scholten, and J. D. Van Elsas.** 1987. A soil chamber for studying the bacterial distribution in the vicinity of roots. Soil Biol. Biochem. **19**:351–352.
6. **Dijkstra, A. F., G. H. N. Scholten, and J. A. Van Veen.** 1987. Colonization of wheat seedling (*Triticum aestivum*) roots by *Pseudomonas fluorescens* and *Bacillus subtilis*. Biol. Fertil. Soils **4**:41–46.
7. **Drahos, D. J., B. C. Hemming, and S. McPherson.** 1986. Tracking recombinant organisms in the environment: β-galactosidase as a selectable non-antibiotic marker for fluorescent pseudomonads. Biotechnology **4**:439–444.
8. **Festl, H., W. Ludwig, and K. H. Schleifer.** 1986. DNA hybridization probe for the *Pseudomonas fluorescens* group. Appl. Environ. Microbiol. **52**:1190–1194.
9. **Griffin, D. M., and G. Quail.** 1968. Movement of bacteria in moist, particulate systems. Aust. J. Biol. Sci. **21**:579–582.
10. **Gurijala, K. R., and M. Alexander.** 1988. Role of sublethal injury in decline of bacterial populations in lake water. Appl. Environ. Microbiol. **54**:2859–2861.
11. **Hagedorn, C., E. L. McCoy, and T. M. Rahe.** 1981. The potential for groundwater contamination from septic effluents. J. Environ. Qual. **10**:1–8.
12. **Hartel, P. G., J. W. Billingsley, and J. W. Williamson.** 1989. Styrofoam cup-membrane assembly for studying microorganism root interactions. Appl. Environ. Microbiol. **55**:1291-1294.
13. **Holben, W. E., J. K. Jansson, B. K. Chelm, and J. M. Tiedje.** 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. **54**:703–711.
14. **Iswandi, A., P. Bossier, J. Vandenabeele, and W. Verstraete.** 1987. Effect of seed inoculation with the rhizopseudomonad strain 7NSK2 on the root microbiota of maize (*Zea mays*) and barley (*Hordeum vulgare*). Biol. Fertil. Soils **3**:153–158.
15. **Madsen, E. L., and M. Alexander.** 1982. Transport of *Rhizobium* and *Pseudomonas* through soil. Soil Sci. Soc. Am. J. **46**: 557–560.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Ogram, A., G. S. Sayler, and T. Barkay.** 1987. The extraction and purification of microbial DNA from sediments. J. Microbiol. Methods **7**:57–66.
18. **Parke, J. L., R. Moen, A. D. Rovira, and G. D. Bowen.** 1986. Soil water flow affects rhizosphere distribution of a seed-borne biological control agent, *Pseudomonas fluorescens*. Soil Biol. Biochem. **18**:583–588.
19. **Postgate, J.** 1989. A microbial way of death. New Sci. **20**:43–47.
20. **Postma, J., J. D. Van Elsas, J. M. Govaert, and J. A. Van Veen.** 1988. The dynamics of *Rhizobium leguminosarum* biovar *trifolii* introduced into soil as determined by immunofluorescence and selective plating techniques. FEMS Microbiol. Ecol. **53**:251–260.
21. **Postma, J., S. Walter, and J. A. Van Veen.** 1989. Influence of different initial soil moisture contents on the distribution and population dynamics of introduced *Rhizobium leguminosarum* biovar *trifolii*. Soil Biol. Biochem. **21**:437–442.
22. **Schaub, S. A., and C. A. Sorber.** 1977. Virus and bacteria removal from wastewater by rapid infiltration through soil. Appl. Environ. Microbiol. **33**:609–619.
23. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Biotechnology **1**:784–791.
24. **Smith, M. S., G. W. Thomas, R. E. White, and D. Ritonga.** 1985. Transport of *Escherichia coli* through intact and disturbed soil columns. J. Environ. Qual. **14**:87–91.
25. **Soby, S., and K. Bergman.** 1983. Motility and chemotaxis of *Rhizobium meliloti* in soil. Appl. Environ. Microbiol. **46**:995–998.
26. **Steffan, R. J., and R. M. Atlas.** 1988. DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. Appl. Environ. Microbiol. **54**:2185–2191.
27. **Thomas, G. W., and R. E. Phillips.** 1979. Consequences of water movement in macropores. J. Environ. Qual. **8**:149–152.
28. **Tiedje, J. M., R. K. Colwell, Y. L. Grossman, R. E. Hodson, R. E. Lenski, R. N. Mack, and P. J. Regal.** 1989. The planned introduction of genetically engineered organisms: ecological considerations and recommendations. Ecology **70**:298–315.
29. **Torsvik, V. L., and J. Goksoyr.** 1978. Determination of bacterial DNA in soil. Soil Biol. Biochem. **10**:7–12.
30. **Trevors, J. T.** 1985. DNA probes for the detection of specific genes in bacteria isolated from the environment. Trends Biotechnol. **3**:291–293.
31. **Trevors, J. T.** 1987. Survival of *Escherichia coli* donor, recipient and transconjugant cells in soil. Water Air Soil Pollut. **34**: 409–414.
32. **Trevors, J. T., T. Barkay, and A. W. Bourquin.** 1987. Gene transfer among bacteria in soil and aquatic environments: a review. Can. J. Microbiol. **33**:191–198.
33. **Trevors, J. T., and G. Berg.** 1989. Conjugal RP4 transfer between pseudomonads in soil and recovery of RP4 plasmid DNA from soil. Syst. Appl. Microbiol. **11**:223–227.
34. **Trevors, J. T., and K. M. Oddie.** 1986. R-plasmid transfer in soil and water. Can. J. Microbiol. **32**:610–613.

34a. **Trevors, J. T., and J. D. Van Elsas.** 1989. A review of selected methods in environmental microbial genetics. Can. J. Microbiol. **35**:895–902.

35. **Trevors, J. T., J. D. Van Elsas, M. E. Starodub, and L. S. Van Overbeek.** 1989. Survival of and plasmid stability in *Pseudomonas* and *Klebsiella* spp. introduced into agricultural drainage water. Can. J. Microbiol. **35**:675–680.
36. **Van Elsas, J. D., A. F. Dijkstra, J. M. Govaert, and J. A. Van Veen.** 1986. Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into two soils of different texture in field microplots. FEMS Microbiol. Ecol. **38**:151–160.

36a. **Van Elsas, J. D., M. Nikkel, and L. S. Van Overbeek.** 1989. Detection of plasmid RP4 transfer in soil and rhizosphere, and the occurrence of homology to RP4 in soil microorganisms. Curr. Microbiol. **19**:375–381.

37. **Van Elsas, J. D., J. T. Trevors, and M. E. Starodub.** 1988. Plasmid transfer in soil and rhizosphere, p. 88–89. *In* W.

Klingmuller (ed.), Risk assessment for deliberate releases. Springer-Verlag, Berlin.

38. **Van Elsas, J. D., J. T. Trevors, and M. E. Starodub.** 1988. Bacterial conjugation between pseudomonads in the rhizosphere of wheat. FEMS Microbiol. Ecol. **53**:299–306.

38a. **Van Elsas, J. D., J. T. Trevors, M.-E. Starodub, and L. S. Van Overbeek.** 1990. Transfer of plasmid RP_4 between pseudomonads after introduction into soil; influence of spatial and temporal aspects of inoculation. FEMS Microbiol. Ecol. **73**:1–12.

38b. **Van Elsas, J. D., J. T. Trevors, L. S. Van Overbeek, and M.-E. Starodub.** 1989. Survival of *Pseudomonas fluorescens* containing plasmids RP4 or pRK2501 and plasmid stability after introduction into two soils of different texture. Can. J. Microbiol. **35**:951–959.

39. **Weller, D. M.** 1984. Distribution of a take-all suppressive strain of *Pseudomonas fluorescens* on seminal roots of winter wheat. Appl. Environ. Microbiol. **48**:897–899.

40. **Yates, J. R., J. H. Lobos, and D. S. Holmes.** 1985. The use of genetic probes to detect microorganisms in biomining operations. J. Ind. Microbiol. **1**:129–135.