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Bacterial conjugation between *Escherichia coli* and *Pseudomonas* spp. donor and recipient cells in soil

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SUMMARY

Experiments conducted in microcosms containing loam soil samples inoculated with either *E. coli* or *Pseudomonas* spp. donor and recipient cells showed that bacterial cells survived and conjugated over a 24-h incubation period. *E. coli* transconjugants were detected 6 h after donor and recipient strains were introduced into sterile soil samples. In non-sterile soil samples, transconjugants were detected between 8 and 24 h incubation. *Pseudomonas* transconjugants were recovered from sterile soil samples between 6 and 12 h after their introduction and as early as 2 h in non-sterile soil. The results show that genetic interactions occur in non-sterile soil in relatively short periods of time at relatively high transfer frequencies (10^{-3} to 10^{-4}). Studies on genetic interactions in soil are becoming necessary in risk assessment/environmental impact studies prior to the release of genetically engineered or modified organisms into uncontained environments.

INTRODUCTION

The use of molecular biology techniques allows the directed change of a microorganism's genome. Some of these microorganisms may have uses in environment biotechnology. Therefore, an understanding of gene transfer in the natural environment is necessary.

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While genetic transfer between laboratory strains of bacteria has been studied extensively in vitro, the same cannot be said for in situ genetic transfer between environmental isolates and/or laboratory strains. Studies have shown that genetic transfer can occur in natural substrates such as soil, surface waters, and sewage [4,5,8,9,17]. However, few of these studies have been quantitative, and even fewer have been made in untreated (i.e., non-sterile environments) [14]. Difficulties occur when working in aquatic and terrestrial microcosms; such as assessing the numbers and types of indigenous microor-

ganisms. Therefore, research must be conducted to develop methodologies for working with natural substrates, and for obtaining quantitative results on gene transfer.

Plasmids have been found in a wide variety of bacterial species [1,3,7,15]. Since plasmids can be transferred to and maintained in organisms without homologous deoxyribonucleic acid (DNA) sequences, it is thought that conjugation may be an important mechanism of gene transfer in the natural environment [10]. Another important feature of plasmid transfer is that non-conjugative plasmids, and chromosomal DNA, can be mobilized into other cells by conjugative plasmids. Conjugation could, therefore, be an important source of horizontal gene transfer to indigenous microorganisms, and such transfer into indigenous species is a major concern in risk assessment and releasing genetically engineered microorganisms into the environment. Whether such transfers will be a problem will depend on numerous factors including the stability of introduced organisms in the natural environment, their reproduction rate(s), ability to transfer genes in such an environment and their effect(s) on the ecosystem.

The aim of this research was to study bacterial gene transfer *via* conjugation in an agriculturally important loam soil incubated under various conditions.

MATERIALS AND METHODS

Bacterial strains

Escherichia coli K-12 C600 Nal^r, Lac^-, F^- plasmidless, and *E. coli* MA527 Lac^+, bearing Sm^r (streptomycin resistance) and Tc^r (tetracycline resistance) on a 60 megadalton (Mdal) conjugative plasmid were used as recipient and donor organisms, respectively. A *Pseudomonas* sp. producing yellow-green fluorescent pigment was provided by J.D. van Elsas, ITAL, Wageningen, The Netherlands. A rifampicin and streptomycin resistant mutant was selected to serve as a recipient in mating experiments [16]. The 36 MDa, conjugative plasmid, RP4 (specifying resistance to carbenicillin, kanamycin, neomycin and tetracycline), was introduced as described by Simon et al. [11] into the original soil isolate. This was used as a donor strain in conjugation experiments.

Media and culture conditions

E. coli C600 was maintained on nutrient agar (Acumedia, Baltimore) amended with 200 μg/ml nalidixic acid. *E. coli* MA527 was maintained on nutrient agar amended with 50 μg/ml tetracycline. The *Pseudomonas* sp. donor strain was maintained on King's B agar with 80 μg/ml each of tetracycline and kanamycin. The *Pseudomonas* sp. recipient strain was maintained on King's B agar with 50 μg/ml of rifampicin. King's B media contains per litre of water: 20 g Bactopeptone (Difco), 1.5 g $MgSO_4 \cdot 7H_2O$, 1.5 g K_2HPO_4, and 10 g glycerol. This was solidified by the addition of 1.5% (w/v) agar (Gibco, Burlington, Canada).

For all mating experiments, both donor and recipient cells were grown 18 to 20 h with shaking at 120 rpm in 10 ml of broth contained in 40 ml bottles (with appropriate inhibitors). *E. coli* cultures were grown in nutrient broth (Gibco) at 37°C with shaking at 120 rpm. *Pseudomonas* spp. were grown in King's B broth at 30°C with shaking at 120 rpm.

Enumeration of the various *E. coli* cell types was accomplished by spread plating on MacConkey agar (Acumedia) containing either 50 μg/ml of tetracycline (*E. coli* MA527), or 200 μg/ml nalidixic acid (*E. coli* C600), or both inhibitors (for *E. coli,* transconjugants). Enumeration of the *Pseudomonas* cell types was done by spreadplating on King's B agar containing either 80 μg/ml each of tetracycline and kanamycin (donor), or 50 μg/ml of rifampicin (recipient), or all three inhibitors (*Pseudomonas* transconjugants).

Conjugation in sterile soil

Two grams of air-dried loam soil (35% sand, 26% clay, 39% silt; pH 6.5) were placed in screw-capped Bijou bottles (7-ml capacity). The bottles were autoclaved for 1 h on each of three consecutive days. Sterile distilled water was added to each soil sample to adjust it to 60% of its water-holding capacity after addition of donor and recipient cell sus-

pensions. These soil aliquots were allowed to equilibrate for 10 to 14 days prior to the addition of cells, to help alleviate any effects due to repeated autoclaving.

Experimental cells were harvested by centrifugation at 4000 × *g* for 7 min. The growth medium was decanted and the cell pellet resuspended in sterile 0.15 M phosphate buffer (pH 7.0). From this suspension, a 1:10 dilution, was made in the same buffer. To each soil sample was added 33 μl of each of the donor and recipient cells 1:10 dilutions. This volume was used in order to add approximately 10^5 to 10^6 cells of each strain per g of soil. Sixty-six μl of buffer was added to control soil samples. The zero time samples were placed on ice immediately after preparation to prevent conjugation. All other samples were incubated at 30°C in the dark. The control samples were incubated for 24 h. At the end of each incubation period, the sample was placed on ice (to stop conjugation) and 5 ml of buffer was added to each bottle. Cells were dislodged from the soil by agitating the bottle and its contents for 3 min on a Mickle high-speed shaker. Soil particles were allowed to settle for 1 min and aliquots of the suspended cells were serially diluted and/or plated on various selective media (see Media and culture conditions section).

Conjugation in non-sterile soil

Matings in non-sterile soil were performed as in sterile soil except samples were not sterilized by autoclaving. Care was taken to not introduce any non-indigenous microorganisms into the soil samples. Plasmid transfer frequencies were calculated as the ratio of transconjugants to donor cells [13,16].

Plasmid isolation

To verify that transfer of the R-plasmids had occurred, plasmid isolation by the alkaline-sodium dodecyl sulfate method [6] were carried out on at least 10 representative transconjugants. Plasmid DNA was detected by horizontal, agarose gel (0.7% w/v) electrophoresis using a Tris (hydroxymethyl) aminomethane-boric acid-EDTA buffer (pH 8.0) [14]. DNA was visualized by staining gels in an aqueous solution of ethidium bromide (1 μg/ml) for 1 h and visualized on a Spectroline 302 nm ultraviolet transilluminator.

RESULTS AND DISCUSSION

Two test systems were used to assess conjugal plasmid transfer and organism survival in soil. One mating system used two strains of *E. coli,* while the other system used two strains of pseudomonads derived from the same soil isolate. The *E. coli* system served as an example of a bacterial species typically not found in the soil environment, while the *Pseudomonas* spp. represented organisms commonly

Table 1

R-plasmid transfer from *E. coli* MA527 to *E. coli* C600 in sterile loam soil incubated at 30°C

Log number CFU/g soil[a]				Plasmid transfer frequency
Time (h)	Donor	Recipients	Transconjugants	
0	5.97	5.18	[0][b]	[0]
2	6.07	5.92	[0]	[0]
6	7.49	7.02	5.15	4.57×10^{-3}
12	8.96	7.86	6.19	1.70×10^{-3}
24	8.54	7.61	6.21	4.68×10^{-3}
Control	[0]	[0]	[0]	[0]

[a] Average of 4 counts from duplicate experiments.

[b] Arithmetic zero [0].

found in soil. Randomly selected transconjugants were screened for plasmid DNA. In all isolates examined, the 36 MDa RP4 and 60 MDa plasmid were observed in *Pseudomonas* and *E. coli* transconjugants respectively (photographs of agarose gels not shown). Plasmid transfer frequencies of 4.57×10^{-3} (Table 1) and zero (Table 2) between *E. coli* cells were observed in sterile soil and non-sterile soil samples after 6 h, respectively.

Transconjugants were recovered from soil samples without any nutrient amendment of the soil, which was previously found to be necessary to obtain *E. coli* transconjugants in sterile sandy loam soil samples [13]. The requirement for nutrient amendment of sandy loam soil samples but not loam samples in order to obtain transconjugants, may be a reflection of the higher levels of organic matter in the loam and/or greater surface areas provided by the higher clay content. It is also noteworthy that in the present study, *E. coli* donor and recipient cells survived better in sterile soil (Table 1) than in the non-sterile soil (Table 2) during the 24 h incubation period.

While conjugation between introduced *E. coli* cells was shown to occur in non-sterile loam soil, this was delayed between 8 to 24 h (Table 2) as opposed to 6 h, in comparison to matings in sterile loam samples (Table 1). However, the relative frequency of plasmid transfer was similar in both sterile and non-sterile soil. It is also noteworthy that the numbers of donor and recipient *E. coli* cells did not increase during 24 h in non-sterile loam samples (Table 2) as observed in the sterile soil samples (Table 1).

In sterile loam soil, both *Pseudomonas* donor and recipient cell numbers increased markedly over a 24 h period (Table 3). Transconjugants (Log. No. 2.27) were detected after 12 h. However, in non-sterile soil samples, transconjugants were recovered 2 h after introduction of the donor and recipient strains (Table 4). Donor and recipient cell numbers did not display any pronounced increases or decreases during the 24-h incubation period. This was noticeably different from the situation in sterile soil (Table 3). Standard laboratory mating on cellulose acetate filters yielded similar frequencies of plasmid transfer (1.08×10^{-3} for *E. coli* and 2.94×10^{-2} for *Pseudomonas* spp.).

Plasmid RP4 encodes for rigid pili which are fragile and easily broken [2]. Therefore, they function more effectively on a surface providing physical support, than in liquids. Since the efficiency of most mating systems on a solid surface is usually greater than or equal to that in a liquid medium, soil, with its abundance of surface areas, should be an effective substrate on which bacteria can conjugate.

Conjugal gene transfer between donor and recipient cells is dependent on their metabolic state, which in turn is controlled by levels of available nutrients [10]. Because of this relationship, any fac-

Table 2

R-plasmid transfer from *E. coli* MA527 to *E. coli* C600 in non-sterile loam soil incubated at 30°C

Log number CFU/g soil[a]				Plasmid transfer frequency
Time (h)	Donor	Recipients	Transconjugants	
0	5.75	5.39	[0][b]	[0]
2	5.86	5.49	[0]	[0]
4	5.85	5.92	[0]	[0]
8	6.03	5.68	[0]	[0]
24	5.88	5.77	3.11	1.70×10^{-3}
Control	[0]	[0]	[0]	[0]

[a] Average of 4 counts form duplicate experiments.

[b] Arithmetic zero [0].

Table 3

RP4 plasmid transfer between *Pseudomonas* isolates in sterile loam soil matings incubated at 30°C

Log number CFU/g soil[a]				Plasmid transfer frequency
Time (h)	Donor	Recipients	Transconjugants	
0	4.37	5.24	[0][b]	[0]
2	4.05	5.29	[0]	[0]
4	3.88	5.60	[0]	[0]
6	4.14	5.87	[0]	[0]
12	5.29	6.79	2.27	9.55×10^{-4}
24	6.38	8.49	3.80	2.63×10^{-3}
48	6.54	8.68	3.04	3.16×10^{-4}
Control	[0]	[0]	[0]	[0]

[a] Average of 4 counts from duplicate experiments.
[b] Arithmetic zero [0].

tor which influences the physiological state of the donor and/or recipient cells might affect gene transfer by conjugation. Such factors include temperature [13], soil moisture, oxygen levels and pH [14]. Conjugation in non-sterile soil is also influenced by competition between introduced bacterial cells and indigenous soil microorganisms.

Van Elsas et al. [16] have also reported that RP4 plasmid transfer between *Pseudomonas* isolates was markedly higher in non-sterile rhizosphere soil (from The Netherlands) of *Triticum aestivum* than in non-rhizosphere soil. The increased frequency of transfer was attributed to the availability of plant root exudates to the bacterial cells and the presence of the root surface, on which conjugation could occur. The decrease in R-plasmid transfer in the presence of other bacteria has been shown in an animal system to not depend on the physical presence of other organisms, implying a chemical effect [12]. However, it is not known if this is true in the soil environment. At the present time, a large data base of information is not available on genetic interactions between different microorganisms in different soil types. However, this information may be neces-

Table 4

RP4 plasmid transfer between *Pseudomonas* isolates in non-sterile loam soil incubated at 30°C

Log number CFU/g soil[a]				Plasmid transfer frequency
Time (h)	Donor	Recipients	Transconjugants	
0	5.79	5.59	[0][b]	[0]
2	5.11	5.06	1.21	1.2×10^{-4}
4	5.80	5.73	1.60	6.31×10^{-5}
6	6.25	6.31	2.67	2.63×10^{-4}
8	6.20	6.62	3.00	6.32×10^{-4}
12	6.52	6.86	4.40	7.59×10^{-3}
24	5.99	5.71	2.62	4.27×10^{-4}
Control	[0]	[0]	[0]	[0]

[a] Average of 4 counts from duplicate experiments.
[b] Arithmetic zero [0].

sary for environmental impact studies/risk assessment prior to using microorganisms in environmental biotechnology applications.

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