Influence of Soil Variables on In Situ Plasmid Transfer from *Escherichia coli* to *Rhizobium fredii*

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A model system was established to determine whether intergeneric plasmid transfer occurs in soil and how various soil variables affect the rate of plasmid transfer. The donor bacterium, *Escherichia coli* HB101 carrying plasmid pBLK1-2 (pRK2073::Tn5), and the recipient bacterium, *Rhizobium fredii* USDA 201, were inoculated into a sterile Adelphia fine-sandy-loam soil. Transconjugants were enumerated by direct plating on antibiotic-amended HM [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; 2-(N-morpholino)ethanesulfonic acid] salts medium. Randomly chosen transconjugants were verified by serological typing and Southern hybridization with a Tn5 gene probe. The maximum transfer frequency was observed after 5 days of incubation (1.8 × 10^-4 per recipient). The influences of clay (0 to 50% addition), organic matter (0 to 15% addition), soil pH (4.3 to 7.25), soil moisture (2 to 40%), and soil incubation temperature (5 to 40°C) on plasmid transfer were examined. Maximum transfer frequencies were noted at a clay addition of 15%, an organic matter addition of 5%, a soil pH of 7.25, a soil moisture content of 8%, and a soil incubation temperature of 28°C. These results indicate that intergeneric plasmid transfer may occur in soil and that soil variables may significantly affect the rate of transfer.

The intentional or unintentional release of genetically engineered organisms into the environment necessitates that we fully understand the stability and fate of introduced genetic elements. Of the many genetically modified organisms currently under development, many will be applied to the soil. Organisms applied to other environments may also ultimately end up in the soil because of processes such as washing from leaf surfaces. It is therefore important to understand the fate of genetically modified organisms introduced into the soil.

One question which needs to be examined prior to the field release of genetically engineered organisms is whether the introduced genetic elements can be transferred to the indigenous soil microbial population. To date, little is known concerning plasmid or gene transfer in soil. One of the first studies demonstrating in situ gene exchange in soil was reported by Weinberg and Stotzky (19). These authors indicated that conjugation between strains of *Escherichia coli* occurred at detectable levels. Another study, by Graham and Istock (6), also reported that linked antibiotic resistance genes were transferred among strains of *Bacillus subtilis* growing together in soil. Those authors also reported the eventual dominance in soil of strains which contained genetic markers from both parents. Timoney et al. (15) studied heavy-metal resistance of *Bacillus* strains in sediment. By examining the heavy-metal resistance patterns of *Bacillus* isolates, those authors suggested that heavy-metal resistance was plasmid mediated. Recently, Trevors and Oddie (17) demonstrated that tetracycline and streptomycin resistance plasmids were transferred between strains of *E. coli* in the soil. The rate of transfer, however, was 5- to 10-fold lower in nonsterile soil than that found in broth matings.

Since little is known concerning plasmid transfer in soil, we initiated a series of studies to determine (i) whether intergeneric plasmid exchange occurs in soil and (ii) to what extent various abiotic factors affect the frequency of plasmid transfer.

**MATERIALS AND METHODS**

**Bacterial strains.** In all experiments, *E. coli* HB101 containing plasmid pBLK1-2 (pRK2073::Tn5), which confers resistance to kanamycin (100 μg/ml), streptomycin (100 μg/ml), and spectinomycin (30 μg/ml), was used as the donor. *Rhizobium fredii* USDA 201 was used as the recipient. In *Rhizobium* transconjugants, the Tn5 derived from plasmid pBLK1-2 confers resistance only to kanamycin (100 μg/ml) and streptomycin (100 μg/ml). *R. fredii* USDA 201 is intrinsically resistant to nalidixic acid at 10 μg/ml. *R. fredii* was obtained from the U.S. Department of Agriculture Agricultural Research Service culture collection in Beltsville, Md., and *E. coli* HB101 (pBLK1-2) was provided by Choong Kim, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md.

**Media and growth conditions.** *E. coli*, which does not use arabinose as a carbon source, was grown for 18 h at 32°C in Luria-Bertani (9) broth supplemented with 100 μg of kanamycin per ml and 30 μg of spectinomycin per ml. Donor *E. coli* cells in soil were enumerated by dilution plating on Luria-Bertani agar medium with kanamycin and spectinomycin. *R. fredii* was grown in tryptone-yeast extract broth (3) with 10 μg of nalidixic acid per ml for 2 days at 28°C. The HM [N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 2-(N-morpholino)ethanesulfonic acid] salts agar medium (4) supplemented with 0.1% arabinose (AIE) (8) was used to enumerate *Rhizobium* sp. recipients in soil. Solid AIE containing 100 μg of kanamycin per ml and 100 μg of streptomycin per ml (AIEKS) was used to enumerate transconjugants.

**Soil.** An Adelphia fine sandy loam soil (Aquic Hapludults) was used for all in situ experiments. The soil pH, initially 3.8, was adjusted to 6.6 by the addition of CaCO₃ (except

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TABLE 1. Soil moisture content as affected by clay and organic matter additions

<table>
<thead>
<tr>
<th>Composition of sample</th>
<th>Water potential (MPa)</th>
<th>% Moisture content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil only</td>
<td>-0.033</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>-0.1</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>-0.5</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>-1.0</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>-1.5</td>
<td>8.2</td>
</tr>
<tr>
<td>Clay</td>
<td>-0.1</td>
<td>20.2</td>
</tr>
<tr>
<td>5%</td>
<td>-0.1</td>
<td>38.0</td>
</tr>
<tr>
<td>15%</td>
<td>-0.1</td>
<td>52.0</td>
</tr>
<tr>
<td>25%</td>
<td>-0.1</td>
<td>87.0</td>
</tr>
<tr>
<td>Organic matter</td>
<td>-0.1</td>
<td>12.6</td>
</tr>
<tr>
<td>2%</td>
<td>-0.1</td>
<td>13.6</td>
</tr>
<tr>
<td>5%</td>
<td>-0.1</td>
<td>22.9</td>
</tr>
</tbody>
</table>

when soil pH was a variable). The organic matter and clay contents of the soil were 1.8 and 15%, respectively.

Because independent variables affect plasmid transfer frequency, the soil clay content was altered by amending the soil with 5, 15, 25, or 50% montmorillonite, or the organic matter content of the soil was adjusted by adding 2, 5, or 15% ground corn stover. Soil pH was adjusted by adding CaCO₃ to the soil to achieve final pH values from 4.3 to 7.25. Water contents were adjusted to a constant soil water potential of -0.1 MPa, except where moisture content was a variable (Table 1). Soil water potential was measured on a ceramic pressure plate. Water potential was kept constant for all treatments, since the addition of clay and organic matter to soil affects the water-holding capacity of soil. For the unamended soil, the moisture contents at -0.033, -0.1, -0.5, -1.0, and -1.5 MPa were also determined (Table 1) to define water availability. Soils were sterilized at 121°C for 1 h five times to eliminate the possibility of the indigenous soil microbial population altering soil variables. The effect of soil temperature on plasmid transfer frequency was examined by incubating mating mixtures at 5, 15, 28, 32, and 40°C.

Conjugation. An initial experiment was conducted to determine the most appropriate time of incubation. Broth cultures (10⁸ cells per ml of each culture) of both donor and recipient were added to replicate milk dilution bottles containing 10 g of soil, and the mixture was incubated at 28°C for 12 days. Periodically, matings were interrupted by the addition of 40 ml of saline-Tween solution (0.85% NaCl, 0.01% Tween 60). Dilution bottles were vigorously agitated for 10 min, and the suspension was serially diluted. A 0.1-ml portion from each of the 10⁸ to 10⁻⁷ dilutions was plated onto AIEKS medium to select for transconjugants. Additionally, 0.1 ml from dilutions 10⁻⁸ to 10⁻⁷ was plated onto AIE and Luria-Bertani agar (with antibiotics) to quantify the number of surviving recipients and donors. Plates containing donor cells were enumerated after 2 days of incubation at 32°C, the recipients were enumerated after 6 days at 28°C, and the transconjugants were enumerated after 8 days at 28°C.

Three replicates for each treatment were used. Frequency of transfer was calculated as the number of transconjugants per number of surviving recipients. Mean comparisons of transfer frequency were calculated by using the least significant difference (P < 0.05).

Transconjugant verification. The identities of 10 randomly chosen transconjugants were verified with strain-specific fluorescent antibodies as described by Schmidt et al. (11). Plasmid transfer to recipients was verified by Southern hybridization. Transconjugants were purified by streaking on AIEKS medium, and total genomic DNA was isolated and purified by CsCl-ethidium bromide density gradient centrifugation as described by Sadowsky et al. (10). Purified genomic DNA was digested with the restriction enzyme XhoI and fragments were separated by horizontal electrophoresis on 0.7% agarose gels in Tris-borate EDTA buffer (9). The DNA was transferred to GeneScreen membranes (DuPont, NEN Research Products). Filters were hybridized to a 32P-labeled Tn5 gene probe produced by nick translation of pSUP1011 (12). Hybridizations were done by using the formamide dextran sulfate procedure described by Mamiatis et al. (9).

RESULTS

Determination of the optimal incubation time. Since little information is available concerning the incubation time required to obtain maximum plasmid transfer frequency in soil, we incubated mating mixtures in sterile soil at 28°C for various times (1 to 12 days) and examined plasmid transfer frequencies. The recipient population declined from an initial value of 2.5 × 10⁸ to 1.3 × 10⁷ CFU/g of dry soil, while the donor population decreased from 4.1 × 10⁸ to 1.7 × 10⁶ CFU/g of dry soil (Fig. 1). The transfer frequency increased from 9.1 × 10⁻⁵ after 1 day of incubation to 1.8 × 10⁻⁴ after 5 days of incubation. The transfer frequency then declined and became nearly constant after 6 days of incubation. Consequently, 5 days of incubation was chosen throughout the studies to interrupt matings.

To determine whether the Kan' Str' colonies on plates were true USDA 201 transconjugants, 10 randomly chosen colonies were examined for their serological reaction with fluorescent antibodies specific for USDA 201 and for the presence of Tn5. All isolates reacted positively with USDA 201 antisera (data not shown). When XhoI-digested genomic DNAs from the same 10 randomly chosen colonies were hybridized to a Tn5 gene probe, all of the transconjugants had four hybridizing fragments (Fig. 2), indicating that the
colonies were bonafide recipients of plasmid pBLK1-2. While all the transconjugants had two hybridizing fragments of the same size (corresponding to the 2.6- and 2.9-kilobase internal XhoI fragments of Tn5), they also possessed two variably sized hybridizing fragments, indicating that Tn5 was randomly inserted in the genome.

**Soil variables. (i) Temperature.** Since soil microorganisms have various temperature optima for growth and, presumably, conjugative plasmid transfer, it was of interest to determine if various soil temperatures would affect transfer frequencies in our model system. Results indicate that transfer frequency was significantly affected by temperature (Fig. 3). The matings demonstrated that the highest plasmid transfer frequency ($5.6 \times 10^{-5}$) was obtained at a temperature of 28°C. The numbers of surviving donors and recipients were highest at 20°C. Interestingly, from 32 to 40°C, the population of *E. coli* declined more rapidly than that of *R. fredii*. The lowest transfer frequencies ($6.5 \times 10^{-7}$ and $5.4 \times 10^{-8}$) were observed for the extreme temperatures of 5 and 40°C. At these temperatures, the numbers of surviving donors and recipients were also lower.

(ii) **Moisture.** Because plasmid transfer is mediated by direct donor and recipient contact, a matrix for cells which permits contact is essential. In soil, water is the matrix via which cells migrate. Inoculation was conducted so as to achieve different moisture contents (2, 5, 8, 14, 20, and 40%). A moisture content of 8% was found to produce the maximum transfer frequency (Fig. 4). This moisture content represents a somewhat dry soil (less than field capacity) (Table 1). Alexander (1) has previously noted that most soil microorganisms grow best at approximately 60% of the water-holding capacity of the soil. Increasing the soil moisture content from 8 to 14% reduced the transfer frequency. The transfer frequency was no longer affected by moisture contents higher than 14% and was quite stable beyond this point. The highest numbers of surviving donors and recipients were recovered at 20% moisture.

(iii) **Soil pH.** In soil, pH not only directly affects the growth of microorganisms, but also affects the solubility of many cations which may indirectly alter growth patterns. To determine if soil pH affects plasmid transfer frequency, six different soil pHs were studied: 4.3, 4.6, 5.25, 6.5, 7.0, and 7.25 (Fig. 5). The highest transfer frequency ($5.5 \times 10^{-5}$) was observed for a pH of 7.25. A soil pH of 5.25 was the minimum pH at which plasmid transfer could be detected (frequency of $4.8 \times 10^{-5}$). We did not find any surviving donors and recipients for any plated dilutions at a pH of 4.3 or 4.6. Hence, while donors and recipients may have been present below a pH of 5.25, no transconjugants were formed, or if formed, they failed to survive.

(iv) **Organic matter content.** Since both donor and recipient cells may differentially adsorb to organic matter and since nutritional factors provided by the organic matter may affect donor and recipient survival, we investigated the effect of soil organic matter content on intergeneric plasmid transfer frequency. The soil originally contained 1.8% organic matter. To this soil, different amounts of organic matter (corn stover) were added (2, 5, or 15% by weight). When the soil was amended with 5% organic matter, transfer frequency

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FIG. 2. Southern hybridization of 32P-labeled Tn5 gene probe to XhoI-digested genomic DNA from *R. fredii* (pBLK1-2) transconjugants. Lanes 1 through 9, Randomly selected transconjugants; lane 10, *R. fredii* control.

![Image](http://aem.asm.org/)

FIG. 3. Effect of incubation temperature on plasmid transfer frequency (---) and on survival of donor (· · · ·) and recipient (· · · ·) populations in soil. Transfer frequency (least significant difference, 0.05), $2.5 \times 10^{-5}$.

![Image](http://aem.asm.org/)

FIG. 4. Effect of soil moisture on plasmid transfer frequency (---) and on survival of donor (· · · ·) and recipient (· · · ·) populations in soil. Transfer frequency (least significant difference, 0.05), $2.2 \times 10^{-5}$.

![Image](http://aem.asm.org/)
FIG. 5. Effect of soil pH on plasmid transfer frequency (---) and on survival of donor (-----) and recipient (------) populations in soil. Transfer frequency (least significant difference, 0.05), 5.8 × 10⁻⁵.

significantly increased from 7.2 × 10⁻⁵ for the nonamended soil to 2.7 × 10⁻⁴ for the amended soil (Fig. 6). Higher concentrations (15%) had no effect on plasmid transfer (6.7 × 10⁻⁵) compared with transfer in the unamended soil. Moreover, the addition of organic matter to the soil did not affect the survival of the donor population. However, a 2% soil-organic-matter addition increased the number of R. fredii recipients.

(v) Clay content. To determine if soil clay content affected plasmid transfer between E. coli and R. fredii, montmorillonite was added to soil at rates of 5, 15, 25, and 50%. This resulted in soil textures of sandy clay loam, sandy clay, sandy clay, and clay, respectively. Survival of both donors and recipients was not affected by the amendment (Fig. 7), and populations remained stable around 1.4 × 10⁸ CFU/g of dry soil for the recipient and 2.0 × 10⁶ CFU/g of dry soil for the donor. The transfer frequency, however, increased from 7.2 × 10⁻⁵ for the original untreated soil to a maximum of 1.9 × 10⁻⁴ when the clay addition was 15%. Transfer frequencies, however, then decreased to 1.3 × 10⁻⁴ for 25% added clay and 1.1 × 10⁻⁴ for 50% added clay.

FIG. 6. Effect of organic matter additions to soil on plasmid transfer frequency (---) and on survival of donor (-----) and recipient (------) populations in soil. Transfer frequency (least significant difference, 0.05), 1.0 × 10⁻⁴.

FIG. 7. Effect of montmorillonite additions to soil on plasmid transfer frequency (---) and on survival of donor (-----) and recipient (------) populations in soil. Transfer frequency (least significant difference, 0.05), 6.7 × 10⁻⁵.

DISCUSSION

From other in vitro or in situ studies, it has been demonstrated that several environmental factors may affect the transfer of genetic information between microorganisms. Most studies, however, have used similar strains of E. coli or Pseudomonas sp. as both the donor and recipient. Our model system, with E. coli as the donor (not a normal soil inhabitant) and R. fredii as the recipient (indigenous in many soils), was very effective for studying the effects of soil variables on intergeneric plasmid transfer. A single time, corresponding to the maximum transfer frequency in sterile soil (5-day incubation), was initially defined as the time to interrupt all soil matings. Krasovsky and Stotzky (7) also reported that they used a 5-day incubation for their studies with sterile soil.

We subsequently studied five different abiotic factors, all of which were found to significantly affect the transfer of a plasmid from E. coli to R. fredii. The positive influence of temperature was also observed by Gauthier et al. (5), who found an increase in transfer frequency between 25 and 30°C from a marine pseudomonad to E. coli in vitro. Altherr and Kasweck (2) also observed a maximum transfer frequency between 20 and 30°C, while Walmsley (18) reported that mating-pair formation by E. coli did not occur below 24°C. In sterile soil and water, plasmid transfer was found by Trevors and Oddie (17) to occur at temperatures as low as 5°C, but only at very low frequencies. The low-transfer frequency observed at 5°C can be attributed either to a loss of donor pili or to the inability of the donor pili to attach to the recipient cell. Since extreme temperatures modify the physiological state of bacteria, it is also possible that the ability for genetic transfer is affected.

Several studies have also found pH to be important in determining in vitro transfer frequencies. In 1981, Stotzky and Krasovsky (13) examined the influence of pH on conjugation in soil. Their results indicate that the number of transconjugants formed at a soil pH of 6.8 to 7.0 was higher than at a soil pH of 6.2 to 6.6. Transconjugant numbers were very low at pHs in the range of 4.7 to 5.5. They later confirmed (7) that maximum transfer frequencies occurred at a soil pH of approximately 7.0. By using our intergeneric model system, we also found that highest transfer frequency was obtained when the soil pH was 7.25. The reduced
transfer frequencies at lower soil pH were probably due to
the reduced number of sufficient donor and recipient cells.
Donors and recipients were not detectable in soil below a pH
of 5.25.

The positive influence of clay additions on conjugation
between *E. coli* strains was first examined by Weinberg and
Stotzky (19). Earlier, Stotzky and Rem (14) demonstrated
that montmorillonite stimulated the respiration of a wide
spectrum of species, primarily by maintaining the pH of the
system favorable for growth. In accordance with these
observations, our studies show that clay amendments,
regardless of their concentration, have a favorable effect on
bacteria survival. It is known that immobilization onto
surfaces such as membrane filters enhances cell contact and
thus conjugation. The high specific surface area (700 to 800
m²/g) and cation exchange capacity (80 to 120 cmol/kg) of
montmorillonite increase the adsorption of cells and their
potential for contact. However, the transfer frequency de-
creased for amendments higher than 15%. This probably
resulted from excessive cell immobilization. Weinberg and
Stotzky (19) have shown that kaolinite (which has a cation
exchange capacity and specific surface area lower than those
of montmorillonite) has less of an effect on plasmid transfer
than montmorillonite does.

While some information is available concerning the influ-
ence of abiotic factors on plasmid transfer, little is known
concerning the influences of moisture and organic matter.
Alexander (1) indicated that maximum bacterial density is
found in soils of fairly low moisture content and that the
optimum moisture level for the activities of aerobic bacteria
often is from 50 to 75% of the water-holding capacity of the
soil. The field capacity (~0.033 MPa) for the Adelphia soil
was 13.8% moisture by weight. We found that the maximum
transfer frequency occurred at 8% moisture (58% of field
capacity). A content of 20% moisture, however, was the
optimum moisture for growth of both donors and recipients.

The current study is the first report we are aware of which
examines the influence of organic matter on intergeneric
plasmid transfer. Addition of organic matter was found to
significantly increase the transfer frequency at the 5% level.
Organic-matter amendments, such as ground corn stover,
increase the cation exchange capacity of soil and the possi-
bility of cell adsorption. Cell-to-cell contact would thus be
expected to be enhanced by organic matter addition to soil.

While this study shows the influence of soil variables on
intergeneric plasmid transfer, we are cognizant of the fact
that sterile soil devoid of competing organisms does not
simulate the normal soil environment. As noted by Trevors
et al. (16), sterile soil represents a compromise between
strict laboratory conditions and in situ experiments. The
current study, however, demonstrates that numerous abiotic
soil factors significantly affect intergeneric plasmid transfer
in soil. The intergeneric model developed in our system
could be used to study plasmid transfer under a variety of
soil conditions.

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