Fate and transport of antibiotic resistance genes in saturated soil columns

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Abstract

Interest in the fate of microorganisms (genetically modified or not) added to soil for a range of applications (e.g., biological fertilizer, bioremediation) has led to the examination of the transport and dispersion of bacteria in soil and ground water environments. The fate of added microorganisms can also be related to the fate of their DNA or any DNA found within soil or groundwater. However, studies have not examined the movement and fate of plasmids themselves in water-saturated soils.

Continuous-flow water-saturated soil columns were used to examine the environmental fate and movement of antibiotic resistance genes. The genes were located on a plasmid pLEP01, which was either introduced into the soil columns directly as a mixture of supercoiled and open circular forms or after linearization by restriction enzymes. Both quantitative and qualitative measurements were done throughout the study. Quantitative measurements included the total DNA and conservative tracer concentration in the column effluent and the transformation frequencies for \textit{E. coli} DH 10B and \textit{Acinetobacter} sp. BD 413 in the presence of column effluent. Qualitative measurements included relative degradation of the introduced DNA by gel electrophoresis and the potential of effluent DNA to transform bacteria leading to the production of antibiotic resistant bacteria. The extent of DNA degradation was found to be proportional to its residence time in the soil column. In addition, tests on the biological activity (bacterial transformation) of the DNA demonstrated that this activity was roughly proportional to the remaining DNA after its degradation. Results suggest a potential for biologically active DNA to be transported over considerable distances in water-saturated soil and ground water.

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1. Introduction

Interest in the fate of genetically modified microorganisms added to soil for a range of applications from biological fertilizer [26] to bioaugmentation of contaminated sites [26] has led to the examination of the fate of free DNA in soil and groundwater environments. When extracellular DNA enters the soil environment, it might be dispersed by rain or irrigation-induced percolation or runoff and by ground water before being completely degraded. While significant work on the dispersal of added microorganisms and their potential to transfer plasmids to indigenous soil microorganisms has been reported [2,12,14,24,25] to our knowledge, this is the first study conducted under saturated flow conditions in order to evaluate the dispersion and transformability of free DNA.
encoding for antibiotic resistance in soil. Several steps have been cited as necessary for DNA to be incorporated into soil bacteria via transformation. Each of the following conditions have to be satisfied for a successful transformation-mediated genetic exchange: (1) contact between genetically and physiologically adapted recipient bacteria and the DNA released into the soil; (2) uptake of donor DNA by recipient cell; (3) escape of DNA from the recipient restriction and modification enzymes; (4) integration of foreign DNA into the recipient genome or autonomous replication; (5) genetic expression of the newly acquired gene. The overall transformation frequencies have been measured during several studies. When the possibility of contact between bacteria and the exogenous DNA was improved, for example by mixing the soil and DNA in microcosms, bacteria were clearly capable of incorporating and expressing the DNA. A question of importance is the bioavailability and quality of the DNA as it moves through the subsurface.

The potential for contact between bacteria and DNA has been considered from two different perspectives. The first is the general physical behavior of DNA in soil and the second is the monitoring of specific genes. DNA degradation rates have been shown to be influenced by soil characteristics when DNA can adsorb onto clays and absorb into soil organic matter and, therefore, essentially out of contact with nucleases. Proteins are also sorbed by soil, and its clay content has a positive effect on protein sorption thus, potentially reducing the availability of nucleases that would be responsible for non-sorbed DNA degradation. Thus, the difference between DNA input and degradation under natural soil conditions leads to significant amounts of indigenous extracellular DNA. DNA concentrations ranged from 5 to more than 35 µg (g of dry soil)\(^{-1}\) depending upon the soil composition can be routinely detected and even extracted from soil. On the other hand, studies concerning the possible release into soil of exogenous extracellular DNA have, for the most part, concentrated on the detection and possible horizontal gene transfer (HGT) to soil bacteria. These results suggest that DNA can be detected up to a few years after being introduced in soil even if the relative amounts are unknown. However, an aspect of the majority of these studies is the lack of a dynamic soil system with water movement.

Many bacteria contain plasmids and these plasmids often code for important genes such as compound degradation, antibiotic resistance, etc. These plasmids are often released into the environment at cell death and certain bacteria are capable of actively secreting large quantities of plasmids. Plasmid extraction and conformation analyses (supercoiled, circular, or linear) can be relatively easily performed, thus providing the means for monitoring their degradation due to nuclease attack, or chemical and physical processes. The different plasmid forms should also have an effect on their sorption to soil as well as their incorporation (transformability) in bacteria either by autonomous replication (such as in Acinetobacter containing homologous sequences). Finally, the linear form of the plasmid can simulate what happens with any linear form of DNA whether its origin is bacterial or other organisms, even plants such as transgenic plants, which often contain bacterially derived genes. Continuous-flow water-saturated soil columns were used to examine the environmental fate and movement of antibiotic resistance genes residing on a plasmid. The genes were located on a plasmid pLEP01, which was introduced into the soil columns either directly in a mixture of its supercoiled and open circular forms or after being linearized by restriction enzymes.

Both quantitative and qualitative measurements were done throughout the study. Quantitative measurements included the total DNA and conservative tracer concentration in the column effluent and the transformation frequencies for E. coli DH 10B and Acinetobacter sp. BD 413 in the presence of column effluent. Qualitative measurements included relative degradation of the introduced DNA by gel electrophoresis and the potential of effluent DNA to transform bacteria leading to a production of antibiotic resistant bacteria.

2. Materials and methods

The surface soil (0–20 cm) used in this study was collected from La Côte St André (France) study area usually planted with corn. This soil is characterized as containing 29.4% sand; 20.9% clay; 47.9% silt; organic matter content of 2%; C.E.C 64 meq. (kg soil)\(^{-1}\); pH 6.98. The soil was recovered at 4°C (humidity 14% and field capacity 30%) and was sieved to a limit of 2 mm to obtain a constant porosity in the columns.

2.1. Columns design

Soil (non-sterile) columns were prepared following the method of Chen et al. by filling glass columns (Pharmacia Biotech) (2.6 ± 0.02 cm diameter × 30 cm length) with 30 g of fresh soil (14% humidity). Final soil packed in column height was 4.2 cm bound on top and bottom by pistons with a polyethylene grill and a polypropylene membrane reinforced by glass fibers of 10 µm thickness. Soil density was estimated at 1.34 g cm\(^{-3}\) and the porosity at 49%. Pistons had Teflon tubes (0.8 mm ID) connected, so that water could be pumped through the system via a peristaltic pump. Columns were saturated in distilled water and fed water continuously at a rate of 0.57 ml min\(^{-1}\) (the same rate used throughout the study) during 2 d in order to stabilize the effluent humic acid concentration based on continuous online optical density (OD260) measurements (Uvaiire spectrometry, LKB, NY, USA) before the addition of the plasmid pLEP01 (described below).
2.2. Plasmid DNA preparation

The plasmid pLEP01 used in this study was extracted from E. coli DH10B, according to the Qiagen Kit (Hilden, Germany) procedure. Plasmid pLEP01 [11] is an ampicillin resistant cloning vector containing the aadA gene, which confers resistance to spectinomycin and streptomycin. The aadA gene is flanked by plastid sequences corresponding to rbcL and accD regions of the plant chloroplast. This gene was described in studies concerning gene transfer in planta from transgenic plants to bacteria [11]. The quantification, purity control, and size measurement of the plasmid were performed by classical methods: spectrophotometry using the optical density at 260 nm wavelength for DNA quantification, 0.8% agarose gels for measuring DNA size and finally the restriction enzyme XbaI (Invitrogen, Pontoise, France) plus agarose gel for purity control. The extracted plasmid concentration was 3300 ng µl⁻¹ (3.3 µg µl⁻¹). The plasmid size was estimated at about 7 kb based on the flow column and stop-mine the hydrodynamic characteristics of the column. In all KCl) replaced the inuent distilled water in order to deter-

2.3. Columns experiments

Two series of experiments were performed: continuous-flow column and stop-flow column studies. The first consisted of injecting the plasmid pLEP01 (3.03 µg µl⁻¹, i.e. 400 µg in 132 µl) in either the original mixture of supercoiled and open circular forms or linear form. The plasmid was injected rapidly (5 s) into the influent line and at least 78 ml of distilled water was pumped through column afterwards. In a separate injection test, 65 ml of a tracer solution (600 ppm KCl) replaced the influent distilled water in order to determine the hydrodynamic characteristics of the column. In all cases, a fraction collector (Gilion, Villers le Bel, France) was used to collect effluent fractions of 0.5 ml downstream from the online optical density spectrophotometer.

The second series involved increasing the residence time of the DNA within the column. With the same experimental setup as described above, after injection of the plasmid into the column, the flow was halted for varying periods of time (1, 3, 10, 24, and 48 h). Then, the flow was restarted. Measurements and fraction collection were the same as that measured above.

2.4. DNA manipulation and transformation

The soil column effluent fractions were collected in order to examine the quality and quantity of the DNA after passage into the soil. Optical density measurements (Kontron Instruments, St-Quentin-Yvelines, France) of the discrete samples taken by the fraction collector were performed to confirm online measurements. Collected fractions were also placed on 0.8% agarose gel in order to determine the DNA in each fraction and to evaluate potential size decrease due to its degradation. DNA was purified from the effluent sample by extraction in a mixture of phenol, chloroform, and isoamyllic (25:24:1). The DNA was subsequently precipitated in absolute ethanol (Normapur, Prolabo, Fontenay sur Bois, France) and rinsed with 70% v/v ethanol in water and then resuspended in distilled water. The DNA suspension was then passed through a purification column (Microspin S-400 HR, Pharmacia Biotech, Orsay, France) to remove trace humic acids.

The DNA extracted from the collected water fractions was also tested for both the presence of the antibiotic resistance gene (aadA) by PCR (see below) and for the ability of this DNA to transform a model soil microorganism Acinetobacter sp. BD413 harboring plasmid pBAB2 [11] which contains homologous sequences to the rbcL and accD regions flanking the aadA gene and E. coli DH10B. The transformation of Acinetobacter sp. BD413/pBAB2 was done based on the procedure described by Palmen et al. [19]. The Acinetobacter strain was initially cultured on Luria Bertani medium (10 g of Bacto tryptone, 5 g of yeast extract and 5 g of NaCl in 1 l of distilled water) containing 20 µg nalidixic acid ml⁻¹ (Sigma Chemical Co., St Louis, MO, USA), then cultured over-night under agitation, before an aliquot was diluted 25-fold into fresh liquid medium and cultured for an additional 2 h to obtain a state of competence as described by Palmen et al. [19]. A control containing a final amount of 1 µg of plasmid pLEP01 in a volume of 40 µl was used to transform 360 µl of competent Acinetobacter sp. pBAB2 cells. The transformation of E. coli was achieved by electroporation, according to standard protocols [6].

2.5. Transformant characterization

PCR amplifications were performed directly on transformant colonies picked from selective-media plates and resuspended in 20 µl of sterile distilled water. Experimental conditions consisted of transferring 29 µl of sterile water, 5 µl of 10 x PCR buffer (Gibco-BRL/Life Technologies Inc, Gaithersburg MD, USA), 2.5 µl of the 1% detergent W-1 solution (Gibco-BRL), 1 µl of 50 mM magnesium chloride, 5 µl of 10 mM deoxynucleoside triphosphate mix (Pharmacia) and 2.5 µl each of 10 µM primers into a PCR tube. Then, 0.25 µl of Taq DNA polymerase (Gibco-BRL) and 1 µl of cell suspensions were added.

Amplifications were carried out in a Perkin-Elmer 7200 thermocycler for 35 cycles, each of which consisted of a denaturation step at 94 ºC for 45 s, an annealing step at 55 ºC for 45 s and an elongation step at 72 ºC for 1 min. PCR was conducted with primers complementary to part of the 16S rDNA gene (pA 5’-AGAGTTTGATCCTGCTG-3’ and pH 5’-AAGAGGTTGATCAGCAGC-3”) The 1600 bp fragment obtained was then digested with HaeIII restriction enzyme (Gibco-BRL) and the resulting patterns compared to...
those of the recipient strain. PCR was also conducted with primers FGPaad1172 (5'-ATTCGGCTGGATTAT-3') and FGPaad1554 (5'-TGACGAGGCTGATACT-3'), which are complementary to part of the aadA gene in order to monitor the presence of the marker gene in the transformant clones.

3. Results

3.1. Continuous-flow column studies

The column had about 17 ml void volume based upon the volume of the soil and bulk porosity (0.49). The average breakthrough time for the conservative tracer (KCl) was about 3 min (Fig. 2), which translates to an effective volume (denoted as V/e) of about 10% of the total volume of water in the soil column. This could be explained as saturated soil or a ground water aquifer, in which mobile and immobile water content depends on the structure of the solid matrix of the medium.

The degradation of the DNA, after passage into the soil column was qualitatively measured by the DNA size distribution on the agarose gel. Visual observation of the gel concluded that none of the forms of DNA were significantly degraded during passage into the soil column in the first series of experiments. Mass balance calculation based on the sum of the DNA in the different fractions collected indicated that almost all of the plasmid injected into the column influent was recovered (Fig. 1), although quantifying DNA by spectrophotometry and agarose gels provides only an approximate mass balance. The major form of the plasmid when harvested from E. coli, as described above, is supercoiled although the circular and some linear forms exist as shown on the agarose gel (Fig. 2, Lane 2). This original plasmid mixture was injected into the soil column as was a linear form produced by cutting the plasmid once with the enzyme SacI. Both experiments used an inert tracer (KCl) to determine the relative retardation of the plasmid. The concentration of the tracer and the two different DNA mixtures are shown as a function of time in Fig. 1. In the case of the column. Lanes 9 and 10: pLEP01 digested by SacI in effluent after 3 min (30 µl). Lanes 11 and 12: pLEP01 digested by SacI in effluent after 10 min (20 µl). Lane 13: pLEP01 digested by SacI in effluent after 100 min (30 µl). Lane 14: pLEP01 digested by SacI in effluent after 150 min (40 µl).

original plasmid mixture, the DNA concentration is the sum of all of the forms present.

3.2. Stop-flow column studies

In order to verify the differences between the reported batch experiments (17,27,28) and the column studies reported here, the column flow was stopped when the peak concentration of the DNA was in the middle of the column. The duration of the stop-flow period ranged from 1 to 48 h. As in the continuous-flow experiments, effluent DNA was monitored for changes in concentration, size and identity using 0.8% agarose gel (Fig. 3). Concentration is illustrated by the differences in the gel DNA signal. No apparent DNA degradation occurred until this period reached 10 h. In order to avoid the effect of decreases in the DNA concentration with time on the measurement of changes in DNA conformation and size, different amounts of the effluent were placed on the gel for different effluent times. These amounts ranged from 20 to 40 µl. The effect of residence time is quite clear as the plasmid is completely degraded after 10 h within the soil column although at 1 and 3 h, the plasmid is fairly visible on the gel (Fig. 3). Although the supercoiled form and one of the linear forms are close together here, as in the previous gels, it could be that a significant amount of the linear form is produced by soil nucleases.

3.3. Effluent transformation

Effluent DNA from all soil column experimental conditions was obtained by purifying the DNA in the collected
fractions. These fractions were pooled at times in order to have sufficient quantities of DNA for transformation studies. Essentially, when the DNA quantity was less than 100 ng total, neither the E. coli nor the Acinetobacter produced transformants as in the case of samples taken after 180 min elution time. All other transformation tests were performed with 300 ng of DNA, even if that required pooling more DNA samples in one case than in another. The purpose was to have a measure of the quality of the DNA to transform the two test bacteria, thus, the quantity of DNA was always the same and only the DNA quality changed in the transformation tests. The two bacteria were also useful in determining the potential effect of soil nucleases in the linearizing of the plasmid. The E. coli used would be able to incorporate, and express antibiotic resistance coded on either circular or supercoiled plasmids, which replicate autonomously, but would be unable to do this for the linear form as no homologous sequences were present. The Acinetobacter sp. BD413/ppBAB2 was capable of homologous recombination on sequences of plasmids, which replicate autonomously, but would be unable to transform the two test bacteria, thus, the quantity of DNA was always the same and only the quality of the DNA to transform the two test bacteria was important. E. coli was studied for its behavior in a water-saturated soil column simulating saturated soil during heavy flooding or a groundwater aquifer. Since the purpose was to compare the plasmid movement and fate to an inert tracer, no attempt was made to homogenize either the soil/water or the flow in the column. The plasmid mixture with a majority of supercoiled plasmid provided a stark contrast to the conservative tracer by leaching out of the soil almost uniformly over time. Perhaps this was the sum of several breakthrough curves for the different forms found in the original plasmid mix, however the supercoiled plasmid was, at least initially, the major form. When the plasmid was linearized by the restriction enzyme (SacI) and then injected into the soil column, it passed through the soil with only a very slight retardation (20% time delay) when compared with the conservative KCl-tracer and at the same time provided a breakthrough curve similar in form to that of the tracer. Differences between the form of the two DNA breakthrough curves might be explained for the tailing effect by differences in the sorption-desorption of the different plasmid forms and for the plasmid breakthrough before the tracer by size-exclusion. However, if the supercoiled form is the major fraction of the natural mixture, it is considered less likely to adsorb than the linear form. The remaining plasmid might be either too tightly sorbed or have been degraded by nucleases in the soil. The apparent ease in which both plasmids passed through the soil column could indicate that none of these forms of plasmid were strongly adsorbed on the soil in the presence of water.

Most previous studies concerning DNA sorption were performed in unsaturated batch soil where most of the target DNA was degraded, even if at the same time a small yet detectable amount of DNA escaped degradation even after several months. If on the other hand, the DNA in

<table>
<thead>
<tr>
<th>Samples</th>
<th>Transformants per 300 ng DNA</th>
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<tbody>
<tr>
<td>pLEP01 control</td>
<td>0.5 × 10^{-3}</td>
</tr>
<tr>
<td>pLEP01 linearized by SacI</td>
<td>0.5 × 10^{-3}</td>
</tr>
<tr>
<td>Effluent (3 min) supercoiled</td>
<td>0.5 × 10^{-3}</td>
</tr>
<tr>
<td>Effluent (3 min) linear</td>
<td>0.5 × 10^{-3}</td>
</tr>
<tr>
<td>Effluent (10 min) supercoiled</td>
<td>0.5 × 10^{-3}</td>
</tr>
<tr>
<td>Effluent (10 min) linear</td>
<td>0.5 × 10^{-3}</td>
</tr>
<tr>
<td>Effluent (100 min) supercoiled</td>
<td>0.5 × 10^{-3}</td>
</tr>
<tr>
<td>Effluent (100 min) linear</td>
<td>0.5 × 10^{-3}</td>
</tr>
<tr>
<td>Effluent (150 min) supercoiled</td>
<td>0.5 × 10^{-3}</td>
</tr>
<tr>
<td>Effluent (150 min) linear</td>
<td>0.5 × 10^{-3}</td>
</tr>
<tr>
<td>Effluent (180 min)</td>
<td>b.d.l.</td>
</tr>
<tr>
<td>After 1 h with no flow in column</td>
<td>0.5 × 10^{-3}</td>
</tr>
<tr>
<td>After 3 h with no flow in column</td>
<td>0.5 × 10^{-3}</td>
</tr>
<tr>
<td>After 10 h with no flow in column</td>
<td>0.5 × 10^{-3}</td>
</tr>
<tr>
<td>After 24 h with no flow in column</td>
<td>0.5 × 10^{-3}</td>
</tr>
<tr>
<td>After 48 h with no flow in column</td>
<td>b.d.l.</td>
</tr>
</tbody>
</table>

b.d.l., below detection limit (no transformants detected).
the saturated columns was not strongly sorbed under saturated conditions, then may be they were not significantly degraded either. The linear form of DNA mimicked the conservative tracer reasonably well with Lanes 9 through 14, in Fig. 2 providing a qualitative view of the plasmid in different fractions collected over time. In addition, to the peaking of concentration of linear form of the plasmid after 10 min (time includes flow in the influent tube), the gel did not indicate any degradation product or smear over time. The supercoiled plasmid was significantly different with a less pronounced plug passing through the column (Lanes 3–7). In addition, the relative concentrations between the various forms did not appear to change significantly with time. The possibility that the supercoiled and open circular plasmids react more with the soil surface than the linear form is unexpected when the three-dimensional form is taken into account. Perhaps, the supercoiled diffused into smaller pores that were not included in the “effective” or mobile volume and thus outside the flow path.

The DNA degradation after passage into the soil column was qualitatively measured by the DNA size distribution on the agarose gel. Visual observation of the gel concluded that both forms of the DNA were not significantly degraded during passage into the soil column. This was to be expected when the relatively low sorption is considered. Nucleases are only going to be of significant quantity in the sorbed phase as the water flowing through the column did not contain any added exogenous nucleases. Other experiments have shown a decrease of the non-sorbed DNA degradation when nucleases are preferentially sorbed on soils. The residence time of the DNA in our column studies was not as long as that reported in batch studies where times reached up to 3 h. The gel results did, however, confirm the DNA concentration in the different fractions as determined by the online spectrophotometer. For example, after the peak of optical density (representing the DNA) had passed through the column, the gel showed no indication of DNA present. As described above, these results are significantly different from that observed in soil batch systems, where the DNA was mixed in an unsaturated soil and required only a couple of hours to degrade. The results of this study showed a potential for biologically active DNA to be transported over considerable distances in water-saturated soil and ground water in continuous-flow. This DNA escapes from degradation by DNases and maintains its biological activity. These results confirm in part the previous reports of DNA degradation in batch systems even if in this case the soil was saturated in water.

Therefore, although the hypothesis that the contact between soil and DNA leads to DNA degradation seems appropriate, when there is saturated flow, the water keeps the DNA moving and prevents DNA degradation at least on the time scale studied here. Nonetheless, the current experiments did not provide data on DNA degradation in continuous-flow systems on the same time scale (up to 48 h) as under non-flow conditions. Hence, the existence of saturated flow cannot yet be considered as a DNA preservation process by keeping DNA separated from adsorbed nucleases. On the other hand, this work does show the potential mobility of non-degraded DNA. Another important aspect is the ability of this transported “free” DNA to transform bacteria and in this experiment to confer antibiotic resistance. That this DNA simulates possible releases of DNA from a variety of sources, such as other bacteria, plants and animals and genetic modified organisms provides arguments for the natural mixing of DNA in the subsurface environment.

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