Natural Transformation and Availability of Transforming DNA to Acinetobacter calcoaceticus in Soil Microcosms

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A small microcosm, based on optimized in vitro transformation conditions, was used to study the ecological factors affecting the transformation of Acinetobacter calcoaceticus BD413 in soil. The transforming DNA used was A. calcoaceticus homologous chromosomal DNA with an inserted gene cassette containing a kanamycin resistance gene, nptII. The effects of soil type (silt loam or loamy sand), bacterial cell density, time of residence of A. calcoaceticus or of DNA in soil before transformation, transformation period, and nutrient input were investigated. There were clear inhibitory effects of the soil matrix on transformation and DNA availability. A. calcoaceticus cells reached stationary phase and lost the ability to be transformed shortly after introduction into sterile soil. The use of an initially small number of A. calcoaceticus cells and nutrients, resulting in bacterial growth, enhanced transformation frequencies within a limited period. The availability of introduced DNA for transformation of A. calcoaceticus cells disappeared within a few hours in soil. Differences in transformation frequencies between soils were found; A. calcoaceticus cells were transformed at a higher rate and for a longer period in a silt loam than in a loamy sand. Physical separation of DNA and A. calcoaceticus cells had a negative effect on transformation. Transformation was also detected in nonsterile soil microcosms, albeit only in the presence of added nutrients and at a reduced frequency. These results suggest that chromosomal DNA released into soil rapidly becomes unavailable for transformation of A. calcoaceticus. In addition, strain BD413 quickly loses the ability to receive, stabilize, and/or express exogenous DNA after introduction into soil.

The use of genetically engineered organisms in the environment has raised concerns about the transfer of their heterologous DNA to indigenous microorganisms. Transfer of genetic information to or between bacteria has been widely demonstrated both in vitro and in natural systems. In natural systems, most of the studies have focused on the conjugal transfer of plasmids (9); some of these transfers represent a molecular mechanism of gene transfer with an extremely broad host range (8). Less is known about other mechanisms of gene transfer such as transformation, and the importance and frequencies of transformation in soil remain to be elucidated. In transformation, the discrimination of DNA uptake presumably is dependent on sequence homology between the incoming DNA and the genome of the recipient cell. Uncertainty about the efficiency of such a genetic barrier to the incoming DNA in soil adds to the need for a better understanding of transformation as a process that is relevant to bacterial populations under natural conditions (44, 45, 48).

Laboratory experiments have shown that natural transformation occurs in many bacterial genera such as Azotobacter, Bacillus, Haemophilus, Pseudomonas, and Acinetobacter (22). Studies in soil extracts, fresh and marine waters, and aquifer material have provided evidence that transformation of bacteria also can occur in natural environments (6, 23, 29, 38, 47). Moreover, Graham and Istock (11) found gene transfer between strains of Bacillus subtilis in sterile soil, which they attributed to transformation, and Lee and Stotzky (16) reported transformation of the same bacterial species in montmorillonite-amended soil. However, data on the factors that affect natural transformation in agricultural soils are scarce (16). Transformation in the natural environment has recently been discussed in an excellent review by Lorenz and Wackernagel (22).

Natural transformation in soil previously has been regarded as unlikely to be of ecological relevance because of the proposed rapid enzymatic degradation of released DNA. However, substantial evidence has built up indicating that DNA can be stabilized by binding to mineral surfaces (18, 19, 25, 33, 34). Most of these studies have focused on the persistence and transfer of DNA in artificial systems containing purified sands and clays (10, 13, 20, 27, 39). The bound DNA was shown to retain its transforming ability, and plasmid DNA introduced into soil has been reported to persist for up to 60 days (34). Following extraction from soil, it could be taken up by bacterial cells via (artificial) transformation, which suggested that it was biologically intact (34).

The gram-negative soil and water bacterium Acinetobacter calcoaceticus (3, 36) is transformable in vitro, in groundwater and river water, and in soil extracts (4, 17, 23, 28, 49). A. calcoaceticus BD413 is transformable with both chromosomal and plasmid DNA and does not discriminate between heterologous and homologous DNA with respect to uptake (6, 28). Competence development by A. calcoaceticus has been well described and is linked to growth (6, 7, 28).

In this study, a small microcosm system was developed to assess the transformation of A. calcoaceticus BD413 with chromosomal DNA in soil. By using this system, the impact of various ecological factors on the transformation frequency of
A. calcoaceticus cells in both sterile and nonsterile soils was studied.

### MATERIALS AND METHODS

#### Bacterial strains

The bacterial strains used in this study, along with their sources and references, are listed in Table 1. *A. calcoaceticus* and *Pseudomonas fluorescens* strains were cultured in Luria-Bertani (LB) broth (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 1 liter of H2O [pH 7.2]) supplemented with antibiotics at 50 μg/ml according to their phenotype (Table 1); rifampin, kanamycin, nalidixic acid, and/or ampicillin was used. Liquid cultures in LB broth were grown at 27°C with shaking (225 rpm) overnight, and LB agar (LBA) plates (1.5% agar, Oxoid no. 3) were incubated at 30°C for 48 h. For plasmid isolation, *Escherichia coli* cultures were grown overnight in LB broth at 37°C with shaking (225 rpm) overnight, and LB agar (LBA) plates were stored at 20% glycerol at −80°C. Minimal medium M9, prepared by the method of Sambrook et al. (35), was supplemented with 0.4% glucose.

For all transformations, 10 ml of an overnight culture of *A. calcoaceticus* BD413 Rpr inoculated from a single colony (absorbance at 600 nm of a 1/10 dilution, ∼0.2) was centrifuged (3,000 g for 3 min), and resuspended in the initial matrix on transformation. Filter transformations were done as described above, except that the filters were incubated for 24 h at 30°C.

### DNA extraction and purification

Plasmid pSKTG DNA was isolated from *A. calcoaceticus* DSM586(pSKTG) by an alkaline extraction method (35). pSKTG was obtained from *E. coli* with the Jetstar Midi plasmid isolation kit (Genomed Inc.). Chromosomal DNA from *A. calcoaceticus* DSM586 (chr::KTG) was isolated by a modified version of the method of Wilson (50). DNA from separate isolations was pooled and restratified with phenol-chloroform and chloroform to ensure uniform quality and high purity. The purity and molecular weight of the DNA were confirmed by UV spectroscopy and agarose gel electrophoresis. The chromosomal DNA used for transformation was of large molecular size mass (20 to 40 kb). High-molecular-weight DNA is efficient for the transformation of *Pseudomonas stutzeri* (5) and *A. calcoaceticus* (22).

### Filter transformations

Sterile water-soaked Millipore GS filters were placed on LBA containing rifampin (50 μg/ml) and air dried for 5 min. The DNA (10 μl; 1 μg/ml) and competent (28) *A. calcoaceticus* BD413 Rpr cells (100 μl; 10^8 CFU) were briefly mixed in an Eppendorf tube and spread evenly on the filter. The filters were incubated for 24 h at 30°C. After incubation, the filters were shaken in 5 ml of saline, and the resulting suspensions were serially diluted. Plating was done in replicate onto LBA supplemented with rifampin for the enumeration of recipient cells and onto LBA supplemented with rifampin and kanamycin for enumerating transformants.

### Combined filter-soil transformations

To study the inhibitory effects of the soil matrix on transformation, filter transformations were done as described above, except that after introduction of *A. calcoaceticus* BD413 Rpr cells and DNA onto the filter, a soil plug held in a small soil microcosm (described below) was put on top. Further, to investigate the effects of spatial separation of recipient cells and donor cells, an alkaline-south blotting with KTG DNA as the probe (Fig. 1b). One clone was selected and used as the source of DNA in the transformation assays.
transforming DNA, the DNA (10 μg) was placed on the filter and the recipient cell suspension (10⁸ CFU) was mixed through the soil plug. Conversely, the transforming DNA (10 μg) was mixed into the soil plug and the A. calcoaceticus cells were placed on the filter. The soil moisture content in the plug was adjusted by carefully pipetting aliquots onto the soil surface; the plugs were then placed on saline–1.5% agarose. The addition of cells and DNA were either simultaneous or serial as outlined below. The transformation systems were incubated at 30°C.

The availability of transforming DNA to the standard 24-h transformation time was assessed with 10⁴ CFU with or without LB broth as a nutrient source.

To study the effect of the soil matrix on transformation, a set of experiments was performed with sterilized portions of the two soils. First, the effect of progressively increasing the incubation period (0, 1, and 30 min, 6 h, and 1, 3, and 7 days) of the recipient cells with an incubation time of 24 h before DNA addition. In this test was used to compare the data and assess the effects of the experimental factors.

Molecular analyses. All molecular analyses were carried out by standard procedures (35). To generate a probe for nptII, plasmid pSKTG was restricted with PstI. Following gel electrophoresis, the 923-bp nptII fragment was isolated with the Gene Clean II kit (Biot101, La Jolla, Calif.) and labelled with digoxigenin-11-dUTP (DIG) by the DIG random-labelling kit (Boehringer).

For colony hybridization, colonies were transferred to Qiabrade uncharged nylon membranes (Qiagen, no. 60234) and processed (35). The membranes were then prehybridized and hybridized as described in the DIG manual (Boehringer).

To localize the KTG gene in the donor strain and transformants of the soil transformation assay was determined. Plasmid-digested genomic DNA, isolated as described above, was run on 0.7% agarose gels and capillary blotted (overnight) to Hybond N (Amersham) uncharged nylon membranes with 0.4 M NaOH. The membranes were treated at 120°C for 20 min and hybridized with the nptII probe as in the colony hybridization.

For PCR amplifications, cells from single colonies from transformant- and recipient-selective plates were transferred to PCR tubes containing 27 μl of water, 5 μl of 10× PCR buffer II (Perkin-Elmer), 5 μl of 25 mM MgCl₂, 10 μl of 4 mM deoxynucleoside triphosphate mix (Pharmacia), 1 μl each of 10 μM primer (IB3 and IB4), and 0.05 μl of T4 gene 32 protein (Boehringer).

The solution was overlaid with mineral oil (Sigma) and heated at 98°C for 10 min. Then 0.25 μl of AmpliTag DNA polymerase (Perkin-Elmer) was added to the tubes at 92°C. Amplification was done by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. The primers used were selected to specifically amplify a 412-bp product of the KTG gene cassette by annealing to a region of the adaB and creβB gene sequences (37). Primer sequences were 5'-TCT CAT GCT GGA GTT CTT CG-3' and 9'-CTG CTG TTC TAT AGG ACT GG-3'. The membranes were treated at 120°C for 20 min and hybridized with the nptII probe as in the colony hybridization.

RESULTS

Filter transformations. To verify if the ability to be transformed had been affected by the resistance to rifampin in A. calcoaceticus BD413 Rp', we compared the transformation frequencies of this strain with that of the wild-type strains BD413 and DSM586. As can be seen in Fig. 2, no major differences between the three strains with respect to transformation efficiency with chromosomal DNA were detected. Sim-
Rpr cells were used. In this study, the maximum transformation frequency for seven independent assays with two or more replicates per assay was (3.7 ± 1.2) × 10^-3. With 10^7 CFU, the filters with different initial bacterial densities showed approximately the same numbers of transformants. After 24 h of incubation with DNA, the filters with different initial bacterial densities increased the transformation frequency up to a maximum level (Fig. 3). With 10 μg of chromosomal DNA per filter, the average transformation frequency for 15 independent assays with two or more replicates per assay was (5.2 ± 3.9) × 10^-3 per recipient. For plasmid DNA (10 μg), the average frequency for seven independent assays with two or more replicates per assay was (1.9 ± 1.1) × 10^-5 per recipient.

In one experiment, nutrient-rich (LBA) and nutrient-poor (minimal medium M9) agar media were used to study the effect of the nutrient supply on transformation efficiency. The average transformation frequencies were similar for the two media; on M9 with 0.4% glucose, they were (2.9 ± 1.2) × 10^-2 for chromosomal DNA and (1.4 ± 0.2) × 10^-5 for plasmid DNA.

To study the effect of temperature on transformation, A. calcoaceticus BD413 Rpr cells were mixed with transforming DNA and incubated for 24 h at different temperatures. Incubation at 10°C gave an average transformation frequency of (6.2 ± 10.8) × 10^-4, incubation at 30°C gave (3.9 ± 5.9) × 10^-3, and incubation at 37°C gave (4.4 ± 0.6) × 10^-3. An incubation temperature of 30°C was used in all further studies in soil.

To verify the nature of the CFU appearing on the transformant-selective plates, randomly selected plates were used for colony lifts which were hybridized with the nptII probe. All colonies from the transformant-selective plates hybridized to the probe, giving a strong signal (Fig. 4b), whereas colonies obtained from the recipient-selective plates did not (Fig. 4a). Randomly picked colonies were also used for specific PCR amplification to detect the KTG cassette. The expected PCR products were observed with all colonies picked from the transformant-selective plates analyzed (Fig. 1a). No products were detected for colonies picked from recipient-selective plates, in the control PCR mixture without added DNA, or when DNA from Paenibacillus polymyxa was used as a target. To further assess the presence of KTG in the transformants, Southern blot analysis with the nptII probe was performed. The selected clones showed hybridization bands at the expected 925 bp (Fig. 1c).

Given the high transformation frequencies obtained and good selectability of A. calcoaceticus BD413 Rpr, this strain was chosen for further studies of transformation in soil. Transformation with a plasmid carrying the frequently used IncQ-

<table>
<thead>
<tr>
<th>TABLE 2. Combined filter-soil microcosm transformations of A. calcoaceticus BD413</th>
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<tbody>
<tr>
<td><strong>Conditions and time (days)</strong></td>
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<tr>
<td></td>
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<tr>
<td>Filter transformation</td>
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<td></td>
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<tr>
<td>Filter transformation with soil on top</td>
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<td>Bacteria in soil and DNA on filter</td>
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<tr>
<td>Bacteria on filter and DNA in soil</td>
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<table>
<thead>
<tr>
<th><strong>Notes</strong></th>
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<tbody>
<tr>
<td>a Transformation period of bacteria in soil (10^8 CFU added).</td>
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<tr>
<td>b Recipient and transformant counts are given as log values. The frequency is given as transformant counts divided by recipient counts. The mean coefficient of variation was 0.3.</td>
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<tr>
<td>c Recipient counts from soil.</td>
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<tr>
<td>d Recipient counts from filter.</td>
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</table>

**FIG. 3.** Filter transformation of A. calcoaceticus BD413 Rpr with increasing concentrations of plasmid or chromosomal DNA with a transformation period of 24 h on LBA plates.
obtained after a short period of bacterial exposure to soil. The soil type clearly affected transformation (Table 3), since the transformation frequencies were always higher in FSL than in ELS. In the latter soil, transformation was often below the limit of detection.

Addition of recipient cells to soil in the presence of LB broth instead of saline resulted in up to fourfold-higher recipient cell counts after 24 h in both soils, indicating a slight stimulus of growth to higher final cell densities (data not shown). With nutrients, the transformation frequencies obtained were significantly enhanced in ELS after 1 and 30 min and 6 h and to a lesser extent in FSL (only at 6 h). For FSL, the addition of LB broth with the transforming DNA resulted in initially (1 and 30 min) reduced and later (6 h) similar transformation frequencies compared to those in the treatment where LB broth was added with the A. calcoaceticus cells (Table 3). In ELS, the transformation frequencies were reduced throughout.

The use of lower initial recipient cell levels (10⁴ CFU) and an increase in the residence time in soil to 24 h (permitting recipient cell growth at the expense of nutrients released in the sterilization process) before DNA addition did not enhance transformation frequencies in either of the two soil types (Table 3). Here, only the presence of added LB broth with the cells resulted in detectable transformation frequencies in FSL but not in ELS, whereas all other treatments resulted in no detectable transformation (data not shown). The transformation period in soil was extended from 24 h to 7 days, with no further increase of transformation frequency after the standard 24 h (Table 3).

Increasing the time of residence of DNA in soil before the addition of A. calcoaceticus cells progressively reduced the transformation frequencies obtained in FSL, whereas transformation in ELS was below the detection limit throughout. By using the high inoculum level (10⁸ CFU), transformation was readily detected for up to 30 min in FSL (albeit at decreasing frequencies); after 6 h (up to 7 days [data not shown]), transformation frequencies were below the limit of detection (Table 3). DNA residing in soil for 1, 3, or 7 days before addition of the low inoculum level (10⁴ CFU) could not be utilized as a genetic source by the inoculant cells in either of the two soils (data not shown). The recipient CFU in soil were stable throughout the experimental period, at roughly 10⁹ to 10¹⁰ CFU per microcosm.

By using the high inoculum level (10⁸ CFU per microcosm), the effects of soil type, incubation time of DNA or recipient cells (0 or 30 min or 1, 3, or 7 days), and nutrients on transformation frequencies were assessed in nonsterile soil microcosms. Irrespective of the presence of nutrients, the inoculant populations survived well, at levels of ca. 10⁷ to 10⁸ CFU per microcosm, over 3 days in the two unamended soils. Survival after 7 days was lower. In the microcosms of both soils without added nutrients, transformation was never detected (Table 4). Also, the presence of nutrients (100 µl of LB broth or maize root exudate) did not enhance the transformation frequency in ELS to a detectable level at any time. However, LB broth stimulated the transformation of A. calcoaceticus cells residing in FSL for 30 min, giving rise to an average transformation frequency of 2.3 × 10⁻⁶ per recipient. Addition of maize root exudate to FSL could mimic this nutrient effect, resulting in a frequency of transformation of 7.5 × 10⁻⁷ (Table 4). Hence, transformation of A. calcoaceticus in soil was clearly stimulated by nutrients, and natural nutrients such as maize root exudates also produced this effect.

Control of recipient and putative transformant colonies. For all soil experiments, randomly picked putative transformant and recipient colonies were assayed by PCR analysis with the
TABLE 3. Transformation of *A. calcoaceticus* BD413 in sterile soil microcosms

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Treatment</th>
<th>Time</th>
<th>Nutrient(s)</th>
<th>FSL</th>
<th>ELS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial cells (10⁶ CFU) added before DNA</td>
<td>0 min&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Saline</td>
<td>2.5 × 10⁻⁵</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LB&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.2 × 10⁻⁵</td>
<td>2.9 × 10⁻⁶</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1 min&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Saline + LB&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.6 × 10⁻⁵</td>
<td>1.5 × 10⁻⁵</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LB</td>
<td>1.2 × 10⁻⁴</td>
<td>1.4 × 10⁻⁷</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>30 min&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Saline + LB</td>
<td>5.6 × 10⁻⁵</td>
<td>2.8 × 10⁻⁵</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LB</td>
<td>5.8 × 10⁻⁶</td>
<td>1.3 × 10⁻⁶</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Saline + LB</td>
<td>7.2 × 10⁻⁵</td>
<td>BD</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>6 h&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Saline</td>
<td>2.5 × 10⁻⁵</td>
<td>6.3 × 10⁻⁷</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LB</td>
<td>2.3 × 10⁻⁴</td>
<td>2.3 × 10⁻⁶</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Saline + LB</td>
<td>3.9 × 10⁻⁶</td>
<td>BD</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1 day&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Saline</td>
<td>1.5 × 10⁻⁶</td>
<td>BD</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LB</td>
<td>BD</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>Saline + LB</td>
<td>BD</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Bacterial cells (10⁴ CFU) added before DNA</td>
<td>1 day&lt;sup&gt;c&lt;/sup&gt;/1 day&lt;sup&gt;d&lt;/sup&gt;</td>
<td>LB</td>
<td>7.3 × 10⁻⁶</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1 day/3 days</td>
<td>LB</td>
<td>2.3 × 10⁻⁶</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1 day/7 days</td>
<td>LB</td>
<td>6.0 × 10⁻⁷</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DNA added before bacterial cells (10⁶ CFU)</td>
<td>1 min&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Saline</td>
<td>2.0 × 10⁻⁵</td>
<td>BD</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>30 min</td>
<td>Saline</td>
<td>4.0 × 10⁻⁶</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>Saline</td>
<td>BD</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>BD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Frequencies given as transformants per recipient. The mean coefficient of variation was 0.3. Average recipient counts with a high inoculum (10⁸ CFU): in FSL with saline, 6.8 × 10⁶; in ELS with LB, 1.0 × 10⁷; in ELS with saline 3.9 × 10⁷; in ELS with LB, 6.0 × 10⁷; with a low inoculum (10⁴ CFU): in FSL with saline, 3.6 × 10⁶; in ELS with LB, 8.5 × 10⁶; in ELS with saline, 2.2 × 10⁷; in ELS with LB, 4.3 × 10⁷.

<sup>b</sup> Incubation time of bacterial cells in soil before DNA was added. After addition, all samples were incubated for 24 h unless otherwise stated.

<sup>c</sup> Residence time of DNA in soil before bacterial cells were added.

<sup>d</sup> Incubation time of bacteria in soil before DNA was added. After addition, all samples were incubated for 24 h unless otherwise stated.

<sup>e</sup> BD, below detection limit (10⁻⁹).

TABLE 4. Transformation of *A. calcoaceticus* BD413 (10⁸ CFU) in nonsterile soil microcosms

<table>
<thead>
<tr>
<th>Time (min)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Nutrient&lt;sup&gt;b&lt;/sup&gt;</th>
<th>FSL soil</th>
<th>ELS soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>Saline</td>
<td>BD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>BD</td>
</tr>
<tr>
<td>30</td>
<td>LB</td>
<td>2.3 × 10⁻⁶</td>
<td>BD</td>
</tr>
<tr>
<td>30</td>
<td>Root exudates</td>
<td>7.5 × 10⁻⁷</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Frequencies are given as transformants per recipient. The mean coefficient of variation was 0.3. Average recipient counts in FSL with saline, 7.5 × 10⁶; in ELS with LB, 1.4 × 10⁷; in ELS with root exudates, 1.5 × 10⁷; in ELS with saline, 4.0 × 10⁷; in ELS with LB, 8.3 × 10⁷ (FSL and ELS experiments were done independently).

<sup>b</sup> Incubation time of bacteria in soil before DNA was added. After addition, all samples were incubated for 24 h.

<sup>c</sup> Medium in which cells were added to soil.

<sup>d</sup> BD, below detection limit (10⁻⁹); ND, not determined.
any detectable transformation, even though the recipient CFU counts after 24 h of transformation were similar for the 10^4- and 10^6-CFU inoculants. Both inocula might have quickly (within 24 h) reached stationary phase in soil, which presumably resulted in reduced competence (28). Cell growth has been reported to be necessary for efficient transformation of \textit{A. calcoaceticus} (7). Moreover, \textit{A. calcoaceticus} cell populations incubated in groundwater for 7 days retained competence only at a drastically reduced level (0.5%). The remaining competence of the population probably was caused by cell lysis and concomitant growth of surviving cells (23).

In vitro transformation experiments with \textit{A. calcoaceticus} have benefited from the capability of this organism to become competent during exponential growth (28). However, natural soils are generally poor in nutrients, which severely limits the possibilities for bacterial growth. Soil sterilization is known to result in the release of nutrients, which presumably differ in quality and quantity between soils (24). To investigate if the sterile soils used provided limiting nutrient conditions for transformation, the effect of extra nutrients (LB broth) was assessed. With the high inoculum level, the addition of nutrients resulted in enhanced transformation in ELS but not in FSL; after the stationary phase was reached (within 24 h), as monitored by stable recipient counts, transformation was no longer detectable. Hence, nutrient deprivation affects transformation in some soils and the addition of nutrients can then enhance transformation frequencies. The low inoculum level (10^4 CFU) with added nutrients resulted in detectable transformants in FSL after 24 h, whereas with the high (10^6 CFU) level, transformants were detectable only up to 6 h of incubation in soil. This is in line with the hypothesis that successful transformation of \textit{A. calcoaceticus} in soil is indeed promoted by bacterial growth.

Adding the LB broth together with \textit{A. calcoaceticus} cells means that the bacteria will be dispersed in soil with nutrients, thereby favoring bacterial growth. However, the presence of a physical distance between DNA and recipient cells might still prevent successful transformation. To study if spatial inaccessibility affected transformation and if it can be enhanced by allowing \textit{A. calcoaceticus} cells to grow into spaces harboring both DNA and LB broth, DNA was also added to the soil as a solution in LB broth. As this treatment resulted in transformations similar to those obtained when the cells were added with the LB, the effect of spatial localization of cells and nutrients was not clear.

The experiments in the nonsterile soil portions corroborated the inferences obtained in experiments in sterile systems. Whereas conditions in ELS were never conducive to the appearance of transformants, FSL was conducive to transformation, albeit only when added nutrients (LB broth or maize root exudate) were present. The conditions (nutrient levels) in some soils or rhizospheres have been reported to be conducive to gene transfer processes, e.g., conjugation (41, 45) or transformation (21, 45). Furthermore, an influence of soil type on conjugation has been reported by Richaume et al. (32). In the present study, FSL allowed a higher transformation rate than did ELS. The higher clay content in FSL (26%) than in ELS (3%) might have affected the transformation frequency by stabilizing the introduced DNA (25).

The availability of transforming DNA to \textit{A. calcoaceticus} cells in FSL decreased quickly, since transformants could no longer be detected after only 30 min of DNA residence in soil before bacterial addition. Adsorption of DNA to sediments has been extensively reported (18, 19, 25, 26, 33, 34), and persistence of bacterial DNA in soil (monitored by PCR) for several weeks has been found (31, 34). However, this does not imply that chromosomal DNA is readily available to bacteria in soil. For instance, Tebbe and Vahjen (40) reported the negative influence of humic acids on transformation, possibly by an effect on the DNA availability. Further, it is not clear if naked DNA added to soil can mimic DNA released by lysing cells. However, in controlled cell lysis studies, it was found that DNA released from lysed cells is almost as efficient as pure DNA for transformation of \textit{A. calcoaceticus} (14).

As introduced chromosomal DNA became unavailable for substantial transformation events within a few hours, the availability of DNA for transformation of \textit{A. calcoaceticus} cells in soil is probably highly restricted. Under the prevailing nutrient-limited conditions in soil, \textit{A. calcoaceticus} cells may be largely refractory to transformation. Hence, the availability of transforming DNA in soil at the time and location that cells become competent probably exerts a great influence on the transformation process.

From the results with sterile and nonsterile soil systems, we therefore conclude that the occurrence of growing \textit{A. calcoaceticus} cells with access to freshly released DNA is a prerequisite for in situ transformation. The stimulation of transformation by maize root exudates suggested that the maize rhizosphere might be a habitat where transformation of \textit{A. calcoaceticus} can take place.

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