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
were competent for transformation (28). For experiments with nutrients, the volume of saline. The bacterial cells were then in the late exponential phase and

3

with chromosomal KTG DNA.

Escherichia coli

Pseudomonas fluorescens

BD413

Braunschweig, Germany.

A. calcoaceticus

sources and references, are listed in Table 1.

TABLE 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
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<tr>
<td>Acinetobacter calcoaceticus</td>
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<tr>
<td>DSM586</td>
<td>DSM</td>
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<tr>
<td>DSM586 Nxr</td>
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<td>DSM586 (chr::KTG) Nxr</td>
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<td>BD413 Rp</td>
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<tr>
<td>BD413(chr::KTG) Rp</td>
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<tr>
<td>BD413(pSKTG) Rp</td>
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<tr>
<td>Pseudomonas fluorescens</td>
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<tr>
<td>R2I(pSKTG)</td>
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<td>Escherichia coli</td>
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<tr>
<td>MC1061(pSKTG)</td>
<td>-37</td>
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<tr>
<td>SM10(396)(pUT/KTG)</td>
<td>-37</td>
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</table>

a DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

b With chromosomal insertion of the KTG cassette.

c Spontaneous rifampin-resistant mutant.

d Spontaneous nalidixic acid-resistant mutant.

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 (at 225 rpm) overnight. 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 (at 225 rpm). The strains were stored in 20% glycerol at

A chromosomal insertion of the KTG gene cassette in A. calcoaceticus BD413 Rpr isolated from soil microcosms, cut with PstI cut; 3, wild-type DSM586; 4, nontransformed colony; 5, colonies transformed with pSKTG DNA; 6, A. calcoaceticus colony transformed with chromosomal KTG DNA; 7, pSKTG DNA; 8, chromosomal KTG DNA; 9, 1-kb ladder. (b) Southern blot of transforming DNA isolated from A. calcoaceticus BD413(pSKTG). Clones of A. calcoaceticus which stably maintained pSKTG were successfully obtained (data not shown).

A chromosomal insertion of the KTG gene cassette in A. calcoaceticus was produced by a mating between Escherichia coli SM10 (lambda pir) with plasmid pUT/KTG as the donor (37) and A. calcoaceticus DSM586 Nxr as the recipient. Both washed donor and recipient cultures (50 μl each) were placed on nitrocellulose filters (Millipore GS; GSWP07080) on LBA for 48 h at 28°C. Following incubation, the cells were resuspended in 1 ml of LB broth, serially diluted, and plated onto LBA with selection for transconjugant CFU (i.e., containing kanamycin and nalidixic acid). The KTG gene cassette was found to be inserted into the chromosome of several clones of A. calcoaceticus DSM586, as evidenced by Southern blotting with KTG DNA as the probe (Fig. 1b). One clone was selected and used as the source of DNA in the transformation assays.

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 reextracted with phenol-chloroform and chloroform to ensure uniform quality and high purity. The purity and molecular weight of the DNA was 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/μl) and competent (28) A. calcoaceticus BD413 Rp cells (100 μl; 10^6 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 transformant cells.

The undiluted recipient cell suspension also was plated onto LBA supplemented with rifampin and kanamycin to check for spontaneous mutations to Km. The transforming DNA was further placed onto LBA to control for sterility and for the eventual presence of donor cells. For all the transformations performed, growth of recipient CFU on the transformant-selective plates was never observed. Moreover, the transforming DNA placed directly on LBA did not give rise to any colony formation.

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 Rp 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

FIG. 1. (a) Agarose gel electrophoresis of PCR-amplified DNA with primer set IB3 plus IB4. Lanes: 1, 1-kb ladder (Gibco-BRL); 2, DNA from Pseudbacillus polymyxa; 3, PCR mix; 4, nontransformed A. calcoaceticus recipient colony; 5, A. calcoaceticus colony transformed with pSKTG DNA; 6, A. calcoaceticus colony transformed with chromosomal KTG DNA; 7, pSKTG DNA; 8, chromosomal KTG DNA; 9, 1-kb ladder. (b) Southern blot of transforming DNA isolated from A. calcoaceticus DSM586 (chr::KTG) hybridized to an nptII probe. Lanes: 1, uncut; 2, PstI cut; 3, wild-type DSM586 PstI cut. (c) Southern blot of DNA from colonies of A. calcoaceticus BD413 Rp isolated from soil microcosms, cut with PstI, and hybridized to an nptII probe. Lanes: 1, untransformed colony; 2 and 3, colonies transformed with chromosomal KTG DNA.
transforming DNA, the DNA (10 μg) was placed on the filter and the recipient cell suspension (10^6 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 the addition of water with the DNA cells (see below). Controls consisted of only bacterial cells placed on the filter or mixed through the soil plug and of only DNA on the filter or in the soil. The time allowed for transformation to occur was 24 h (routinely) or 7 days.

**Soil microcosms.** Two different soils, Flevo silt loam (FSL) and Ede loamy sand (ELS), were sampled from soil microplots in Wageningen, The Netherlands. Both soils have been described before (43). The soils were sieved (4-mm mesh), and portions were sterilized by gamma irradiation with a 10^6Co source (4 megarad) and stored at 4°C. Other portions were used directly in the experiments (nonsterile soil). At the start of each experiment, the soils were packed evenly in autoclaved polypropylene cylinders of 1 cm^3 (made from 15-mL polypropylene centrifuge tubes [Greiner no. 187261]). The density of the soils after A. calcoaceticus cells, DNA, and water had been added was 1.40 g/cm^3 for FSL and 1.59 g/cm^3 for ELS. The cylinders were 7 mm tall. The final moisture content was 35% for FSL and 18% for ELS, which corresponds to 60% of their water-holding capacity. For transformation studies, A. calcoaceticus cells in 100 μl of saline or LB broth and/or DNA (in water) were added to the soil by carefully pipetting aliquots onto the soil surface; the plugs were then placed on saline-1.5% agarose. The additions of cells and DNA were either simultaneous or serial as outlined below. The transformation systems were incubated at 30°C. After different incubation and transformation times (see below), the soil plugs were shaken in 4.75 ml of 0.1% sodium pyrophosphate (tetrasodium dihydrogen decahydrate [Merck]) supplemented with 100 μl of 5-mg/ml DNase I (Boehringer, Mannheim, Germany) and 1 g of sterile gravel (2 to 4 mm in diameter) and diluted as for the filter transformations. DNase I was used to prevent transformations during the soil-processing procedure (see below). The plating, incubation, and enumeration procedures for the soil transformations were the same as the procedures for the filter transformations.

For all transformations in soil, controls consisted of adding only A. calcoaceticus cells or only transforming DNA to the soil plug. As there were no detectable spontaneous Km^r mutants of the A. calcoaceticus recipient strain, the recipient counts were performed with the same microcosm as the transformant counts, except for the combined filter and soil transformations. Cycloheximide (100 μg/ml) was added to LBA plates for sampling of nonsterile soil.

As outlined in the following section, different combinations of inoculum levels (10^4 or 10^8 CFU), nutrients (100 μg of LB broth or maize root exudate [obtained as described in reference 46] per microcosm) or maize root exudate [obtained as described in reference 46] per microcosm) or maize root exudate [obtained as described in reference 46] per microcosm) or no nutrients, and incubation times (0 min to 7 days) before transformation were investigated. Also, the effect of prolonged transformation (3 to 7 days) compared to standard transformations was assessed.

**Transformation in soil microcosms: experimental factors.** 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 inoculation level by the different treatments and incubation times (0, 1, and 3 days) of the recipient strain, the recipient cells or only transforming DNA to the soil plug. As there were no detectable spontaneous Km^r mutants of the A. calcoaceticus recipient strain, the recipient counts were performed with the same microcosm as the transformant counts, except for the combined filter and soil transformations. Cycloheximide (100 μg/ml) was added to LBA plates for sampling of nonsterile soil.

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**Filter transformations.** A. calcoaceticus BD413 wild type (wt), the spontaneous mutant BD413 R^p, and DSM586 N^r with chromosomal (chrom) or plasmid DNA (10 μg per 10^6 CFU) with a transformation period of 6 or 24 h on LBA plates.

**Bacterial strains.** 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 gene fragment was isolated with the Gene Clean II kit (Biot101, La Jolla, Calif.) and labelled with digoxigenin-11-dUTP (DIG) by the DIG random-labeling kit (Boehringer).

For colony hybridization, colonies were transferred to Quabane uncharged nylon membranes (Qiagen, no. 60234) and processed (35). The membranes were then prehybridized and hybridized as described in the DIG manual (Boehringer). Two 5-min washes were done at room temperature with 2× SSC (0.3 M NaCl, 0.03 M sodium citrate)-0.1% (wt/vol) sodium dodecyl sulphate. Chemiluminescent detection with the CSPD substrate (2a) for alkaline phosphatase was done as described in the Boehringer manual.

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**RESULTS.**

Filter transformations. To verify if the ability to be transformed had been affected by the resistance to rifampin in A. calcoaceticus BD413 R^p, 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 or more replicates per assay was (1.9 the average frequency for seven independent assays with two CFU, with an average of (3.7 cell densities showed approximately the same numbers of incubation with DNA, the filters with different initial bacterial transformation frequencies (data not shown). After 24 h of filters, different levels of competent which should initially be mixed with transforming DNA on the transformation mixture of strain BD413 Rpr for up to 7 days did not substantially enhance the transformation frequencies (Table 2).

To assess the optimum number of A. calcoaceticus cells which should initially be mixed with transforming DNA on the filters, different levels of competent A. calcoaceticus BD413 Rp' cells were used. In this study, the maximum transformation frequencies were obtained with A. calcoaceticus cells added to the filter at 10⁵ and 10⁶ CFU, whereas lower (e.g., 10⁴ CFU) as well as higher (10⁷ CFU) bacterial densities decreased the transformation frequencies (data not shown). After 24 h of incubation with DNA, the filters with different initial bacterial cell densities showed approximately the same numbers of CFU, with an average of (3.7 ± 1.0) × 10⁵.

Increasing the concentrations of both plasmid DNA and chromosomal DNA increased the transformation frequency up to a maximum level (Fig. 3). With 10 μg of chromosomal DNA per filter on LBA, 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 Rp' 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 Rp', this strain was chosen for further studies of transformation in soil. Transformation with a plasmid carrying the frequently used IncQ-

<table>
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<tr>
<th>Conditions and time (days)</th>
<th>FSL</th>
<th>ELS</th>
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<tr>
<td></td>
<td>Recipient counts</td>
<td>Transformant counts</td>
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<td>Filter transformation</td>
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<tr>
<td></td>
<td>7</td>
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<td>9.4</td>
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<tr>
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<td>9.7</td>
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<td>Bacteria on filter and DNA in soil</td>
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<td>9.9</td>
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<tr>
<td></td>
<td>7</td>
<td>9.4</td>
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</table>

TABLE 2. Combined filter-soil microcosm transformations of A. calcoaceticus BD413

* Transformation period of bacteria in soil (10⁶ CFU added).
* 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.
* Recipient counts from soil.
* Recipient counts from filter.
Based on 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 $10^9$ to $10^{10}$ (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 ($10^6$ 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 ($10^4$ 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 $10^9$ to $10^{10}$ CFU per microcosm.

By using the high inoculum level ($10^6$ 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 $10^7$ to $10^8$ 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 ($100 \mu l$ 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 $2.3 \times 10^{-4}$ per recipient. Addition of maize root exudate to FSL could mimic this nutrient effect, resulting in a frequency of transformation of $7.5 \times 10^{-7}$ (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

![FIG. 4. Colony hybridization of chromosomally transformed *A. calcoaceticus* hybridized to an nptII probe (DIG nonradioactive labelling and chemiluminescent detection). Recipient-selective (a) and transformant-selective (b) plates from filter transformations and recipient-selective (c) and transformant-selective (d) plates from soil microcosm transformations are shown.](http://aem.asm.org/Downloadedfrom)
TABLE 3. Transformation of A. calcoaceticus BD413 in sterile soil microcosms

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<th>Conditions</th>
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<th>Nutrient(s)</th>
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<td>LB</td>
<td>1.2 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>Saline + LB</td>
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<td></td>
<td></td>
<td>LB</td>
<td>2.3 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>Saline + LB</td>
<td>1.5 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bacterial cells (10&lt;sup&gt;4&lt;/sup&gt; CFU) added before DNA</td>
<td>1 day&lt;sup&gt;e&lt;/sup&gt;/1day&lt;sup&gt;e&lt;/sup&gt;</td>
<td>LB</td>
<td>7.3 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1 day/3 days</td>
<td>LB</td>
<td>2.3 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1 day/7 days</td>
<td>LB</td>
<td>6.0 × 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>DNA added before bacterial cells (10&lt;sup&gt;6&lt;/sup&gt; CFU)</td>
<td>1 min&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Saline</td>
<td>2.0 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>30 min</td>
<td>Saline</td>
<td>4.0 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>Saline</td>
<td>BD</td>
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</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 (10<sup>8</sup> CFU): in FSL with saline, 6.8 × 10<sup>5</sup>; in FSL with LB, 1.0 × 10<sup>20</sup>; in ELS with saline, 3.9 × 10<sup>5</sup>; in ELS with LB, 6.0 × 10<sup>5</sup>; with a low inoculum (10<sup>4</sup> CFU): in FSL with saline, 3.6 × 10<sup>3</sup>; in FSL with LB, 8.5 × 10<sup>3</sup>; in ELS with saline, 2.2 × 10<sup>5</sup>; in ELS with LB, 4.3 × 10<sup>5</sup>.

<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> Transformation period after both DNA and bacteria have been added to soil.

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

<sup>e</sup> Bacterial cells suspended in LB medium.

<sup>f</sup> Bacterial cells suspended in saline, DNA added in LB medium.

<sup>g</sup> BD, below detection limit (10<sup>-9</sup>).

DISCUSSION

The maximum in vitro transformation frequency of A. calcoaceticus BD413 Rp<sup>d</sup> with chromosomal DNA obtained with 10 μg of DNA and 10<sup>9</sup> CFU per filter for 24 h was 10<sup>-2</sup>. Species of only a few bacterial genera like Azotobacter, Hae¬mophilus, Bacillus, Neisseria, and Streptococcus have been reported to reach such high transformation frequencies (15, 22).

Similar optimized conditions have been reported by Cruze et al. (7) and Palmen et al. (28) for transformation of A. calcoaceticus in liquid media and by Ahlquist et al. (1) for transformation on solid media. The optimized conditions were extended stepwise to small-scale soil microcosms. Soil microcosms of various types have been extensively used (2, 42) to study the persistence, survival, and transport of introduced bacteria, and they have often been found to be practical and realistic tools to mimic the soil environment under controlled conditions. The use of the small microcosms developed allowed for the quick assessment of the influence of selected factors on transformation of A. calcoaceticus in soil.

Use of the high inoculum level (10<sup>8</sup> CFU) in saline in the sterile soil microcosms with DNA added after longer intervals resulted in transformation frequencies soon dropping to below the detection limit whereas recipient CFU counts increased about 40-fold. This suggests that A. calcoaceticus cells progressively lose competence in soil or that they become physically refractory to transformation. Postma (30) reported that 10<sup>8</sup> CFU of Rhizobium sp. introduced into ELS and FSL reached stationary phase after 24 h whereas 10<sup>8</sup> CFU took 4 days to reach this phase. Hence, we added 10<sup>8</sup> CFU of A. calcoaceticus and extended the transformation period up to 7 days to allow for more extensive bacterial growth which might stimulate transformation. However, these adjustments did not result in

TABLE 4. Transformation of A. calcoaceticus BD413 (10<sup>8</sup> CFU) in nonsterile soil microcosms

<table>
<thead>
<tr>
<th>Time (min)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Nutrient&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Transformation frequency&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>Saline</td>
<td>BD&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>30</td>
<td>LB</td>
<td>2.3 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>30</td>
<td>Root exudates</td>
<td>7.5 × 10&lt;sup&gt;-7&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 × 10<sup>5</sup>; in FSL with LB, 1.4 × 10<sup>5</sup>; in FSL with root exudates, 1.5 × 10<sup>5</sup>; in ELS with saline, 4.0 × 10<sup>5</sup>; in ELS with LB, 8.3 × 10<sup>5</sup> (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<sup>-9</sup>); ND, not determined.
any detectable transformation, even though the recipient CFU counts after 24 h of transformation were similar for the 10⁴- and 10⁵-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 *A. calcoaceticus* (7). Moreover, *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 *A. calcoaceticus* have benefitted 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 (10⁸ CFU) with added nutrients resulted in detectable transformants in FSL after 24 h, whereas with the high (10⁸ 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 *A. calcoaceticus* in soil is indeed promoted by bacterial growth.

Adding the LB broth together with *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 *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 transformation frequencies 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 *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 *A. calcoaceticus* (14).

As introduced chromosomal DNA became unavailable for substantial transformation events within a few hours, the availability of DNA for transformation of *A. calcoaceticus* cells in soil is probably highly restricted. Under the prevailing nutrient-limited conditions in soil, *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 *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 *A. calcoaceticus* can take place.

**ACKNOWLEDGMENTS**

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