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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

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TABLE 1. Bacterial strains used in this study

Strain	Source or reference
Acinetobacter calcoaceticus	
DSM586	DSM[a]
DSM586 Nx^r	This work[b]
DSM586 (*chr*::KTG) Nx^r	This work[c]
DSM586(pSKTG) Nx^r	This work
BD413	12
BD413 Rp^r	This work[d]
BD413 (*chr*::KTG) Rp^r	This work[c]
BD413(pSKTG) Rp^r	This work
Pseudomonas fluorescens	
R2f(pSKTG)	37
Escherichia coli	
MC1061(pSKTG)	37
SM10(λ*pir*)(pUT/KTG)	37

[a] DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.
[b] Spontaneous nalidixic acid-resistant mutant.
[c] With chromosomal insertion of the KTG cassette.
[d] Spontaneous rifampin-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 H_2O [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, 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 (at 225 rpm). The strains were stored in 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 Rp^r inoculated from a single colony (absorbance at 600 nm of a 1/10 dilution, >0.2) was centrifuged (3,000 × *g* for 5 min), resuspended in 0.85% NaCl (saline), recentrifuged (3,000 × *g* for 2 min), and resuspended in the initial volume of saline. The bacterial cells were then in the late exponential phase and were competent for transformation (28). For experiments with nutrients, the washing and resuspension were done with LB broth. The final density of the *A. calcoaceticus* washed cultures was 10^9 CFU/ml; to establish a less dense inoculum (10^4 CFU per g of soil), the cultures were serially diluted.

Construction of sources of transforming DNA. The gene cassette KTG (37), chosen to serve as a selectable marker in transformations with both plasmid and chromosomal DNA, consisted of the *nptII* gene (kanamycin resistance), the *aadB* gene (gentamicin resistance) and a truncated *Bacillus thuringiensis* subsp. *morrisoni cryIVB* gene. The IncQ plasmid pSKTG (37), which contained the KTG cassette, was electroporated into *A. calcoaceticus* DSM586 cells by using plasmid DNA extracted from *P. fluorescens* R2f(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 Nx^r as the recipient. Both washed donor and recipient cultures (50 μl each) were placed on nitrocellulose filters (Millipore GS; GSWP04700) 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 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/μl) and competent (28) *A. calcoaceticus* BD413 Rp^r 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 transformant cells.

The undiluted recipient cell suspension also was plated onto LBA supplemented with rifampin and kanamycin to check for spontaneous mutations to Km^r. 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 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^r 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

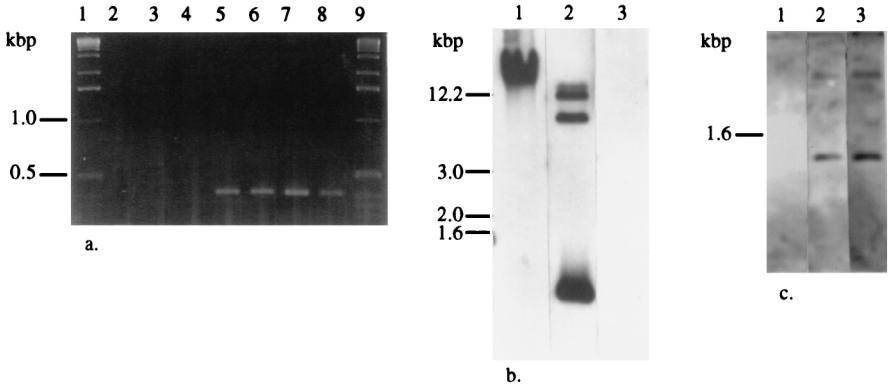

FIG. 1. (a) Agarose gel electrophoresis of PCR-amplified DNA with primer set IB3 plus IB4. Lanes: 1, 1-kb ladder (Gibco-BRL); 2, DNA from *Paenibacillus polymyxa*; 3, PCR mix; 4, nontransformed *A. calcoaceticus* recipient colony; 5, *A. calcoaceticus* colony transformed with pSKTG DNA; 6, *A. calcoaceticus* 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, *Pst*I cut; 3, wild-type DSM586 *Pst*I cut. (c) Southern blot of DNA from colonies of *A. calcoaceticus* BD413 Rp^r isolated from soil microcosms, cut with *Pst*I, and hybridized to an *nptII* probe. Lanes: 1, untransformed colony; 2 and 3, colonies transformed with chromosomal KTG DNA.

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transforming DNA, the DNA (10 μg) was placed on the filter and the recipient cell suspension (10^8 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 addition of water with the DNA or 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 ^{60}Co source (4 megarads) 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 the different soil incubation and transformation times (see below), the soil plugs were shaken in 4.75 ml of 0.1% sodium pyrophosphate (tetrasodium diphosphate 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), DNA (10 μg per microcosm), nutrients (100 μl of LB broth 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 the standard 24-h transformation time was assessed with 10^4 CFU with or without LB broth as a nutrient source.

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 incubation times (0, 1, and 30 min, 6 h, and 1, 3, and 7 days) of the recipient cells (10^8 CFU in saline) in soil before their contact with the transforming DNA was studied. After DNA addition, all soil portions were incubated for 24 h (transformation time) before plating. This setup also was used with 10^4 CFU of recipient cells with an incubation time of 24 h before DNA addition. In this experiment, the transformation times tested were 24 h as well as 3 and 7 days. The effect of addition of nutrients to (sterile) soil was investigated by performing the transformation assay with recipient cells (10^4 or 10^8 CFU) in LB broth (100 μl) instead of saline. LB broth was also added together with the DNA instead of with the bacterial cells (10^8 CFU).

The availability of transforming DNA to *A. calcoaceticus* BD413 cells was further investigated by incubating DNA in (sterile) soil for increasing periods (0, 1, and 30 min, 6 h, and 1, 3, and 7 days) before addition of bacterial cells (10^8 CFU). In the 0-min treatment, the DNA was mixed with the recipient cells in an Eppendorf tube before distribution into the soil. The experiment also was performed with 10^4 inoculant CFU with DNA residence times in soil of 1, 3, and 7 days; here, transformation was allowed to proceed for 24 h or 3 or 7 days for the 1-day DNA incubation, for 24 h or 3 days for the 3-day DNA incubation, and for 24 h for the 7-day DNA incubation.

A second set of experiments with nonsterile portions of FSL and ELS were performed with the same setups outlined above, except that the low inoculum levels (10^4 CFU) and the 7-day incubations were not used. Maize root exudate (100 μl) obtained as a liquid (46) was used in one experiment to translate the effect of added LB broth to nutrient sources common in soil. The sterility of the exudate was confirmed by plating onto LBA.

DNase I tube transformation control. To prevent transformation from occurring in the dilution and plating procedures, the soil plugs were diluted in 0.1% sodium pyrophosphate with added DNase I. To control the efficiency of DNase I in degrading transforming DNA and preventing transformation, an assay was done as follows. DNA (10 μg) was added to FSL or ELS plugs, and the soils were immediately suspended in 4.75 ml of 0.1% sodium pyrophosphate with or without 100 μl of DNase I and 10^8 CFU of *A. calcoaceticus*. Plating was done as for the filter transformations. *A. calcoaceticus* cell suspension (100 μl) was also added to the soil plugs, and 10 μl of DNA (10 μg) was added to 0.1% sodium pyrophosphate with or without DNase I (100 μl) shortly before suspension of the soils. Plating was done as for the filter transformations. Transformant colonies were never detected in any samples after the DNase I treatment.

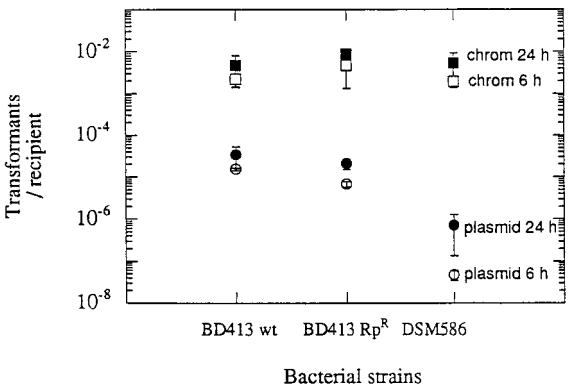

FIG. 2. Filter transformation of *A. calcoaceticus* BD413 wild type (wt), the spontaneous mutant BD413 Rp^r, and DSM586 Nx^r with chromosomal (chrom) or plasmid DNA (10 μg per 10^8 CFU) with a transformation period of 6 or 24 h on LBA plates.

Statistics. All experimental parameters described are given per system (soil plug or filter). All experiments on filters and with soil were done in triplicate and repeated once to check their reproducibility. As discussed by Palmen et al. (28), the data are presented as mean values for single experiments ± standard deviation (SD). The variability of the data is given as the coefficient of variation (SD/mean); it ranged from 0.1 to 0.3. All transformation frequencies are given as CFU of transformants per CFU of recipient, in accordance with the results of Palmen et al. (28). A Student *t* 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 *Pst*I. Following gel electrophoresis, the 923-bp *nptII* gene fragment was isolated with the Gene Clean II kit (Bio101, La Jolla, Calif.) and labelled with digoxigenin-11-dUTP (DIG) by the DIG random-labelling kit (Boehringer).

For colony hybridization, colonies were transferred to Qiabrane 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 sulfate. Chemiluminescent detection with the CSPD substrate (2a) for alkaline phosphatase was done as described in the Boehringer manual.

The localization of the KTG insert in the donor strain and transformants of the soil transformation assay was determined. *Pst*I-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 membrane was 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 $MgCl_2$, 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 AmpliTaq 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 *aadB* and *cryIVB* gene sequences (37). Primer sequences were 5′-TCT CAT GCT GGA GTT CTT CG-3′ for IB3 and 5′-CTG CTG TTC TAT AGG ACT GG-3′ for IB4. The expected product was generated both with pSKTG and with the KTG insert in the *A. calcoaceticus* genome (Fig. 1a).

RESULTS

Filter transformations. To verify if the ability to be transformed had been affected by the resistance to rifampin in *A. calcoaceticus* BD413 Rp^r, 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-

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

Conditions and time (days)[a]	FSL[b]			ELS[b]		
	Recipient counts	Transformant counts	Frequency	Recipient counts	Transformant counts	Frequency
Filter transformation						
1	9.9	7.1	1.4×10^{-3}	10	7.7	4.7×10^{-3}
7	9.4	7.1	4.6×10^{-3}	10.3	7.4	1.3×10^{-3}
Filter transformation with soil on top						
1	9.9	6.9	9.2×10^{-4}	10	7.3	1.7×10^{-3}
7	9.4	7.0	3.4×10^{-3}	10.3	7.1	7.8×10^{-4}
Bacteria in soil and DNA on filter[c]						
1	9.2	6.2	1.1×10^{-3}	9.4	5.8	2.2×10^{-4}
7	9.7	6.5	6.2×10^{-4}	9.4	6.1	5.2×10^{-4}
Bacteria on filter and DNA in soil[d]						
1	9.9	4.5	3.9×10^{-6}	10	5.0	8.1×10^{-6}
7	9.4	4.6	1.6×10^{-5}	10.3	4.6	2.1×10^{-6}

[a] Transformation period of bacteria in soil (10^8 CFU added).
[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.
[c] Recipient counts from soil.
[d] Recipient counts from filter.

ilarly, plasmid pSKTG transformed the wild type and the Rp^r derivative of strain BD413 equally well, at frequencies about 100-fold below those obtained with chromosomal DNA. Strain DSM586 showed a significantly ($P < 0.05$) lower transformability with plasmid pSKTG. The transformation frequency with both chromosomal DNA and plasmid DNA increased for all strains up to 24 h (Fig. 2). Further incubation of the transformation mixture of strain BD413 Rp^r 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^r cells were used. In this study, the maximum transformation frequencies were obtained with *A. calcoaceticus* cells added to the filter at 10^7 and 10^8 CFU, whereas lower (e.g., 10^6 CFU) as well as higher (10^9 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 $(3.7 \pm 1.0) \times 10^{10}$.

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 $(5.2 \pm 3.9) \times 10^{-3}$ per recipient. For plasmid DNA (10 μg), the average frequency for seven independent assays with two or more replicates per assay was $(1.9 \pm 1.1) \times 10^{-5}$ 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 $(2.9 \pm 1.2) \times 10^{-2}$ for chromosomal DNA and $(1.4 \pm 0.2) \times 10^{-5}$ for plasmid DNA.

To study the effect of temperature on transformation, *A. calcoaceticus* BD413 Rp^r cells were mixed with transforming DNA and incubated for 24 h at different temperatures. Incubation at 10°C gave an average transformation frequency of $(6.2 \pm 10.8) \times 10^{-4}$, incubation at 30°C gave $(3.9 \pm 5.9) \times 10^{-3}$, and incubation at 37°C gave $(4.4 \pm 0.6) \times 10^{-3}$. 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^r, this strain was chosen for further studies of transformation in soil. Transformation with a plasmid carrying the frequently used IncQ-

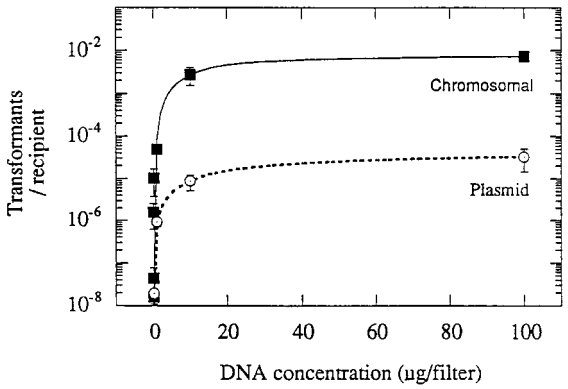

FIG. 3. Filter transformation of *A. calcoaceticus* BD413 Rp^r with increasing concentrations of plasmid or chromosomal DNA with a transformation period of 24 h on LBA plates.

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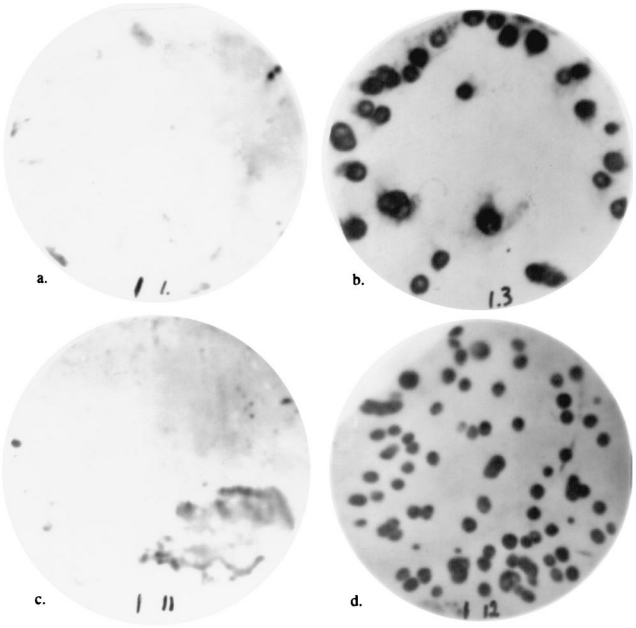

FIG. 4. Colony hybridization of chromosomally transformed *A. calcoaceticus* hybridized to an *nptII* probe (DIG nonradioactive labelling and chemiluminescent detection). Recipient-selective (a) and transformant-selective (b) plates from filter transformations and recipient-selective (c) and transformant-selective (d) plates from soil microcosm transformations are shown.

based origin of replication was not extended to soil, as the frequencies on the filters were at least 100-fold lower than those obtained with chromosomal DNA. A lowered transformation efficiency with plasmid DNA also has been reported by Palmen et al. (28). Chromosomal DNA containing the KTG gene cassette was therefore used as the source of transforming DNA throughout the soil microcosm studies.

Combined filter-soil microcosm transformations. As shown in Table 2, the placement of sterile soil onto the filter containing the transformation mix reduced the transformation frequencies obtained somewhat, in particular with ELS, for which the difference was significant ($P < 0.05$). With both soils, there were significant (inhibitory) effects of spatial separation of the transforming DNA and *A. calcoaceticus* cells (on the filter versus in the soil) on the transformation frequencies (Table 2). Placing DNA on the filter (with recipient cells in soil) significantly ($P < 0.05$) enhanced the transformation frequencies compared to adding it to the soils (recipient cells on the filter) (Table 2). In the experimental setup used, there were only small differences between the two soils with respect to the effects tested. Hence, both soils reduced transformation frequencies of *A. calcoaceticus* on the filter, as compared to those obtained on undisturbed filters, presumably mainly by a spatial separation effect.

Soil microcosm transformations. Studies in microcosms were first performed with sterile soils to assess the effects on transformation of soil type, incubation time of DNA or *A. calcoaceticus* cells in soil, nutrients, and transformation period. Incubation of *A. calcoaceticus* cells (10^8 CFU) for progressively increasing times in soil before addition of DNA clearly decreased the transformation frequencies in both soils (Table 3), and after 1 day of residence in soil (before DNA addition), transformation was no longer detectable in either of the two soils. Maximum transformation frequencies without added nutrients (about 10^{-4} for FSL and about 10^{-7} for ELS) were 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 (10^4 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 (10^8 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 (10^8 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. 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 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

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TABLE 3. Transformation of *A. calcoaceticus* BD413 in sterile soil microcosms

Treatment			Transformation frequency[a]	
Conditions	Time	Nutrient(s)	FSL	ELS
Bacterial cells (10^8 CFU) added before DNA	0 min[b]	Saline	2.5×10^{-5}	BD[g]
		LB[e]	1.2×10^{-5}	2.9×10^{-6}
		Saline + LB[f]	1.6×10^{-5}	1.5×10^{-6}
	1 min[b]	Saline	1.2×10^{-4}	1.4×10^{-7}
		LB	5.6×10^{-5}	2.8×10^{-5}
		Saline + LB	5.8×10^{-6}	1.3×10^{-6}
	30 min[b]	Saline	7.2×10^{-5}	BD
		LB	2.5×10^{-5}	6.3×10^{-7}
		Saline + LB	3.9×10^{-6}	BD
	6 h[b]	Saline	4.8×10^{-7}	BD
		LB	2.3×10^{-6}	2.3×10^{-6}
		Saline + LB	1.5×10^{-6}	BD
	1 day[b]	Saline	BD	BD
		LB	BD	BD
		Saline + LB	BD	BD
Bacterial cells (10^4 CFU) added before DNA	1 day[b]/1day[c]	LB	7.3×10^{-6}	BD
	1 day/3 days	LB	2.3×10^{-6}	BD
	1 day/7 days	LB	6.0×10^{-7}	BD
DNA added before bacterial cells (10^8 CFU)	1 min[d]	Saline	2.0×10^{-5}	BD
	30 min	Saline	4.0×10^{-6}	BD
	6 h	Saline	BD	BD

[a] Frequencies given as transformants per recipient. The mean coefficient of variation was 0.3. Average recipient counts with a high inoculum (10^8 CFU): in FSL with saline, 6.8×10^9; in FSL with LB, 1.0×10^{10}; in ELS with saline 3.9×10^9; in ELS with LB, 6.0×10^9; with a low inoculum (10^4 CFU): in FSL with saline, 3.6×10^9; in FSL with LB, 8.5×10^9; in ELS with saline, 2.2×10^9; in ELS with LB, 4.3×10^9.
[b] Incubation time of bacterial cells in soil before DNA was added. After addition, all samples were incubated for 24 h unless otherwise stated.
[c] Transformation period after both DNA and bacteria have been added to soil.
[d] Residence time of DNA in soil before bacterial cells were added.
[e] Bacterial cells suspended in LB medium.
[f] Bacterial cells suspended in saline, DNA added in LB medium.
[g] BD, below detection limit (10^{-8}).

KTG-specific primer set IB3 and IB4. All putative transformants analyzed showed the expected bands, confirming their identity as transformants. Colonies growing on recipient-selective plates, as well as colonies presumed to represent indigenous bacteria, never gave rise to PCR products (data not shown). In addition, hybridization of transformant and recipient colonies obtained from soil with the *nptII* probe showed that only transformant colonies could produce hybridization signals (Fig. 4c and d).

DISCUSSION

The maximum in vitro transformation frequency of *A. calcoaceticus* BD413 Rp^r with chromosomal DNA obtained with 10 μg of DNA and 10^8 CFU per filter for 24 h was 10^{-2}. Species of only a few bacterial genera like *Azotobacter*, *Haemophilus*, *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 (10^8 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 10^8 CFU of *Rhizobium* sp. introduced into ELS and FSL reached stationary phase after 24 h whereas 10^4 CFU took 4 days to reach this phase. Hence, we added 10^4 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 (10^8 CFU) in nonsterile soil microcosms

Treatment		Transformation frequency[a]	
Time (min)[b]	Nutrient[c]	FSL soil	ELS soil
30	Saline	BD[d]	BD
30	LB	2.3×10^{-6}	BD
30	Root exudates	7.5×10^{-7}	ND[d]

[a] Frequencies are given as transformants per recipient. The mean coefficient of variation was 0.3. Average recipient counts in FSL with saline, 7.5×10^7; in FSL with LB, 1.4×10^8; in FSL with root exudates, 1.5×10^8; in ELS with saline, 4.0×10^8; in ELS with LB, 8.3×10^8 (FSL and ELS experiments were done independently).
[b] Incubation time of bacteria in soil before DNA was added. After addition, all samples were incubated for 24 h.
[c] Medium in which cells were added to soil.
[d] BD, below detection limit (10^{-8}); ND, not determined.

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any detectable transformation, even though the recipient CFU counts after 24 h of transformation were similar for the 10^4- and 10^8-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 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 (10^8 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.

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