Induced Natural Transformation of *Acinetobacter calcoaceticus* in Soil Microcosms

KAARE M. NIELSEN,^{1*} ATLE M. BONES,¹ AND JAN D. VAN ELSAS²

Unigen—Center for Molecular Biology, Norwegian University of Science and Technology, N-7005 Trondheim, Norway,¹
and Research Institute for Plant Protection, IPO-DLO, NL-6700 GW Wageningen, The Netherlands²

Received 21 April 1997/Accepted 1 August 1997

Factors affecting natural transformation of *Acinetobacter calcoaceticus* BD413 with homologous chromosomal DNA in a silt loam soil microcosm were investigated. Inducible transformation of declining populations of noncompetent *A. calcoaceticus* cells was detectable for up to 6 days when a simple carbon source, salts, and freshly added DNA were used. In two different experimental setups, the residence time in soil of induced cells could be increased to either 11 or 24 h before DNA addition without reduced transformation frequency; 200- to 1,000-fold fewer transformants were observed following the addition of water. These observations suggest that *A. calcoaceticus* remains transformable for several hours after its activation by nutrients in soil. Increasing the levels of phosphate salts significantly enhanced the numbers of transformants without increasing the recipient counts correspondingly. Variable levels of ammonium or divalent cations (Mg^{2+} and Ca^{2+}) did not have a similar major influence. Soil moisture content significantly affected the transformation frequency of *A. calcoaceticus* cells, with a general tendency of higher frequencies in drier soil. A minimal frequency was observed at around 35% soil moisture. The data indicate that *A. calcoaceticus* cells in soil which cannot be detectably transformed are easily induced by nutrients to undergo natural transformation with chromosomal DNA. Access to nutrients seems to be critical for the development and maintenance of competence in soil, which is also affected by abiotic factors like moisture level and phosphate salt concentration.

Natural transformation, the uptake of extracellular DNA by bacteria, may represent a horizontal gene transfer mechanism of importance in the environment (7, 15, 38). The significance of this mechanism can only be understood by identifying bacterial species that become competent for transformation, the genetic and cellular pathways involved, and the environmental factors influencing and regulating the process. Natural transformation has been detected in over 25 bacterial genera, many of which are ubiquitous soil bacteria (2, 15, 22). Recently, transformation of *Escherichia coli* in spring water was shown (1), and the process is probably more widespread than previously thought (31). Natural transformation has been reported in several environmental settings, e.g., fresh and marine waters and sediments (3, 26, 32, 33), river epilithon (39), sterile soil, soil extracts, and amended mineral soil (8, 9, 16). However, reports on natural transformation with chromosomal DNA in natural (nonsterile) soils are scarce (13, 17). Expression of competence factors is often required for natural transformation to occur (15). Only a few bacteria that express natural competence in the environment have been detected (28), and little information exists on the factors that affect the development and regulation of their in situ competence (6, 20, 21, 37).

The soil bacterium *Acinetobacter calcoaceticus* is easily grown into a naturally competent state during exponential phase, and it is transformable with chromosomal DNA at high frequencies (11, 24, 25). In a previous study (17), a soil microcosm for the study of transformation of *A. calcoaceticus* BD413 was developed and it was concluded that competent cells added to soil ceased to be transformable within 24 h. Addition of Luria-Bertani (LB) medium or root exudates was able to

prolong the period during which cells remained transformable or to enhance the transformation frequency.

In this study, the microcosm was used to assess whether development of competence in *A. calcoaceticus* cells residing in soil is inducible in situ. The effects on transformation frequencies of the addition of nutrients, divalent cations, and phosphate or ammonium salts and of the incubation time of bacteria in soil and soil moisture were studied in a silt loam. Also, a direct in vitro assay was developed to screen more directly for acquired competence in *A. calcoaceticus* cells in soil after nutrient stimulation.

MATERIALS AND METHODS

Bacterial strain, media, and DNA. Inocula of *A. calcoaceticus* BD413 Rp^r (10^9 CFU ml⁻¹, 0.85% NaCl) were prepared as described by Nielsen et al. (17). Samples from the soil microcosms were plated either on recipient-selective LB (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 1 liter of H₂O [pH 7.2]) agar (LBA) plates (1.5% agar) supplemented with 100 µg of cycloheximide per ml and 50 µg of rifampin per ml or on transformant-selective plates which were, in addition, supplemented with 50 µg of kanamycin per ml. Chromosomal DNA extracted from *A. calcoaceticus* BD413 containing the KTG gene cassette (30) was used as transforming DNA (17). The KTG cassette consisted of the *nptII* gene (kanamycin resistance), the *aadB* gene (gentamycin resistance), and a truncated *Bacillus thuringiensis* subsp. *morrisoni* cryIVB gene (30).

Soil microcosm and transformation procedure. A Flevo silt loam (FSL) soil obtained from microplots in Wageningen, The Netherlands, was used in all microcosms. FSL soil has been described previously (30, 35). After sieving (4-mm mesh), the soil was used directly or stored in plastic bags at 4°C. Microcosms consisted of autoclaved polypropylene cylinders of 1-cm³ volume, to which 1.2 g of soil was added as described before (17). The pH of the FSL soil was 7.2 (35). An optimum pH for natural transformation of *A. calcoaceticus* of between 5.6 and 7.9 has been indicated previously (28).

Except where otherwise stated as a result of the treatments applied (see "Experimental factors," below), a standard procedure for transformation studies in soil was used throughout. *A. calcoaceticus* cells suspended in sterile 0.85% NaCl were added to the soil microcosms (10^8 CFU per g of dry soil) by careful pipetting of a 100-µl aliquot onto the surface of each microcosm. Final density of the soil was 1.4 g/cm³. The cylinders containing the inoculated soil portions were placed on sterile water agarose (1.5%) in petri dishes. The soil microcosms were incubated for 24 h before addition of nutrient solution (100 µl), or water (100 µl) as a control. A nutrient solution of 5× M9L (5× M9 salts [29] plus 2%

* Corresponding author. Mailing address: Unigen—Center for Molecular Biology, MTFs, Norwegian University of Science and Technology, N-7005 Trondheim, Norway. Phone: 47.73.598680. Fax: 47.73.598705.

TABLE 1. Natural transformation of *A. calcoaceticus* BD413 with increasing time of residence in soil microcosms before addition of nutrient and transforming DNA

Time (days) ^a	Nutrient							
	Water		5× M9L			5× M9L-25P		
	CFU/sampled soil portion of:		CFU/sampled soil portion of:		Transformation frequency	CFU/sampled soil portion of:		Transformation frequency
	Recipients	Transformants	Recipients	Transformants		Recipients	Transformants	
1	4.3 × 10 ⁷	26	5.9 × 10 ⁻⁷	1.7 × 10 ⁸	10,136	5.9 × 10 ⁻⁵	ND ^c	ND
2	1.5 × 10 ⁷	BD ^d	<6.6 × 10 ⁻⁸	8.6 × 10 ⁷	1,374	1.6 × 10 ⁻⁵	ND	ND
3	2.2 × 10 ⁶	BD		3.4 × 10 ⁷	2.3	6.8 × 10 ⁻⁸	ND	ND
4	1.6 × 10 ⁶	BD		6.1 × 10 ⁶	BD	<1.6 × 10 ⁻⁷	4.8 × 10 ⁶	7
5	8.5 × 10 ⁵	BD		4.8 × 10 ⁶	BD		2.5 × 10 ⁶	68
6	8.2 × 10 ⁵	BD		1.8 × 10 ⁶	BD		1.1 × 10 ⁶	68
7	ND	ND		ND	ND		6.8 × 10 ⁵	BD
								1.5 × 10 ⁻⁶
								2.7 × 10 ⁻⁵
								6.2 × 10 ⁻⁵
								<1.5 × 10 ⁻⁶

^a Time of addition of nutrients and transforming DNA (after 1 h) after bacterial inoculation (10⁸ CFU).^b Transformation frequency is given as transformant CFU divided by recipient CFU. The mean coefficient of variation (CFU) was 0.4.^c ND, not determined.^d BD, below detection limit.

lactic acid) was used unless otherwise stated. One hour after nutrient addition, 15 µg of DNA per g of dry soil was added. Nutrients and DNA were added as described for the inoculum. Microcosms without added DNA or cells or with added calf thymus DNA (Sigma, St. Louis, Mo.) were used as controls. After another 24 h of incubation, the cores of the soil microcosms (25% of the total dry weight of the soil) were sampled by using the wide end of a 1-ml Eppendorf tip, transferred to a 1.5-ml Eppendorf tube with 0.9 ml of 0.1% sodium pyrophosphate (tetra-sodiumdiphosphate-decahydrate; Merck) supplemented with 50 µl of a 5-mg/ml DNase I stock solution (Boehringer, Mannheim, Germany), and Vortex mixed with 0.2 g of sterile gravel (2 to 4 mm in diameter). The solution was either plated directly on LBA plates (with selection as described above for the recipient- and transformant-selective plates) or serially diluted in 0.1% sodium pyrophosphate before plating.

CFU were enumerated after a 72-h incubation period at 27°C. All experimental procedures and incubations were done at 20°C except for the plated soil samples and parts of the competence assay as described below. A temperature optimum for natural transformation of *A. calcoaceticus* of between 14 and 37°C has been reported previously (28). Transformation frequencies are given as the number of *A. calcoaceticus* CFU growing on transformant-selective LBA plates divided by the number of CFU on recipient-selective plates. All CFU counts refer to the sampled soil portions of 0.3 g (dry weight) of soil.

Experimental factors. (i) Nutrient addition. To determine a suitable additive for stimulation of the competence of *A. calcoaceticus* cells residing in soil, different nutrient sources were added to the inoculated soil portions. These were 0.1× tryptic soy broth (Oxoid), LB, 5× LB, minimal medium M9 (29) with 0.4% lactic acid (Merck) adjusted to pH 6 with NaOH, 0.4 to 4% lactic acid, 1× to 10× M9, 5× M9L, 5× M9L-25P (5× M9L with 25× M9 phosphate salts), trace element solution (1 g of MnSO₄·H₂O, 0.25 g of ZnSO₄·7H₂O, 0.25 g of CuSO₄·5H₂O, 0.25 g of H₃BO₃, and 0.05 g of Na₂MoO₄·2H₂O in 1 liter of H₂O), and multifruit and vegetable juices (Krings Fruchtsaft AG, Mönchengladbach, Germany) diluted 1/10 or 1/100 in water. Water added to the microcosms

was used as a control. To study the effects of divalent cations on transformation, different concentrations of MgSO₄ (0, 0.081, 0.81, 8.1, or 81 mM) and CaCl₂ (0, 0.0068, 0.068, 0.68, or 6.8 mM) were included in the 5× M9L media. For the studies involving variable phosphorus (P) and nitrogen (N) concentrations, 0, 1, 5, 10, or 25 times the standard P or N concentration in the M9 salts was included in the 5× M9L media. The pH was adjusted to 6.2 with 10 M NaOH. The carbon source was 2% lactic acid throughout.

(ii) Increasing the residence time of bacteria in soil before transformation. The effects of varying the incubation time (1 [standard], 2, 3, 4, 5, or 6 days) of *A. calcoaceticus* in soil before the addition of nutrients (5× M9L) or water (control) and DNA were studied. In addition, 5× M9L with a 25-fold concentration of phosphate salts in M9 medium was used at selected time points (4, 5, 6, or 7 days).

(iii) Transformation period after nutrient-DNA addition. To assess how long the *A. calcoaceticus* cells in the nutrient- or water-treated soil microcosms remained transformable, DNA additions were done at 0, 1 (standard), 3, 6, 11, or 24 h after nutrient or water amendment.

(iv) Soil moisture. The effects of variable soil moisture levels were studied. Soil microcosms were dried at 37°C for 48 h. Sterile water to adjust soil moisture was added either before, with, or after the bacterial inoculum. The addition of varying amounts of water (0, 50, 100, 200, or 300 µl), 100 µl of inoculant and 5× M9L or water, and DNA gave final moisture contents of 18, 22, 27, 35, and 44%. The percentage of moisture was calculated as wet weight of soil minus dry weight of soil divided by dry weight of soil multiplied by 100. The soil dry weight was determined after soil samples were dried at 105°C for 2 days.

In situ competence assay. Assessment of the competence of *A. calcoaceticus* cells in soil was done as follows: microcosms with cells incubated for 24 h were amended with 100 µl of 5× M9L or water (control) and incubated for 0, 1, 3, 6, 9, 24, or 48 h at 20°C. After incubation, the microcosms were sampled as described above, and, following Vortex mixing, 15 µg of DNA was added and the

TABLE 2. Natural transformation of *A. calcoaceticus* BD413 with increasing time of incubation in soil microcosms after nutrient input and before addition of transforming DNA

Time (h) ^b	Nutrient ^a					
	Water			5× M9L		
	CFU/sampled soil portion of:		Transformation frequency ^c	CFU/sampled soil portion of:		Transformation frequency
	Recipients	Transformants		Recipients	Transformants	
0	3.9 × 10 ⁷	49	1.2 × 10 ⁻⁶	1.6 × 10 ⁸	10,234	6.5 × 10 ⁻⁵
1	4.9 × 10 ⁷	30	6.2 × 10 ⁻⁷	1.6 × 10 ⁸	11,480	7.3 × 10 ⁻⁵
3	6.4 × 10 ⁷	65	1.0 × 10 ⁻⁶	2.3 × 10 ⁸	19,338	8.6 × 10 ⁻⁵
6	3.9 × 10 ⁷	32	8.3 × 10 ⁻⁷	2.3 × 10 ⁸	21,205	9.3 × 10 ⁻⁵
11	6.7 × 10 ⁷	63	9.5 × 10 ⁻⁷	1.9 × 10 ⁸	14,597	7.6 × 10 ⁻⁵
24	6.2 × 10 ⁶	BD ^d	<1.6 × 10 ⁻⁷	8.7 × 10 ⁷	BD	<1.1 × 10 ⁻⁸

^a Nutrients were added to the soil microcosms 24 h after inoculation with bacteria (10⁸ CFU).^b Time of introduction of transforming DNA after nutrient addition.^c Transformation frequency is given as transformant CFU divided by recipient CFU. The mean coefficient of variation (CFU) was 0.4.^d BD, below detection limit.

FIG. 1. CFU of *A. calcoaceticus* BD413 in a silt loam incubated with increasing concentrations of phosphate salts (P) or ammonium (N) in modified 5× M9L medium. Symbols: ○, N recipients; ●, N transformants; ◇, P recipients; ◆, P transformants. Vertical bars represent SDs.

solution was incubated at 27°C for 2 h. The suspension was then treated with DNase I and plated as described for the standard procedure of transformation.

Samples were taken before and after the 2-h period of incubation as controls to measure bacterial growth. Increases in recipient cell numbers were not detected in the 2-h competence assay for either 5× M9L- or water-amended microcosms. Increases in recipient cell numbers in samples from soil microcosms which were amended with water and subsequently treated with 100 µl of fresh 5× M9L immediately before the 2-h incubation period were not detected either, suggesting that activation of the cells during the 2-h period was not the cause of the enhanced transformation frequencies.

Controls. In addition to the specific controls described for each experiment, a number of controls were performed by the standard procedure of transformation, unless otherwise stated, to determine if plate transformation had occurred.

To determine the availability of DNA incubated in soil to competent *A. calcoaceticus*, bacterial cells grown in vitro were added to soil amended with DNA (DNA incubated in soil for 5 min to 24 h) and immediately sampled and plated.

To determine the availability of DNA incubated in soil to nutrient-stimulated soil-grown *A. calcoaceticus* cells, suspensions from soil portions containing *A. calcoaceticus* stimulated with nutrients were mixed (1/1) with suspensions from soil portions containing DNA (incubated for 5 min to 24 h in soil) and immediately sampled and plated.

To determine the availability of fresh DNA to nutrient-stimulated soil-residing *A. calcoaceticus*, soil portions containing bacterial cells were suspended, mixed with DNA, and immediately plated.

All plate transformation controls were negative. In addition, our experimental procedures did not suggest that plate transformations had occurred. CFU were found to be dilutable, indicating that high amounts of DNA and cells on the undiluted transformant-selective plates did not induce plate transformants. DNase I (50 µg ml⁻¹) was included in all soil suspensions (as described in reference 17); free DNA occurring in these suspensions should therefore be rendered rapidly unavailable for transformation. Further, sampling of bacterial cells was done at least 24 h after DNA addition to the soil microcosms. Previous studies showed that chromosomal DNA incubated in soil for over 6 h is not available to *A. calcoaceticus* as transforming DNA (17).

Identification of putative transformants. To assess the identity of putative transformant colonies, PCR amplifications were performed directly on colonies picked from restreaked transformant- and recipient-selective (control) plates by the method developed by Hofmann and Brian (10) with experimental conditions as described previously (17). The primers specifically amplified a 412-bp sequence of the KTG gene cassette by annealing to regions of the *aadB* (primer 1) and *cryIVB* (primer 2) genes (30).

To compare metabolic patterns of putative transformants with that of the inoculum strain, GN-Biolog microplates (Biolog, Inc., Hayward, Calif.) were utilized according to the manufacturer's instructions, with a 24-h incubation time and visual reading.

Statistics. The data are presented as mean values for single experiments ± standard deviations (SDs) (24). All experiments were done in triplicate and repeated at least once. The variability of the data (CFU counts) given as the coefficient of variation (SD/mean) ranged from 0.3 to 0.4. The limit of detection is given as the reciprocal of the recipient cells (CFU). The Student *t* test was used to compare the data with regard to significance (*P* of <0.05 was regarded as significant).

FIG. 2. Transformation frequencies of *A. calcoaceticus* BD413 incubated in a silt loam with increasing moisture content. Symbols: ●, water added before bacterial cells; ◆, water added with bacterial cells; ○, water added after bacterial cells. The coefficient of variation (CFU) was 0.4.

RESULTS

Nutrient addition. To identify suitable conditions for promoting competence of *A. calcoaceticus* in soil, different nutrient combinations were used. The highest transformation frequencies of *A. calcoaceticus* cells were observed following the addition of 5× LB and 5× M9L (both 3.4×10^{-5}) to the soil with a 24-h incubation period (after nutrient-DNA addition). Other nutrient supplies, e.g., 0.1× tryptic soy broth, 1× to 10× M9 salts, LB, 0.4 to 4% lactic acid, and diluted fruit and vegetable juices, gave significantly (*P* < 0.05) lower frequencies (data not shown). As a control, added water was used, which gave a transformation frequency of 3.3×10^{-7} , with a detection limit of 2×10^{-8} . Addition of trace elements in the 5× M9L did not alter the frequency significantly (*P* < 0.05). Given the defined composition of 5× M9L and the high transformation frequencies obtained with it, this medium was utilized in further studies. As in vitro transformation of *A. calcoaceticus* is known to be dependent on divalent cations (24), the effects of increased concentrations of Mg²⁺ (0.081 to 81 mM) and Ca²⁺ (0.0068 to 6.8 mM) in the 5× M9L medium were elucidated. Enhanced transformation frequencies could not be detected over the concentration range examined (data not shown). The middle value in the concentration range equalled the concentration in the minimal medium used for growing *A. calcoaceticus* BD413 to competence (24).

Increasing the residence time of bacteria in soil. To assess how long *A. calcoaceticus* cells could reside in soil and still be induced in situ to undergo natural transformation, the bacterial cells were incubated for up to 7 days before the addition of nutrients or water and transforming DNA. As shown in Table 1, transformants were detectable for up to only 1 day after addition of water and up to 6 days when 5× M9L-25P was added. CFU counts of the *A. calcoaceticus* cells in soil declined 250-fold over the 7-day period of incubation (Table 1).

Transformation period after nutrient stimulation. We assessed how long the *A. calcoaceticus* cells in soil induced to competence maintained their ability to be transformed. Cells were incubated for increasing periods of time in soil with nutrients before DNA was added. If DNA was added within the first 11 h after stimulation, the transformation frequencies varied from 6.5×10^{-5} to 9.3×10^{-5} when 5× M9L was added and from 8.3×10^{-7} to 12×10^{-7} when water was added

TABLE 3. Direct assay for in situ competence of *A. calcoaceticus* BD413 with increasing time of incubation in soil microcosms after nutrient addition

Time (h) ^b	Nutrient ^a					
	Water			5× M9L		
	CFU/sampled soil portion of:		Transformation frequency ^c	CFU/sampled soil portion of:		Transformation frequency
	Recipients	Transformants		Recipients	Transformants	
0	6.4 × 10 ⁷	9	1.5 × 10 ⁻⁷	5.8 × 10 ⁷	BD ^d	<1.7 × 10 ⁻⁸
1	6.4 × 10 ⁷	21	3.3 × 10 ⁻⁷	6.0 × 10 ⁷	7	1.2 × 10 ⁻⁷
3	4.8 × 10 ⁷	8	1.7 × 10 ⁻⁷	7.5 × 10 ⁷	380	5.1 × 10 ⁻⁶
6	3.4 × 10 ⁷	16	4.8 × 10 ⁻⁷	7.4 × 10 ⁷	1,274	1.7 × 10 ⁻⁵
9	5.3 × 10 ⁷	7	1.3 × 10 ⁻⁷	1.1 × 10 ⁸	2,194	2.0 × 10 ⁻⁵
24	5.2 × 10 ⁷	BD	<1.9 × 10 ⁻⁸	2.0 × 10 ⁸	1,043	5.3 × 10 ⁻⁶
48	1.9 × 10 ⁷	BD		1.6 × 10 ⁸	BD	<6.3 × 10 ⁻⁹

^a Nutrients were added to the soil microcosms 24 h after inoculation with bacteria (10^8 CFU).

^b Time of sampling after nutrient addition for 2-h incubation with transforming DNA.

^c Transformation frequency is given as transformant CFU divided by recipient CFU. The mean coefficient of variation was 0.35.

^d BD, below detection limit.

(Table 2). However, transformants were no longer detected if the DNA was introduced 24 h or more after addition of nutrients or water. Between 11 and 24 h after 5× M9L addition, the number of transformants dropped over 6,000-fold, to below detection (Table 2).

Effects of variable P and N concentrations. To investigate the effects of variable P and N concentrations, a 25-fold range, from 0 to 25 times the concentration in M9 medium (29), was used. As shown in Fig. 1, increasing the N concentration exerted (with one exception) a transformation-inhibiting effect, from a frequency of 3.1×10^{-6} to 7.4×10^{-8} . Increasing amounts of P enhanced the transformation frequencies of *A. calcoaceticus* significantly ($P < 0.05$), from 4.4×10^{-6} to 1.2×10^{-4} .

Effects of soil moisture content. Increasing the soil moisture content affected the transformation frequencies of *A. calcoaceticus* significantly (Fig. 2). Irrespective of the various water addition treatments, the highest transformation frequencies were obtained at low soil moisture levels. As shown in Fig. 2, the mode of water addition to adjust the soil moisture also influenced the transformation frequencies observed. Surprisingly, in all treatments there was a minimum frequency observed at 35% soil moisture (which is approximately 60% of the soil's water-holding capacity), after which the frequency increased again (Fig. 2).

In situ competence assay of *A. calcoaceticus* cells in soil. An assay was developed to directly study the ability of *A. calcoaceticus* cells residing in soil to take up and express exogenous DNA. Cells in soil incubated for 24 h and subsequently treated in situ for 0 to 48 h with 5× M9L or water were suspended in 0.1% sodium pyrophosphate and exposed to transforming DNA for 2 h. As can be seen in Table 3, *A. calcoaceticus* cells from the water-treated soil microcosms showed competence for up to only 9 h, whereas cells activated in situ with 5× M9L showed competence for up to 24 h, with significantly enhanced transformation frequencies after only 1 h (100- to 1,000-fold). Between 24 and 48 h after nutrient amendment, a sharp 900-fold reduction in the number of transformants was seen (Table 3). This reduction was not followed by a similar reduction in the recipient cell numbers (twofold).

Analysis of transformants. The characteristic colony morphology of *A. calcoaceticus* cells (circular, convex, dense, slightly yellow colonies, Fig. 3b to d) on the selective medium used made discrimination of these from the natural background feasible. Randomly-picked colonies from transfor-

mant-selective plates were restreaked and PCR amplified with the KTG-specific PCR amplification system. All colonies presumptively identified as *A. calcoaceticus* transformants revealed the expected 412-bp band of the KTG cassette in the transforming DNA (data not shown). Randomly picked colonies were also shown to be *A. calcoaceticus* by their metabolic patterns, which were identical to that of the inoculant on GN-Biolog plates. A few bacterial colonies emerged on transformant-selective plates as a result of growth of naturally Rp^+ and Km^r bacteria from the nonsterile FSL soil used (Fig. 3). GN-Biolog screening of background bacterial colonies revealed *Moraxella* spp. as the predominant species.

DISCUSSION

The aim of this study was to examine the key abiotic factors that affect natural transformation of *A. calcoaceticus* cells residing in soil. In a previous study (17), a silt loam was found to be more conducive to gene transfer than a loamy sand. Furthermore it was observed that *A. calcoaceticus* did not maintain detectable transformability after incubation for 24 h or more in sterile or nonsterile soils. Freshly added DNA and the presence of growing or metabolically active *A. calcoaceticus* cells were suggested to be prerequisites for detectable in situ transformation. In this study, we investigated whether selected factors in a silt loam could enhance, prolong, or induce natural transformation of *A. calcoaceticus* cells that had entered the stationary phase in soil and were nontransformable.

Inducible in situ transformation of the declining populations of *A. calcoaceticus* cells was indeed found for up to 6 days. Palmen et al. (25) found 4-day-old stationary *A. calcoaceticus* cells from batch culture to easily develop competence when diluted into fresh medium. Thus, both in vitro and in soil, presumably nontransformable *A. calcoaceticus* cells in the stationary phase can be readily induced to develop competence by nutrient upshifts favoring their growth and activity.

The induced bacterial cells maintained their ability to be transformed for 11 to 24 h following stimulation, after which a 900- to 6,000-fold drop in transformation frequencies was observed, suggesting that growth- and activity-limiting conditions perturbed transformation after 24 h. These results together with the in situ competence assay support the conclusion that *A. calcoaceticus* cells can indeed become competent in soil (in situ) and that their level of competence is influenced by their metabolic state. The few transformants obtained after addition

FIG. 3. LBA plates with soil suspensions obtained from microcosms with added *A. calcoaceticus*. (a) Transformant-selective plate inoculated with an undiluted soil suspension from a microcosm to which only *A. calcoaceticus* cells had been added. (b) Transformant-selective plate inoculated with an undiluted soil suspension from a microcosm to which both *A. calcoaceticus* and DNA were added. (c) Transformant-selective plate inoculated with a diluted soil suspension from a microcosm to which both *A. calcoaceticus* and DNA were added. (d) Recipient-selective plate inoculated with a diluted soil suspension from that shown in panel a.

of water were most likely caused by a redistribution of nutrients, DNA, or cells in the microcosm due to the induced water flow.

The combined addition of simple organic compounds and phosphate salts to soil induced transformation of *A. calcoaceticus* in our microcosm. Similar nutrient amendments have also been reported to enhance transformation frequencies of *Pseudomonas stutzeri* on agar medium with soil extract (14). Although speculative, several factors can contribute to the transformation-enhancing effects of phosphate. Improved availability of DNA may occur, as added phosphate may interact with the same sites on soil particles as does the phosphate backbone of DNA (12, 18, 23). Transmembrane helices of poly- β -hydroxybutyrate in association with polyphosphate have

been suggested to assist in the uptake of DNA in *Escherichia coli* (27). Polyphosphate is accumulated by *A. calcoaceticus* cells introduced into rich medium after P starvation, and polyphosphate granules have been associated with DNA fibrils (34). Prolonged high metabolic activity due to accumulated polyphosphate may occur (5). Decreased binding of bacterial cells to soil particles is also feasible (4). However, such an effect is unclear, as the addition of divalent cations (Mg^{2+} and Ca^{2+}) did not influence the transformation frequencies substantially. The amounts of available divalent cations in the FSL soil might have been nonlimiting for transformation, as FSL is calcium rich (35).

The moisture level in soil may influence the localization and physiology of the introduced cells and the distribution of nu-

trients and transforming DNA. Localization of bacterial cells in soil pores of different sizes due to the three different rewetting and bacterial inoculation procedures applied was expected. Addition of water before the bacterial cells presumably will locate the *A. calcoaceticus* cells in larger pores. A mixed water-cell suspension will locate cells over a broader range of soil pore sizes, whereas addition of cells before water locates them in smaller pores. The generally enhanced transformation frequency observed in drier soils might suggest an improved efficient concentration of DNA, cells, or nutrients, altered cell physiology, or enhanced competitive ability of *A. calcoaceticus* cells. The minimum transformation frequency observed at 35% soil moisture was possibly related to a maximal stimulation of the competitive activity of the indigenous aerobic microorganisms, with consequently less favorable physiological conditions or availability of DNA for *A. calcoaceticus*.

We conclude that (i) poorly transformable *A. calcoaceticus* cells can be induced to undergo natural transformation with chromosomal DNA in soil, (ii) competence in *A. calcoaceticus* cells is maintained for several hours after its induction in soil, and (iii) transformation is affected by abiotic factors like soil moisture level and phosphate concentration.

High phosphorus and nutrient levels (19) in agricultural soils may occur due to the spread of manure slurries. This soil management practice can thus result in a soil situation conducive to in situ transformation of *A. calcoaceticus*. Our studies (reference 17 and this work) have elucidated several factors and conditions of importance for the development of in situ competence of *A. calcoaceticus*. Further studies with rhizosphere microcosms (36) and field trials will clarify the effects of the soil fauna and flora and further elucidate the influence of bacterial population dynamics and activity on natural transformation in terrestrial environments.

ACKNOWLEDGMENTS

This work was supported by grants from the Norwegian Research Council (MU:101649/720) to K.M.N. and A.M.B. and from the EU-IC programme to J.D.v.E.

REFERENCES

- Baur, B., K. Hanselmann, W. Schlimme, and B. Jenni. 1996. Genetic transformation in freshwater: *Escherichia coli* is able to develop natural competence. *Appl. Environ. Microbiol.* **62**:3673–3678.
- Carlson, C. A., L. S. Pierson, J. J. Rosen, and J. L. Ingraham. 1983. *Pseudomonas stutzeri* and related species undergo natural transformation. *J. Bacteriol.* **153**:93–99.
- Chamier, B., M. G. Lorenz, and W. Wackernagel. 1993. Natural transformation of *Acinetobacter calcoaceticus* by plasmid DNA adsorbed on sand and groundwater aquifer material. *Appl. Environ. Microbiol.* **59**:1662–1667.
- Filip, Z. 1973. Clay minerals as a factor influencing the biochemical activity of soil microorganisms. *Folia Microbiol.* **18**:56–74.
- Fixter, L. M., and M. K. Shervani. 1991. Energy reserves in *Acinetobacter*, p. 273–294. In K. J. Townner, E. Bergogne-Berezin, and C. A. Fewson (ed.), *The biology of Acinetobacter*. Plenum Press, New York, N.Y.
- Frischer, M. C., J. M. Thurmond, and J. A. Paul. 1993. Factors affecting competence in a high frequency of transformation marine *Vibrio*. *J. Gen. Microbiol.* **139**:753–761.
- Fry, J. C., and M. J. Day (ed.). 1990. Bacterial genetics in natural environments. Chapman and Hall, London, England.
- Gallori, E., M. Bazzicalupo, L. Dal Canto, P. Nannipieri, C. Vettori, and G. Stotzky. 1994. Transformation of *Bacillus subtilis* by DNA bound on clay in non-sterile soil. *FEMS Microbiol. Ecol.* **15**:119–126.
- Graham, J. B., and C. A. Istock. 1978. Genetic exchange in *Bacillus subtilis* in soil. *Mol. Gen. Genet.* **166**:287–290.
- Hofmann, D. A., and D. A. Brian. 1991. Sequencing PCR DNA amplified directly from a bacterial colony. *BioTechniques* **11**:30–31.
- Juni, E. 1972. Interspecies transformation of *Acinetobacter*: genetic evidence for a ubiquitous genus. *J. Bacteriol.* **112**:917–931.
- Khanna, M., and G. Stotzky. 1992. Transformation of *Bacillus subtilis* by DNA bound on montmorillonite and effect of DNase on the transforming ability of bound DNA. *Appl. Environ. Microbiol.* **58**:1930–1939.
- Lee, G. H., and G. Stotzky. 1990. Transformation is a mechanism of gene transfer in soil. *Korean J. Microbiol.* **28**:210–218.
- Lorenz, M. G., and W. Wackernagel. 1991. High frequency of natural genetic transformation of *Pseudomonas stutzeri* in soil extract supplemented with a carbon/energy and phosphorus source. *Appl. Environ. Microbiol.* **57**:1246–1251.
- Lorenz, M. G., and W. Wackernagel. 1994. Bacterial gene transfer by natural genetic transformation in the environment. *Microbiol. Rev.* **58**:563–602.
- Lorenz, M. G., K. Reipschlag, and W. Wackernagel. 1992. Plasmid transformation of naturally competent *Acinetobacter calcoaceticus* in non-sterile soil extract and ground water. *Arch. Microbiol.* **157**:355–360.
- Nielsen, K. M., M. D. M. van Weerelt, T. N. Berg, A. M. Bones, A. N. Hagler, and J. D. van Elsas. 1997. Natural transformation and availability of transforming DNA to *Acinetobacter calcoaceticus* in soil microcosms. *Appl. Environ. Microbiol.* **63**:1945–1952.
- Ogram, A. V., M. L. Mathot, J. B. Harsch, J. Boyle, and C. A. Pettigrew, Jr. 1994. Effects of DNA polymer length on its adsorption to soils. *Appl. Environ. Microbiol.* **60**:393–396.
- Opperman, M. H., M. Wood, and P. J. Harris. 1989. Changes in microbial populations following the application of cattle slurry to soil at two temperatures. *Soil Biol. Biochem.* **21**:263–268.
- Page, W. J. 1985. Genetic transformation of molybdenum-starved *Azotobacter vinelandii*: increased transformation frequency and recipient range. *Can. J. Microbiol.* **28**:389–397.
- Page, W. J., and G. A. Grant. 1987. Effect of mineral iron on the development of transformation competence in *Azotobacter vinelandii*. *FEMS Microbiol. Lett.* **41**:257–261.
- Paget, E., and P. Simonet. 1994. On the track of natural transformation in soil. *FEMS Microbiol. Ecol.* **15**:109–118.
- Paget, E., L. J. Monrozier, and P. Simonet. 1992. Adsorption of DNA on clay minerals: protection against DNase I and influence on gene transfer. *FEMS Microbiol. Lett.* **97**:31–40.
- Palmen, R., B. Vosman, P. Buijman, C. K. Breek, and K. J. Hellingwerf. 1993. Physiological characterisation of natural transformation in *Acinetobacter calcoaceticus*. *J. Gen. Microbiol.* **139**:295–305.
- Palmen, R., B. Vosman, and K. J. Hellingwerf. 1994. Physiological regulation of competence induction for natural transformation in *Acinetobacter calcoaceticus*. *Arch. Microbiol.* **162**:344–351.
- Paul, J. H., M. E. Frischer, and J. M. Thurmond. 1991. Gene transfer in marine water column and sediment microcosms by natural plasmid transformation. *Appl. Environ. Microbiol.* **57**:1509–1515.
- Reutch, R. N., and H. L. Sadoff. 1988. Putative structure and functions of a poly- β -hydroxybutyrate-calcium polyphosphate channel in bacterial plasma membranes. *Proc. Natl. Acad. Sci. USA* **85**:4176–4180.
- Rochelle, P. A., M. J. Day, and J. C. Fry. 1988. Occurrence, transfer and mobilization in epilithic strains of *Acinetobacter* of mercury-resistance plasmids capable of transformation. *J. Gen. Microbiol.* **134**:2933–2941.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed., appendix A.3. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Smit, E., and J. D. van Elsas. 1992. Conjugal gene transfer in the soil environment: new approaches and developments, p. 79–94. In M. Gauthier (ed.), *Gene transfers and environment*. Springer-Verlag, Berlin, Germany.
- Smith, J. M., C. G. Dowson, and B. G. Spratt. 1991. Localized sex in bacteria. *Nature* **349**:29–31.
- Stewart, G. J., and C. D. Sinigalliano. 1990. Detection of horizontal gene transfer by natural transformation in native and introduced species of bacteria in marine and synthetic sediments. *Appl. Environ. Microbiol.* **56**:1818–1824.
- Stewart, G. J., C. D. Sinigalliano, and K. A. Garko. 1991. Binding of exogenous DNA to marine sediments and the effect of DNA/sediment binding on natural transformation of *Pseudomonas stutzeri* strain ZoBell in sediment columns. *FEMS Microbiol. Ecol.* **85**:1–8.
- Toerien, D. F., A. Gerber, L. H. Lötter, and T. E. Cloete. 1990. Enhanced biological phosphorus removal in activated sludge systems. *Adv. Microb. Ecol.* **11**:173–230.
- van Elsas, J. D., A. F. Dijkstra, J. M. Govaert, and J. A. van Veen. 1986. Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into two soils of different texture in field microplots. *FEMS Microbiol. Ecol.* **38**:151–160.
- van Elsas, J. D., J. T. Trevors, and M. E. Starodub. 1988. Bacterial conjugation between *Pseudomonads* in the rhizosphere of wheat. *FEMS Microbiol. Ecol.* **53**:299–306.
- Veal, D. A., H. W. Stokes, and G. Daggard. 1992. Genetic exchange in natural microbial communities. *Adv. Microb. Ecol.* **12**:383–430.
- Wellington, E. M. H., and J. D. van Elsas (ed.). 1992. Genetic interactions among microorganisms in the natural environment. Pergamon Press, Oxford, England.
- Williams, H. G., M. J. Day, J. C. Fry, and G. J. Stewart. 1996. Natural transformation in river epilithon. *Appl. Environ. Microbiol.* **62**:2994–2998.