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EFFECTS OF AIR-DRYING AND WETTING CYCLES ON THE TRANSFORMING ABILITY OF DNA BOUND ON CLAY MINERALS

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Summary—Chromosomal DNA from *Bacillus subtilis* and plasmid pHV14 bound on montmorillonite (M) and kaolinite (K) homoionic to Ca were subjected to repeated cycles of air-drying and wetting. After each cycle the ability of the bound DNA to transform competent cells was evaluated. Chromosomal DNA bound on clay retained its transforming efficiency after three to four air-drying and wetting cycles, whereas plasmid DNA lost the transforming ability after one to two cycles. This loss was neither due to the desorption of DNA from the complexes nor to the negative effects on DNA bound on clay of the acidic pH, that develops in the water film surrounding the clay surface during the air-drying. The clay—DNA complexes required 6 h of wetting to regain their transforming ability. Free DNA retained its transforming ability for longer than bound DNA and did not require 6 h of wetting to regain its transforming ability. These results indicate that the different behaviour of DNA—clay complexes after air-drying is related to the molecular form of the DNA and to a conformational change in the DNA molecule bound on clay. © 1997 Elsevier Science Ltd

INTRODUCTION

Transformation is a mechanism of gene transfer in soil (Graham and Istock, 1978; Gallori *et al.*, 1994; Lorenz and Wackernagel, 1994). Results obtained by several authors have demonstrated that adsorption and binding of DNA on clay and sand particles protects the DNA against degradation by nucleases without inhibiting its transforming ability (Lorenz and Wackernagel, 1987; Lorenz *et al.*, 1988; Romanowsk *et al.*, 1991; Khanna and Stotzky, 1992; Paget *et al.*, 1992; Chamier *et al.*, 1993). The transforming ability of chromosomal and plasmid DNA bound on Ca-montmorillonite persisted for 15 days in non-sterile soil under moist conditions (Gallori *et al.*, 1994), demonstrating that bound DNA is partially resistant to enzymatic attack by soil micro-organisms.

Bacterial DNA can accumulate in soil microaggregates, especially where most of the bacterial colonies are confined (Paget *et al.*, 1992). The results by Ogram *et al.* (1994) indicated that the adsorption of calf thymus DNA on soil particles increases with decreasing DNA fragment length. It was hypothesized that the smaller DNA fragments adsorbed within soil pores might be more protected against degradation by nucleases than larger DNA fragments unable to enter pores or be adsorbed on surface sites. It should be noted that calf thymus DNA is not representative of extracellular DNA in soil.

The effects of some environmental factors on the adsorption and binding of DNA on clays and the transforming ability of the clay–DNA complexes were investigated by Khanna and Stotzky (1992). Adsorption of chromosomal DNA was pH-dependent, with the greatest adsorption below the isoelectric point (pH 5.5). However, the transformation frequency of the clay–DNA complexes was highest at pH 7. The optimal temperature for transformation by the clay–DNA complexes was 37°C.

Few data are available on the effect of water content on the transfer of genetic information in soil. The water content of soil fluctuates throughout the year, and such fluctuations markedly affect microbial activity and the fate of extracellular molecules, such as proteins and DNA (Orchard and Cook, 1983; Stotzky, 1986; Taylor and Parkinson, 1990; Ciardi *et al.*, 1993; Tate, 1995). Bacteria are aquatic organisms, and the transfer of genetic information in soil is higher when the soil water tension is near or at the optimum value for their growth (Stotzky, 1989).

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The hydration of DNA was studied by Falk *et al.* (1963a); Falk *et al.* (1963b). Between 0 and 65% relative humidity, water was adsorbed by the PO_4^{2-} Na^+ portion of the DNA backbone; the P-O-C and C-O-C oxygens also become hydrated below 65% relative humidity. Above 65% relative humidity the C = O and N bases become hydrated. The hydration of all DNA sites was completed at 80% relative humidity and further hydration of DNA was accompanied by swelling (Falk *et al.*, 1963a). It was also suggested that DNA could exist in different secondary structural forms (A or B) depending on its relative humidity status (Falk *et al.*, 1963a; Falk *et al.*, 1963b; Blackburn and Gait, 1990).

The aim of our work was to study the effect of moisture changes on the transforming ability of chromosomal and plasmid DNA bound on kaolinite (K) and montmorillonite (M) homoionic to Ca. The two types of clay were chosen on the basis of their different abilities to retain water, and the DNA molecules utilized are representative of the extracellular forms which may occur in soil (Lorenz and Wackernagel, 1994; Ogram *et al.*, 1994). *Bacillus subtilis* was used because it is a common inhabitant of soil and its transformation by DNA, both free and bound on either sand or clay, has been reported (Graham and Istock, 1978; Lorenz *et al.*, 1988; Khanna and Stotzky, 1992; Gallori *et al.*, 1994; Lorenz and Wackernagel, 1994).

MATERIALS AND METHODS

Bacteria and media

B. subtilis strains PB 168 (*trp* C2) and PB 19 (prototroph) were grown on Tryptose Blood Agar Base (TBAB, Difco) or in minimal medium (MM) (Davis and Mingioli, 1950) containing the required amino acid (tryptophan) at a final concentration of 25 $\mu g\ ml^{-1}$. The strains were maintained on slants of nutrient agar (NA, 1.5%, w/v, Difco) supplemented with 10^{-5} M $MnCl_2.4H_2O$. The phenotypes of the strains were verified regularly.

Preparation of DNA

Chromosomal DNA from donor strain PB 19 was prepared as described by Khanna and Stotzky (1992) and its size ranged from 10 to 40 Kb. Plasmid pHV14 (Ehrlich, 1978), expressing chloramphenicol resistance (Cm^r) in *B. subtilis*, was prepared and purified with Qiagen tip 500 (Qiagen Inc., CA, U.S.A.). Its molecular size was 7.4 kb.

Preparation of competent cells

Recipient cells of *B. subtilis* PB168 were made competent as described by Khanna and Stotzky (1992).

Clay minerals

Montmorillonite (M) (Crook County, Wyoming, U.S.A.) is a 2:1 expanding clay mineral, characterized by high surface area (800–850 $m^2\ g^{-1}$) and adsorptive property (CEC = 76.4 cmol kg^{-1}). Kaolinite (K) (Zettliz, Germany) is a 1:1 non-expanding clay mineral, characterized by low surface area (30–50 $m^2\ g^{-1}$) and adsorptive property (CEC = 5–10 cmol kg^{-1}).

Preparation of homoionic clays

The < 2 μm fractions of M and K were separated by differential centrifugation and made homoionic to Ca, as described by Fusi *et al.* (1989).

Preparation of clay–DNA complexes

The clay–DNA complexes were prepared by the addition of chromosomal (15, 30 or 60 μg) or plasmid DNA (5, 10 or 30 μg) to 100 μl of M–Ca and K–Ca suspension [22 mg ml^{-1} deionized distilled water (dd H_2O at pH 6.0)] in 1 ml of dd H_2O. After 120 min of shaking (40 rev min^{-1}), maximum adsorption at equilibrium was obtained (Gallori *et al.*, 1994) and the mixture was centrifuged at 40,000 *g* for 20 min at 20°C. The DNA concentration in the supernatants was determined by measurement of the absorbance at A_{260}, as described by Gallori *et al.* (1994). This procedure was repeated until no more DNA was detected (A_{260}) in the supernatants.

Sterilization

With the exception of bacterial suspensions and clays all materials used in the experiments were sterilized by autoclaving.

Air-drying and wetting cycles

Centrifuge tubes (Ultra-Clear, Beckman, U.S.A. 13 × 51 mm) containing pellets of clay–DNA complexes were air-dried for 48 h at 28°C in a thermostatic cell with air recycling and then rewetted by adding 100 μl of dd H_2O. The tubes were then covered with parafilm® (American National Can™ Greenwich, CT) and allowed to stand at 28°C for 24 h before a new air-drying cycle. Air-drying and wetting cycles were repeated several times. The transforming ability of the DNA either bound on clay or present in the supernatants, after each air-drying and wetting cycle, was determined by the sacrifice of samples (see Transformation experiments). After air-drying and wetting we added 1 ml of dd H_2O to the pellets, then the samples were centrifuged at 40 000 **g** for 20 min at 20°C and DNA concentrations of the supernatants were determined by UV absorption, as described above (see Preparation of clay-DNA complexes). The complexes were then washed and centrifuged until no more DNA was detected (A_{260}) in the supernatants. The pellets so obtained were utilized in the trans-

formation experiments. Three replicates were carried out for each air-drying and wetting cycle.

Agarose gel electrophoresis

The supernatants, obtained after each air-drying and wetting cycle, of both plasmid and chromosomal DNA–clay complexes were concentrated by lyophilization, resuspended in 0.5 ml of dd H_2O and then electrophorized on 0.6% (w/v) agarose gel (Gallori *et al.*, 1994) in the presence of pure DNA (pHV14 and PB19) and standard molecular weight markers (High range, BioRad).

Bacterial transformation

The pellets of bound clay–DNA complexes were added to 1 ml of competent cells of *B. subtilis* PB168 (containing approx. $5–10 \times 10^8$ cells) in centrifuge tubes (polystyrene tubes Ultra Clear ™, Beckman, U.S.A. 13 × 51 mm) and rotated at 70 rev min^{-1} for 30 min at 37°C. Then the contents of the tubes were vortexed with saline, and serial diluitions were plated on appropiate selective media for the enumeration of transformants and viable cells. In experiments with chromosomal DNA, selection was on MM agar whereas with plasmid DNA, TBAB plus chloramphenicol (20 μg ml^{-1}) was used. Competent cells only treated with DNA (chromosomal and plasmid) were plated on the same media as controls. All plates were incubated for 24–36 h at 37°C. Transformants and total viable cells were then enumerated, and transformation frequencies were expressed as the numbers of Trp^+ (chromosomal DNA) or Cm^r (plasmid DNA) transformants per number of total viable cells (Gallori *et al.*, 1994). Transformation of desorbed DNA after each air-drying and wetting cycle was carried out by mixing 0.5 ml of the supernatant from DNA–clay complex with 1 ml of competent cells of *B.subtilis*. Then the procedure was similar to that reported for DNA bound on clay.

Microstructure studies

The microstructure studies of clay and clay–DNA complexes were carried out to obtain the Pore Size Distribution (PSD), in the range of 10–300 Å of radius, by nitrogen adsorption at 28°C with Carlo Erba Sorptomatic 1900 apparatus (D'Acqui *et al.*, 1996).

X-ray diffraction analysis

Oriented samples of the clay–DNA complexes were prepared by pipetting suspensions of the complexes onto glass slides followed by air-air-drying at room temperature. X-ray diffraction patterns were obtained with a PW1410/20 Philips diffractometer with iron-filtered CoKα radiation (Greaves and Wilson, 1969).

Statistics

Statistical analysis include standard errors of the means (SEM).

RESULTS AND DISCUSSION

The percentage of the DNA tightly bound on clay minerals was higher for plasmid than chromosomal DNA, 35 and 13.5% vs 23.1 and 9.9% for M and K, respectively (Table 1). These results agree with those found for soil particles interacting with linear DNA (Ogram *et al.*, 1994): the DNA adsorption increased with a decrease in the size of DNA molecules. It was suggested that the larger DNA molecules were excluded from small pores of soil aggregates, resulting in a lower adsorption than for smaller DNA molecules (Ogram *et al.*, 1994). The X-ray diffraction analysis of clay–chromosomal DNA or –plasmid DNA complexes showed that DNA adsorption did not cause interlayer expansion (data not shown).

Chromosomal DNA bound on M retained the ability to transform competent cells of *B. subtilis* through three cycles of air-drying and wetting, whereas free or K-bound chromosomal DNA retained the transforming ability through four cycles (Fig. 1; Fig. 2). More marked differences were observed in the case of plasmid DNA. In fact, free plasmid DNA maintained its transforming capacity after five air-drying and wetting cycles, while plasmid DNA bound on either M or K lost its ability to transform competent cells after only two and one cycles, respectively (Fig. 1; Fig. 3). These results may indicate that the different behaviour of DNA–clay complexes is related to the molecular form of

Table 1. Percentage of chromosomal (PB19) and plasmid (pHV 14) DNA bound on Ca saturated M and K clays after various cycles of air-drying and wetting

Complex (n = 3)	DNA added to clay (μg)	DNA tightly bound to clay (%)[a]	DNA tightly bound to clay after each cycle[a] (%)				
			1	2	3	4	5
PB19-M	60	23.1 ± 1	15.5 ± 1.3	10.0 ± 1.5	6.9 ± 1.8	4.5 ± 2	4.0 ± 2.2
PB19-K	60	9.9 ± 0.8	8.4 ± 0.9	7.8 ± 1	4.9 ± 1.2	4.9 ± 1.2	4.9 ± 1.2
pHV14-M	30	35.0 ± 1.2	31.8 ± 1	31.8 ± 1	31.8 ± 1	31.8 ± 1	31.8 ± 1
pHV14-K	30	13.5 ± 0.8	12.5 ± 1	11.7 ± 1	10.8 ± 1	10.0 ± 1	10.0 ± 1

n—number of replicates
[a]With respect to the DNA amount added to the clay.

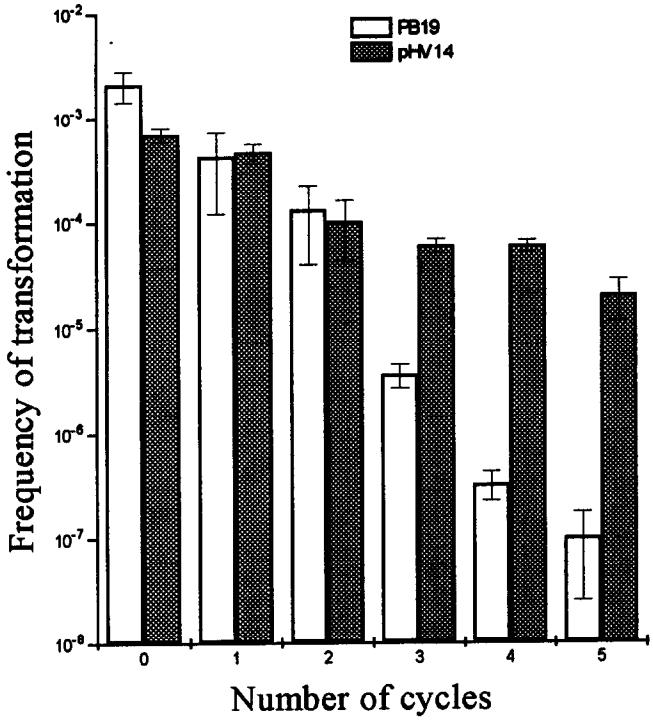

Fig. 1. Transformation frequency of *B. subtilis* PB168 by chromosomal DNA from *B. subtilis* PB19 and plasmid DNA (pHV14) after various air-drying and wetting cycles.

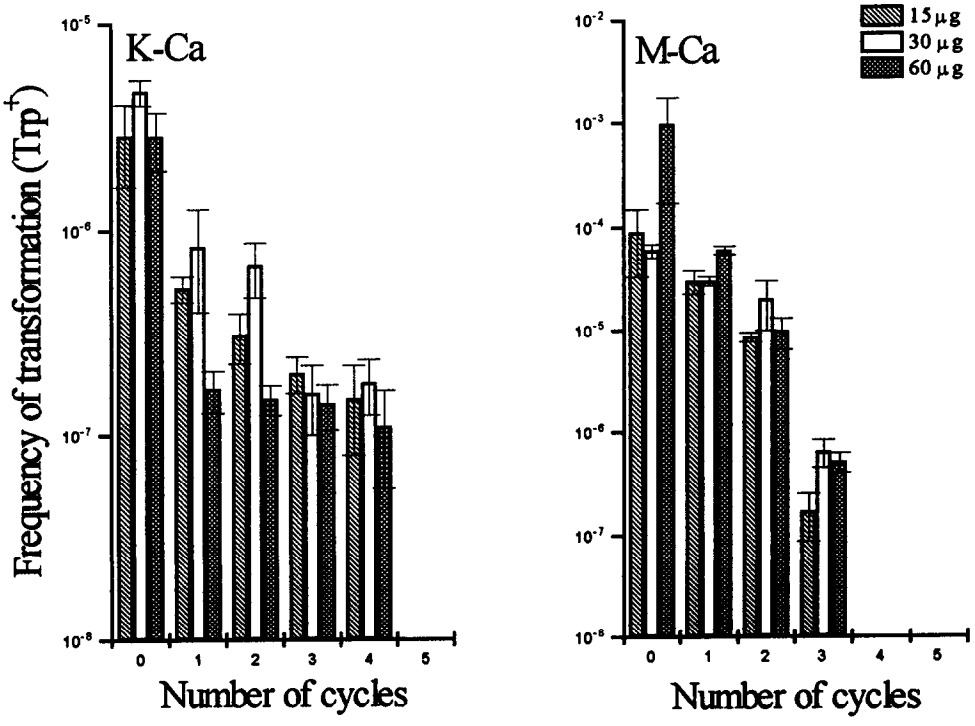

Fig. 2. Transformation frequency of *B. subtilis* PB168 by chromosomal DNA from *B. subtilis* PB19, bound on either kaolinite (K) or montmorillonite (M) homoionic to Ca, after various air-drying and wetting cycles. The amounts of DNA reported in the legend are those initially added to M or K; those effectively bound can be calculated from Table 1.

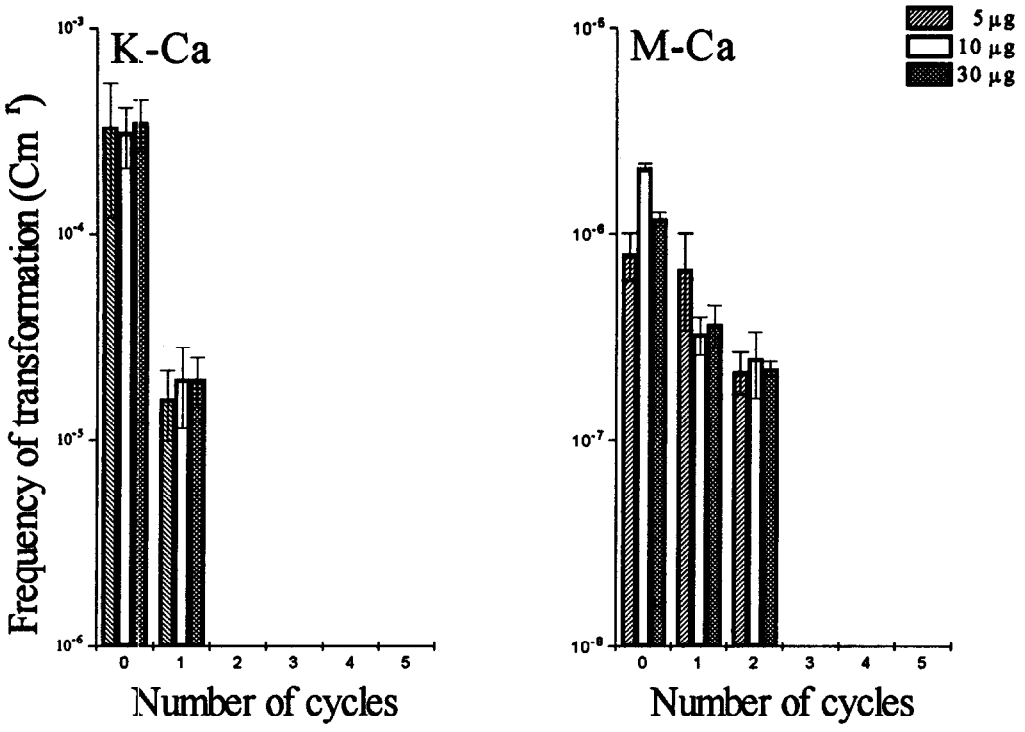

Fig. 3. Transformation frequency of *B. subtilis* PB168 by plasmid DNA (pHV14), bound on either kaolinite (K) or montmorillonite (M) homoionic to Ca, after various air-drying and wetting cycles. The amounts of DNA reported in the legend are those initially added to M or K; those effectively bound can be calculated from Table 1.

the DNA and not to the kind of clay utilized. Probably the longer chromosomal DNA molecule could have more chances to maintain its transformation activity in contrast to the smaller multimeric plasmid DNA molecule when molecular damage, due to dry conditions occurs. Furthermore, we have to consider that plasmid DNA is present with a small number of multimeric transformant forms (Canosi *et al.*, 1978; Mottes *et al.*, 1979) and clays preferentially adsorb the monomeric form which is not efficient in transforming competent bacterial cells (Gallori *et al.*, 1994).

Frequencies of transformation (FT) obtained by chromosomal DNA bound on M were 300-fold higher than those obtained by complexes with K (Fig. 2). The opposite behaviour was shown by plasmid DNA bound on M with 100-fold lower FT than those obtained by complexes with K (Fig. 3). The higher amount of chromosomal DNA bound on M than K may be responsible for the FT differences observed between the chromosomal DNA–M and –K complexes. The FT behaviour of plasmid DNA–M and –K complexes might be influenced by the type of clay. In fact M presents a higher microporosity, due to the arrangement of clay crystallites, than K. The DNA molecules could penetrate into these micropores and become less accessible to the competent bacterial cells.

The FT obtained by plasmid and chromosomal DNA bound on clay did not show any significant difference when the amount of DNA utilized to prepare the complexes was increased. This indicates that even the lowest amount of DNA employed achieved the DNA saturation level for the transformation of the competent cells of *B. subtilis* PB168. The DNA concentration used in our experiments ranged from 1 to 30 $\mu g\,mg^{-1}$ of clay; such concentrations are much higher than those (0.01–0.2 $\mu g\,mg^{-1}$ of soil) present in soil (Paget *et al.*, 1992).

The FT frequencies obtained by plasmid and chromosomal DNA bound on non-sterile clays were not significantly different in respect to the FT of DNA–sterile clay complexes. These results confirmed those reported by Gallori *et al.* (1994) for the FT of clay–DNA complexes in the presence of either sterile or non-sterile soil.

The acidic pH, which develops on the water film surrounding the clay surface after air-drying, may have had a negative influence on the transforming ability of DNA bound on clay. In order to verify this hypothesis, solutions of both free plasmid and chromosomal DNA were incubated at pH 4.5 (obtained by adding 0.5 M HCl). After 3 days, this DNA was utilized to transform competent cells of *B.subtilis* PB168. The FT obtained (1.3×10^{-3} and 1.8×10^{-4} for chromosomal and plasmid DNA, re-

spectively) indicated that both DNA forms maintained their transforming ability. The pH value of 4.5, 2 units lower than the surface pH measured under wet conditions, was selected in view of the acidifying effect of cations such as Ca^{2+} saturating clay under air-drying conditions (Harter and Ahlrichs, 1967; Mortland and Raman, 1968).

To investigate if air-drying and wetting cycles could cause the desorption of DNA bound on clay, we monitored the amount of chromosomal or plasmid DNA released after each cycle. The results indicated that free DNA may be relaesed from M and K complexes after one or two air-drying and wetting cycles (Table 1). The desorbed DNA obtained from the exhaustive washing of clay–DNA complexes after each air-drying and wetting cycle was not able to transform competent *B. subtilis* cells. To evaluate if both plasmid and chromosomal DNA released from complexes after the air-drying and wetting cycle could be due to molecular breaking, supernatants collected after each cycle were concentrated by lyophilization then resuspended in dd H_2O (0.5 ml) and electrophorized on agarose gel; runs of pure plasmid and chromosomal DNA and standard molecular weight marker were also carried out. The presence of DNA was observed after the first cycle and the DNA size remained unchanged according to the electrophoretic patterns (data not shown).

Changes in the configuration of the DNA structure which occurred during air-drying could be responsible for the loss of the transforming ability of DNA bound on clay. Indeed after air-drying, both chromosomal and plasmid DNA bound on clay needed a period of 6 h after wetting to recover their transforming ability, while free DNA was immediately able to transform competent cells. Under the severe air-drying conditions, characterized by a clay moisture variation (w/w) from 30 to 0.013% for M and from 31 to 0.06% for K, the DNA molecule may change its configuration (Blackburn and Gait, 1990) so that it reaches a more close-packed conformation and may become inaccessible to the competent cell. The 6 h wetting time may be necessary for the DNA molecule bound on the clay surface to recover its original configuration and to be accessible again to the competent cell. The difference in the rehydration period necessary for free and bound DNA to recover the active configuration could be attributed to water competition between the clay and the DNA molecules bound on clay minerals. When the conformation of the DNA molecule becomes more close-packed, its negative electric density charge may increase due to the decrease in molecular volume, with a greater electrostatic interaction with the positively-charged clay edges. The DNA polyanions may interact with clay by other mechanisms. The counterion Ca^{2+} may act as a bridge between the negatively-charged clay surface and the DNA molecule. Phosphate groups of the DNA may be chemiadsorbed, e.g. through replacement of OH groups in the clay edges as occurs for some inorganic and organic anions (McBride, 1989). The occurrence of H bonds between DNA and the clay surface could be another type of interaction. Whatever mechanism occurs, polymer adsorption on clay results in a structure of alternating adsorbed and non-adsorbed segments in the DNA fragment (Romanowsky *et al.*, 1991). In agreement with this hypothesis, TEM observations by Paget and Simonet (1994) showed that only some regions of DNA were adsorbed on clay domains.

In conclusion, air-drying and wetting cycles negatively affect the transforming ability of DNA when the nucleic acid is bound on clay. This effect is more marked for plasmid DNA; probably its smaller size and different structure makes it more sensitive to the negative effects of air-drying and wetting cycles. The results emphasize that water content is fundamental in the soil environment, not only for bacteria living on soil (Stotzky, 1989), but also for the mechanisms of horizontal genetic exchange.

Further research is needed, both to verify the hypothesis that conformational tightening of the bound plasmid DNA upon air-drying produces a more close-packed molecule and to gain better insights into the type of interaction between the DNA molecule and clay.

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