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ORIGINAL PAPER

Adsorption of pure and dirty bacterial DNA on clay minerals and their transformation frequency

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Abstract Several studies have investigated the adsorption of *pure* DNA on soil particles and its transformation ability. However, the presence of not purified (*dirty*) rather than pure DNA is more common in the extracellular soil environment. For this reason, we have investigated the adsorption and binding of dirty DNA on montmorillonite (M) and kaolinite (K) and their transforming ability in comparison to pure DNA. After lysis of *Bacillus subtilis* cells, induced by KCl, dirty DNA was characterized by the presence of cellular wall debris (*cwd*) and other constitutional organic components (*coc*). The dirty DNA was then divided into two fractions, one with cellular wall debris (DNA +cwd) and the other without cellular wall debris (DNA −cwd). *B. subtilis* BD 1512 (Cm^r) and BD 170 (Cm^s) were selected as donor and recipient bacteria, respectively, for adsorption and transformation studies. Both cwd and coc seem to facilitate the adsorption of DNA to clay minerals, whereas only cwd promote the DNA binding on clays, protecting also the DNA fragments below 400 bp against nucleases. Both dirty DNA fractions adsorbed and bound on clay minerals were able to transform competent cells.

Keywords DNA–clay complexes · Dirty and pure DNA · Natural transformation

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Introduction

Adsorption and binding of *pure* DNA to soil colloids (clay minerals and humic substances) protect the DNA molecule against enzymatic degradation without inhibiting its transforming ability (Crecchio and Stotzky 1998; Crecchio et al. 2005; Demanèche et al. 2001; Khanna and Stotzky 1992). Several environmental factors (pH, salt concentration, cation valency, moisture, temperature, sterile and nonsterile conditions) can affect both adsorption and binding of pure DNA to soil colloids and the transforming activity of its adsorbed and bound fractions (Gallori et al. 1994; Khanna and Stotzky 1992; Pietramellara et al. 2001). The effect of the pure DNA molecular characteristics on its binding to soil colloids has been also studied (Muller et al. 1993; Pietramellara et al. 1997, 2001). However, DNA released into the soil environment, both after cell death and by active extrusion by growing cells, rarely is present as a pure molecule. Generally, upon death and lysis of the bacterial cells, the DNA is released in the extracellular environment together with proteins, glucides, lipids, which could be considered as constitutional organic components (*coc*), and with RNA and cellular wall debris (*cwd*) (Fang and Hoh 1998; Tamayo et al. 1999). Setlow and Setlow (1995) have pointed out an increased resistance against enzymatic degradation of both proteins and DNA molecules when they are associated together. Lipids seem also to protect the DNA from enzyme degradation (Tamayo et al. 1999). Some polysaccharides in the cell are strongly associated with DNA after cell death and lysis, as evidenced by DNA purification protocols (Fang and Hoh 1998).

The aim of this study was to compare the behavior of pure vs dirty DNA about their adsorption and binding on clay minerals and their resistance to enzyme degradation. Another aim was to study the frequency of transformation

(F_t) of pure and dirty DNA not adsorbed, adsorbed, and bound on clay minerals. The dirty DNA was divided in dirty DNA with cellular wall debris (DNA +cwd) and without cellular wall debris (DNA −cwd), characterized by the presence and absence of cwd, respectively. *Bacillus subtilis* was chosen as the DNA source because it is ubiquitous and naturally competent in soil (Dubnau and Davidoff-Abelson 1971).

Materials and methods

Clay minerals

The characteristics of montmorillonite (M) (Crook County, Wyoming, USA) and kaolinte (K) (Zettlitz, Germany) are reported in Table 1. The <2 μm fraction of the two clays were made homoionic to Ca^{2+} as described by Fusi et al. (1989).

Bacteria and culture media

The donor strain, *B. subtilis* BD 1512 (trp^-, thr^-, Cm^r) was grown on selective PY medium (antibiotic medium *n*° 3, Oxoid) containing 10 μg ml^{-1} chloramphenicol (Cm). The phenotypes of the strains were verified regularly. Recipient cells of *B. subtilis* BD 170 (his^-, leu^-, met^-, Cm^s) were grown on nonselective PY medium, made competent and stored in liquid nitrogen as described by Dubnau and Davidoff-Abelson (1971).

Preparation of DNA

Pure chromosomal DNA was extracted and purified by using the FastDNA®Kit BIO 101 (BIO 101, Carlsbad, USA) and the Fast-Prep®Instrument (Ceccherini et al. 2007). Two types of dirty genomic DNA were used, one with cwd (DNA +cwd), directly released after cell lysis of *B. subtilis* BD 1512 induced by 100 mM KCl (Svarachorn et al. 1989), and one without cwd (DNA −cwd), obtained from the dirty DNA +cwd suspension after cwd precipitation and separation by centrifugation (20,800×*g* for 5 min at 20°C). Both pure and dirty DNA fractions were stored in deionized distilled water (dd H_2O) at −20°C. Aliquots of all the samples were also utilized without clays as controls.

Index of DNA purity

Absorbance of DNA solutions by UV spectrophotometer at 230, 260, 280, and 320 nm was carried out to determine the purity of the DNA molecule as affected by the presence of coc (proteins, carbohydrates, lipids) and inorganic contaminants (clay minerals) (Castro et al. 1995; Glasel 1995; Kim and LaBella 1987).

The DNA index of purity (IP) was determined by the following two equations:

$$\mathrm{IP} = (A_{260} - A_{320})/(A_{280} - A_{320}) \quad (1)$$

$$\mathrm{IP} = (A_{260} - A_{320})/(A_{230} - A_{320}) \quad (2)$$

The first equation takes into consideration contamination by proteins, whereas the second considers the presence of carbohydrates and lipids without distinguishing their contribution. Both equations take into consideration the interferences by clay particles in suspension by subtracting the clay adsorbance at 320 nm from the other adsorbance values.

The RNA presence was assessed by cross digestion with RNase A (Qiagen) and DNase I (Roche) followed by electrophoresis on 1× TAE agarose gel (0.8% w/v), containing 1:10,000 ethidium bromide (10 mg ml^{-1}), at 100 V for 80 min.

Preparation of clay-DNA complexes

Figure 1 shows the procedure used to prepare the clay–DNA complexes and the various DNA fractions (A-DNA not adsorbed, B-DNA adsorbed, and C-DNA bound on clay minerals). The clay-DNA complexes for adsorption isotherms were prepared by adding 0.25, 0.5, 1.0, 2.5, and 5.0 μg of pure or dirty chromosomal DNA of *B. subtilis* BD 1512 as solutions, to either 200 μl of Ca^{2+}–M or Ca^{2+}–K suspension (0.25 mg, 50 μl^{-1}) in 2 ml centrifuge tubes and adding dd H_2O to a final volume of 1 ml. The tubes were rotated for 90 min at 20°C on a wheel at 40 rpm to have the maximum adsorption of DNA on clay minerals at equilibrium (Khanna and Stotzky 1992).

The clay–DNA complexes for transformation assays were prepared as reported above utilizing 25 μg of pure or

Table 1 Properties of montmorillonite and kaolinite

Clay characteristics	Montmorillonite (Wyoming)	Kaolinite (Zettliz)
Si:Al	2:1 Expanding clay	1:1 Nonexpanding clay
External surface area (ESA)	800–850 $m^2\ g^{-1}$ clay	30–50 $m^2\ g^{-1}$ clay
Cation exchange capacity (CEC)	76.4 cmol kg^{-1}	5–10 cmol kg^{-1}
Surface charge capacity (SCC)	1.3×10^{-3} cmol m^{-2}	3.2×10^{-3} cmol m^{-2}
pH (2 mg clay ml^{-1} dd H_2O)	6.8	5.8

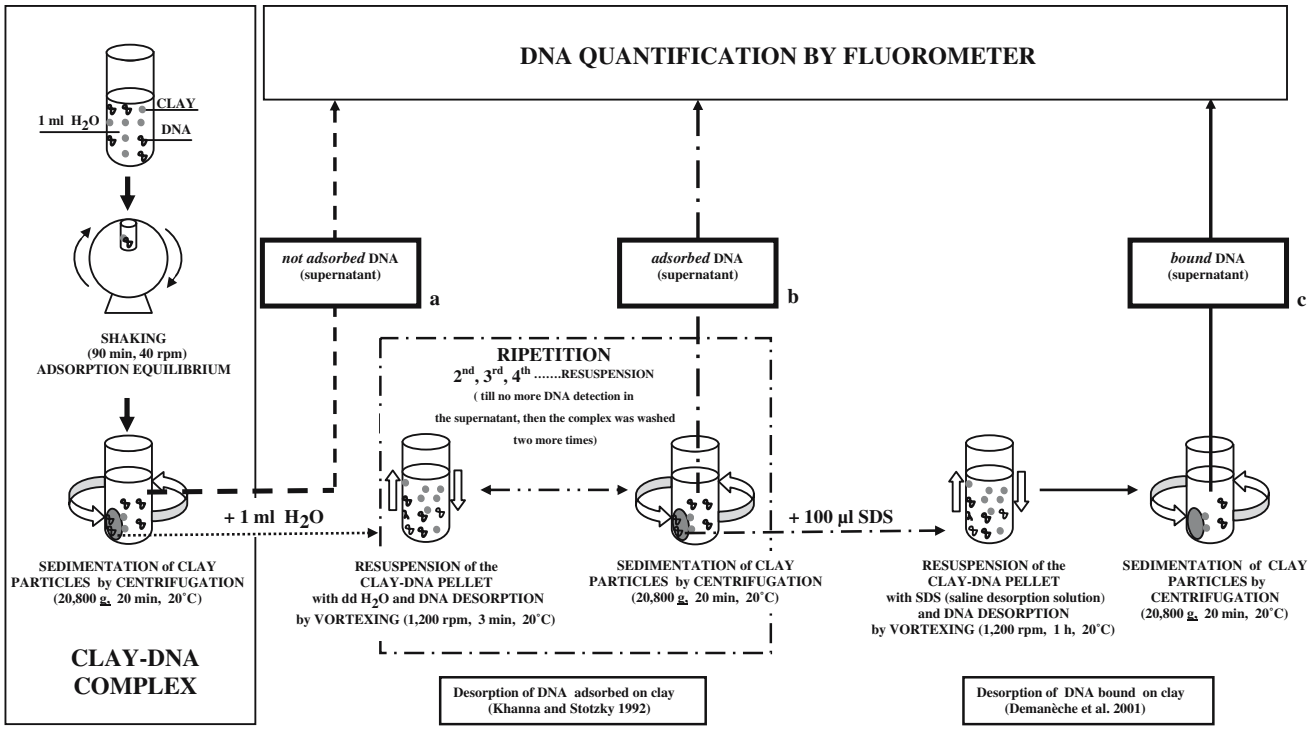


Fig. 1 The procedure used to prepare clay–DNA complexes and to determine the amounts of DNA not adsorbed, adsorbed, and bound on clay

dirty chromosomal DNA of *B. subtilis* BD 1512 and 400 µl of Ca^{2+}–M or Ca^{2+}–K suspension (0.25 mg 50 μl^{-1}).

Quantification of DNA

The fraction of *DNA not adsorbed on clay* was that in the supernatant of the complex at the adsorption equilibrium. The clay–DNA complex was centrifuged (20,800×*g* for 20 min at 20°C) to sediment the clay particles in suspension (Khanna and Stotzky 1992), and then the supernatant was separated from the clay–DNA pellet and analyzed by fluorometer (Hoefer™ DyNA Quant™ 200, Amersham Pharmacia Biotech), using bisbenzimide-dye (Hoechst H 33258), to determine the amount of not adsorbed DNA (Fig. 1).

The pellet of the clay–DNA complex was then resuspended in 1 ml of dd H_2O to desorb the fraction of *DNA adsorbed on clay* by vortexing (1,200 rpm at 20°C for 3 min). The complex suspension was centrifuged (20,800×*g* at 20°C for 20 min) to sediment the clay particles. The supernatant containing the desorbed DNA was separated from the pellet and analyzed by fluorometer. The desorption treatment was repeated until no DNA was detected in the supernatants, and the pellet was then washed two more times (Fig. 1). The total amount of DNA adsorbed on clay was then obtained from the addition of all the DNA aliquots detected after each washing.

The remaining DNA, *DNA bound on clay*, was desorbed by adding 50 µl of a saline desorption solution (sds) (17 mM lactic acid, 3 mM KH_2PO_4, 27 mM Na_2HPO_4, 0.23 mM $MgSO_4$, 11 mM NH_4Cl, 19 µM $CaCl_2$, 0.5 µM $FeSO_4$, 86 mM sodium pyrophosphate, and 57 mM EDTA) to the clay–DNA pellet previously resuspended in 50 µl sds, followed by vortexing the suspension (1,200 rpm at 20°C for 1 h) (Demanèche et al. 2001). The complex was centrifuged (20,800×*g* at 20°C for 20 min) to sediment the clay particles in suspension in the supernatant. The supernatant was then separated from the pellet and analyzed by fluorometer.

DNA transformation frequency

Competent cells of the *B. subtilis* recipient strain BD 170 (Cm^s) were transformed with 25 µg of *B. subtilis* BD1512 (Cm^r) pure and dirty±cwd DNA not adsorbed, adsorbed, or bound on clay minerals. The aliquots of the competent cells used for all transformation assays contained 10^7 cells/ml and were taken from the same stock-culture solution.

The overall amount of DNA was 25 µg to be sure that the fraction bound, adsorbed, and not adsorbed on clays was not the limiting factor in the transformation assays (Khanna and Stotzky 1992). The F_t of all DNA fractions was expressed per microgram (µg) of DNA so as to compare the F_t of all fractions. As controls, we also carried out transformations with 25 µg of pure and both dirty DNAs (±cwd) that have not interacted with clay minerals.

The different fractions of DNA were added to 1 ml of competent cells in 2-ml centrifuge tubes, and the suspen-

sions were slightly shaken for 60 min at 37°C. Then, serial dilutions in physiological solution (NaCl, 8.5 g l^{-1}) were plated on selective PY agar plus chloramphenicol (20 μg ml^{-1}). All plates were incubated for 24–36 h at 37°C. Transformants and total viable cells were then enumerated, and F_t were expressed as the number of Cm^r transformants per number of total viable cells (Gallori et al. 1994).

DNA enzymatic digestion

Both pure and dirty (±cwd) DNAs (200 ng) were digested with 10 U of endonuclease Alu I as recommended by the manufacturer (Roche) in a volume of 30 μl. Then, the integrity of the DNA molecule was monitored by gel electrophoresis loading the whole volume of the digestion mixture on 1% agarose gel in 1× TAE stained with ethidium bromide. Electrophoresis was carried out at 100 V for 80 min.

Sterilization

To prevent donor and recipient bacteria contamination and DNA degradation by contaminants, all materials used in the experiments, except bacterial suspensions and DNA solutions, were previously sterilized by autoclaving (1.1 atm, 20 min).

Statistical analysis

Standard errors of the means (SEM) were calculated.

Results and discussion

Index of DNA purity

According to Eq. 1, which takes into account the presence of proteins, such as constitutional coating proteins (ccp), the dirty DNA ±cwd and the pure DNA were characterized by index of purity (IP) values of 1.9 and 2.0, respectively. According to Glasel (1995), the range of ccp in DNA preparations with IP values ranging from 1.7 to 2.0 can vary from 70 to 0%. However, these percentages are only indicative because IP values also depend on the amino acid composition of proteins, molecular size, and base composition of the DNA molecule, and the ionic strength and pH value of the solution (Glasel 1995; Harlow and Lane 1988; Muller et al. 1993; Wilfinger et al. 1997).

The presence of carbohydrates and lipids in the DNA fraction was evaluated by Eq. 2, and the content of these constituents was inversely proportional to the DNA IP values. The calculated IP values were 1.8, 1.6, and 1.0 for pure DNA, dirty DNA −cwd and +cwd fractions, respectively. It is important to underline that this approach does not permit to distinguish the contribution of carbohydrates from that of lipids.

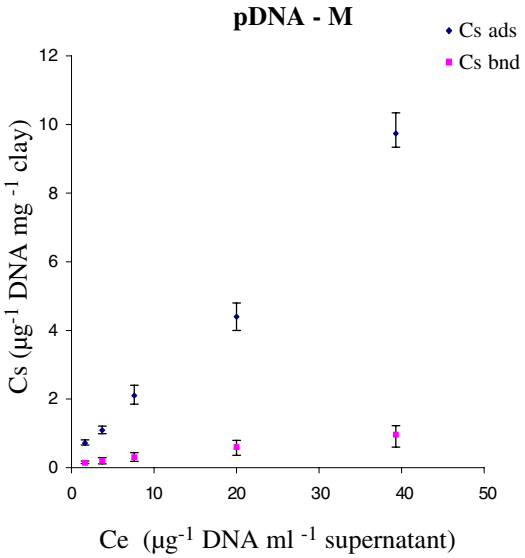
Both IP determinations, even if they did not accurately determine the quantitative presence of proteins, lipids, and carbohydrates in the DNA fraction, were indicative of the presence of these constituents, and thus, suitable for the aim of this study, which was only to discriminate between the behavior of pure and dirty DNA molecules interacting with clay minerals.

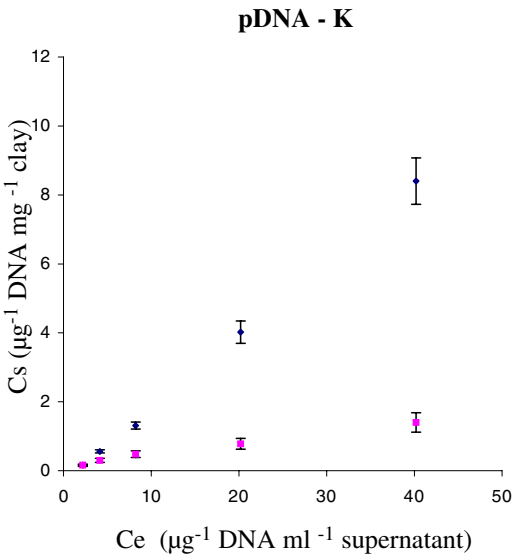
DNA adsorption and binding on clay minerals

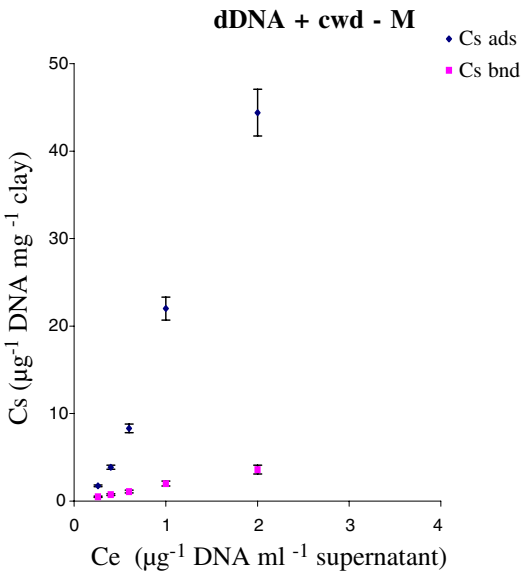
The adsorption isotherms (Fig. 2) clearly pointed out marked differences between the adsorption of pure and dirty (±cwd) DNA on both clay minerals. Both dirty DNAs were adsorbed on clay minerals in higher amounts than pure DNA. This trend was not observed for the DNA bound onto clay minerals because the amount of DNA bound to clays was higher for pure than dirty DNA −cwd. The differences between the amount of DNA adsorbed and that bound to clays was greater for both dirty DNAs than for pure DNA. Finally, the amount of pure and both dirty (±cwd) DNAs adsorbed on clay minerals was higher for M than K, whereas the opposite behavior was detected for the amounts of DNA bound on clays.

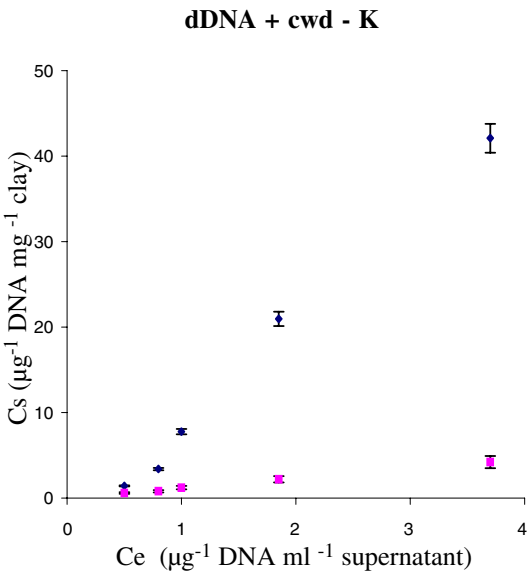
The DNA constitutional organic components (coc) could positively affect the adsorption of the dirty DNA molecule on clay minerals (Fig. 2) by influencing DNA size, conformation, and flexibility (Griffith et al. 1994; Gurlie and Zakrzewska 2000; Liu et al. 1996; Wiethoff et al. 2002). The presence of coc might also have promoted the condensation of the DNA molecule (Bloomfield 1998; Griffith et al. 1994; Gurlie and Zakrzewska 2000; Tavares and Sellstedt 2001), that is a more compact structure with a lower number of available binding sites but with a higher charge density, than the relaxed structure, and this might have increased the amount of dirty DNA adsorbed on clays (Ogram et al. 1994; Pietramellara et al. 2001). It is known that bacterial cell lysis can release DNA in solution in a condensed form, coiled, or globular for chromosomal DNA and circular or circular covalently closed for plasmid DNA (Bloomfield 1998; Gallori et al. 1994; Khanna et al. 1998; Koltover et al. 2000; Romanowski et al. 1992). It is also possible that during the DNA incubation with clays until the adsorption equilibrium, the presence of coc might have promoted the aggregation and condensation of several

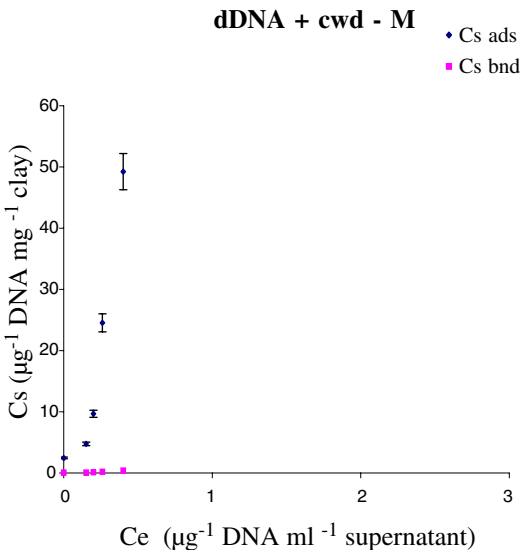
Fig. 2 Amount of pure (*p*) and dirty (*d*) DNA with or without cellular wall debris (±cwd) from *B. subtilis* BD1512 adsorbed (*ads*) or bound (*bnd*) on montmorillonite (*M*) and kaolinite (*K*). Cs is the amount of DNA adsorbed or bound on clay at equilibrium (μg^{-1} DNA mg^{-1} clay); *Ce* is the amount of DNA not adsorbed on clay present in the supernatant solution at equilibrium (μg^{-1} DNA ml^{-1} supernatant)

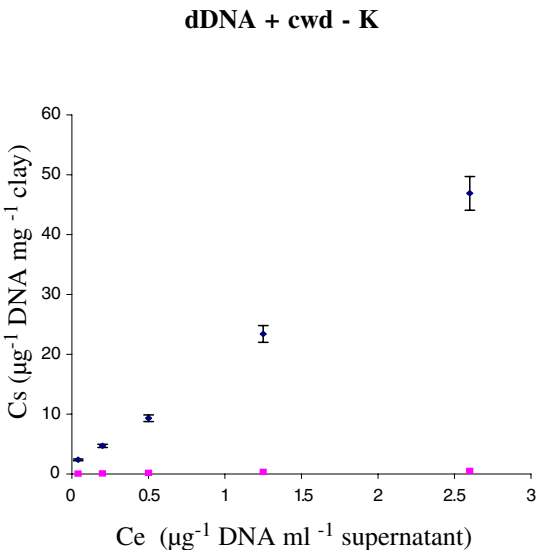

pDNA - M
Cs ads
Cs bnd
Cs (µg-1 DNA mg -1 clay)
Ce (µg-1 DNA ml -1 supernatant)


pDNA - K
Cs (µg-1 DNA mg -1 clay)
Ce (µg-1 DNA ml -1 supernatant)


dDNA + cwd - M
Cs ads
Cs bnd
Cs (µg-1 DNA mg -1 clay)
Ce (µg-1 DNA ml -1 supernatant)


dDNA + cwd - K
Cs (µg-1 DNA mg -1 clay)
Ce (µg-1 DNA ml -1 supernatant)


dDNA + cwd - M
Cs ads
Cs bnd
Cs (µg-1 DNA mg -1 clay)
Ce (µg-1 DNA ml -1 supernatant)


dDNA + cwd - K
Cs (µg-1 DNA mg -1 clay)
Ce (µg-1 DNA ml -1 supernatant)

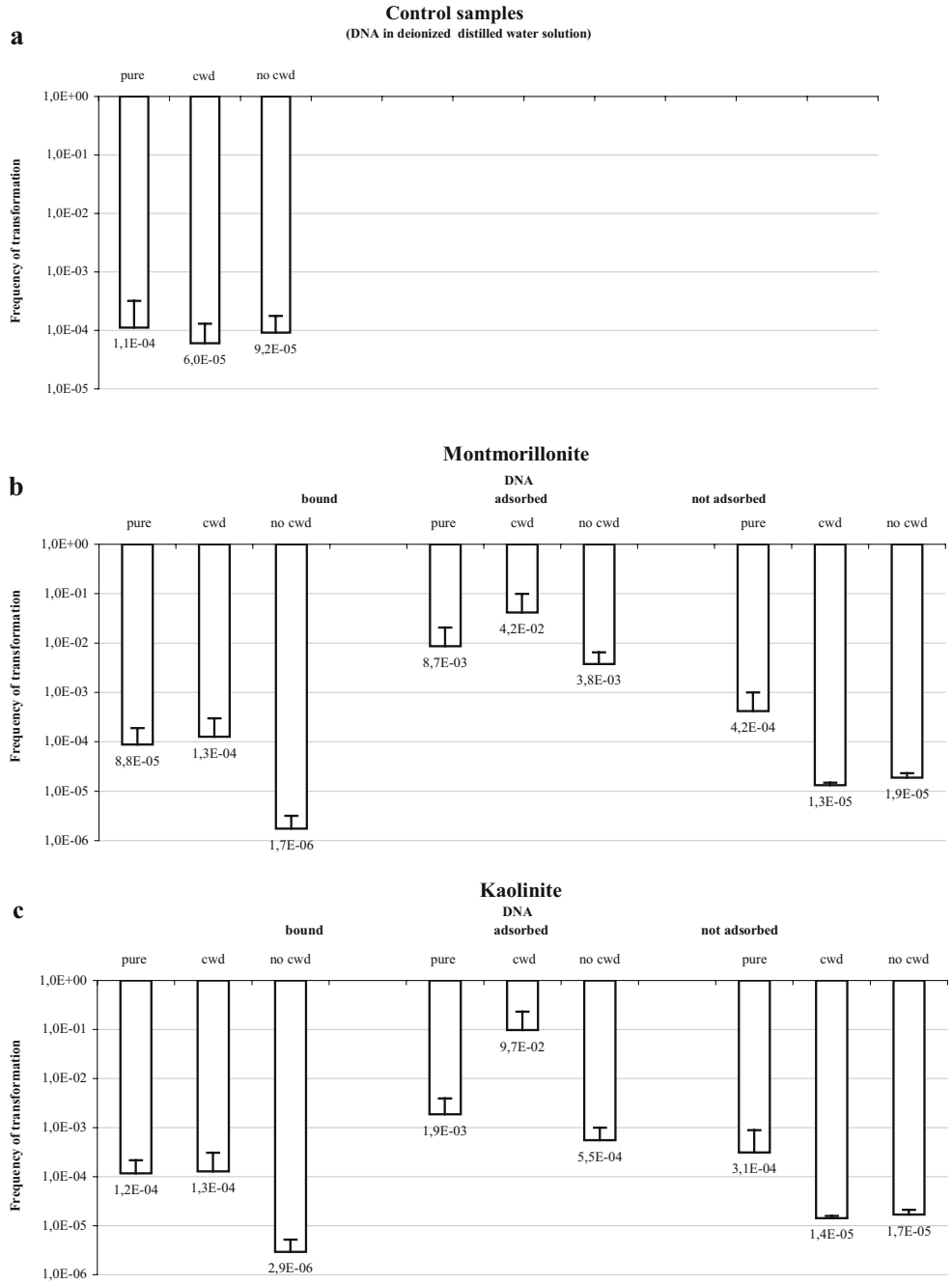


Fig. 3 Frequencies of transformation (F_t) per μg of pure and dirty DNA ±cwd from *B. subtilis* BD1512: **a** DNA control samples that have not had any interaction with clay minerals, **b** DNAs not adsorbed, adsorbed, or bound at equilibrium on montmorillonite (*M*), or **c** on kaolinite (*K*). The values are expressed per μg^{-1} of DNA

chromosomal dirty DNA (±cwd) molecules of the solution (Bloomfield 1998). These multimolecular condensed DNA forms could have successively been adsorbed on clay minerals (Khanna et al. 1998), and this mechanisms may be also responsible for the higher amount of both dirty DNAs adsorbed on clays with respect to pure DNA. The strength of the bound between DNA and clays depend on both the types of binding site on clay minerals and the number of bonds per DNA molecule (Paget and Simonet 1994; Pietramellara et al. 2001). Therefore, the compact structure of single or multimolecular dirty DNA (±cwd) condensed forms with a low number of binding sites could also explain the drastic reduction in the amount of DNA bound on clays, especially for the dirty DNA −cwd.

The coc, together with the cwd, could also promote the dirty DNA adsorption on clay minerals (Fig. 2) by acting as a bridge between the DNA molecule and the clay mineral surface. Among the coc, proteins should play the major role

in the dirty DNA adsorption on clay minerals because they are rapidly adsorbed on clay minerals, and most of them are not readily desorbed (Ding and Henrichs 2002; Tavares and Sellstedt 2001). The cwd of the *B. subtilis* cell wall is prevalently composed of phospholipids with structural proteins on the inner side and with a heteropolymeric matrix of peptidoglycan covalently linked to anionic polymers such as teichoic or teichuronic acid on its outer side (Stephenson et al. 2000). The presence of teichoic or teichuronic acid confers to the external surface of the cellular wall a high density of negative charges (Stephenson et al. 2000). The bacterial cellular wall may act as a bridge as its phospholipids can interact with the proteins (ccp) associated to the DNA molecules (Escriba et al. 1997) and with Ca^{2+} saturating clays through its carboxyl groups. The possibility that phosphate or carboxyl groups of teichoic or teichuronic acid interact with the Ca^{2+} saturating clays (Stephenson et al. 2000) might be rejected considering that the cell lysis method of Svarachorn et al. (1989) based on induction of cellular autolysins enzymes prevalently degrade the peptidoglycan layer of the *B. subtilis* cell membrane.

The dominance of clay edge charges to planar surface charges on K, and the opposite on M, can explain the higher amount of bound DNA by K than M and the opposite behavior for the adsorbed DNA (Ogram et al. 1994; Pereira and Williams 2001).

DNA transformation frequency

The highest F_t values were shown by the dirty DNA +cwd fraction adsorbed on either M or K clays. Both pure DNA and dirty DNA ±cwd fractions adsorbed on M and K showed higher F_t values than those of the respective not adsorbed and bound fractions and those of the respective control samples (Fig. 3a–c). These results indicated the positive effect of adsorption of DNA by clay minerals on DNA-transforming capacity. An hypothesis could be that the weak bounds involved in the adsorption of DNA on clay minerals (Khanna and Stotzky 1992), and the consequent modification induced on the DNA conformation (Khanna et al. 1998; Paget and Simonet 1994; Pereira and Williams 2001), might make the DNA molecule more accessible to competent cells. Frequencies of transformation did not depend on the amount of DNA present onto clays but on the fraction of DNA available to bacterial competent cells (Demanèche et al. 2001).

The higher F_t of dirty DNA +cwd adsorbed or bound on clays than those of pure and dirty DNA −cwd (Fig. 3b and c) evidenced the positive effect of cwd on F_t when the DNA was adsorbed or bound on clays. The presence of cwd might attenuate the structure collapse from 3D to 2D of dirty DNA, especially if in multimolecular aggregate form, when adsorbed or bound onto Ca^{2+} saturated clay surface (Bloomfield 1998; Koltover et al. 2000), thus, facilitating the DNA uptake by bacterial competent cells. Comparing the F_t values of pure and dirty DNA −cwd adsorbed or bound on clays (Fig. 3b and c), it is also clear that coc in presence of clay minerals negatively affected bacterial transformation, probably making the DNA molecule less available to competent cells. On the contrary, in the absence of clay minerals, coc positively affected bacterial transformation as evidenced by the higher F_t of dirty DNA −cwd among the control samples (Fig. 3a). A possible explanation could be that in the absence of any interaction between dirty DNA −cwd and clay minerals, the coc might increase the affinity of the DNA molecule for the DNA specific receptor sites of the external surface of the competent bacterial cell wall.

Among the not adsorbed DNA fractions (Fig. 3b and c), the pure DNA showed the higher transformation frequency, whereas dirty DNA ±cwd evidenced similar F_t values. In addition, by comparing the F_t of control and not adsorbed pure and dirty DNAs (±cwd), only the not adsorbed DNA −cwd showed a significant reduction in the F_t values. As the adsorption equilibrium is based on a dynamic state with equal rates of DNA adsorption and desorption by clays, the main difference between the not adsorbed and control samples is that the former contained molecules which have interacted with clay minerals. The repeated interaction with

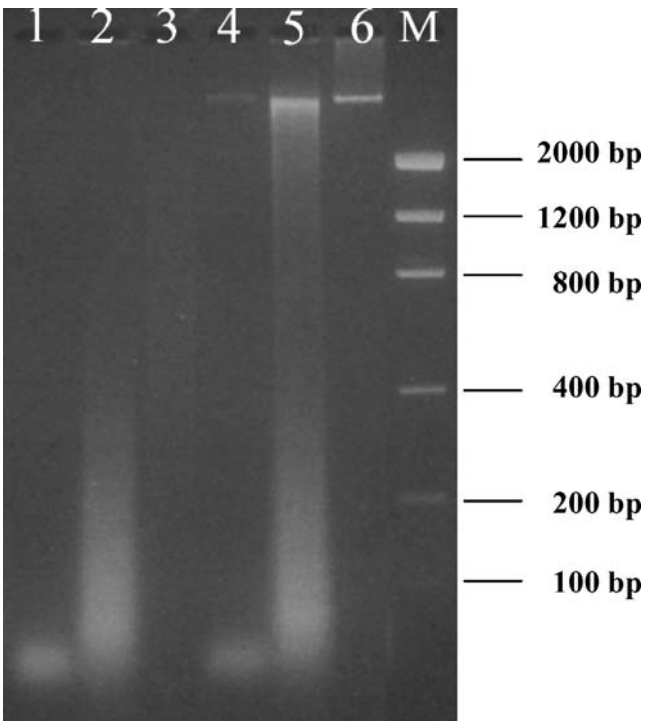


Fig. 4 Agarose gel electrophoresis of DNA digested with endonuclease Alu I (*lanes 1 to 3*) or not digested (*lanes 4 to 6*). *Lanes 1 and 4*: dirty DNA −cwd; *Lanes 2 and 5*: dirty DNA +cwd; *Lanes 3 and 6*: pure DNA; *M* Low Mass Ladder (Fermentas)

the clay surface might have changed the conformation of the dirty DNA −cwd not adsorbed with reduction of its affinity to the DNA specific receptor sites on the external surface of the competent cells.

DNA enzymatic digestion

High molecular weight DNA was present in the supernatant after lysis of bacterial cells by KCl, as evidenced by electrophoresis on agarose gel (Fig. 4, lanes 4, 5, 6). A smear of shorter fragments was also observed in the dirty DNA +cwd fraction (lane 5), probably as the result of the activity of intracellular DNases released after cell lysis.

To asses the effect of coc and cwd on enzyme degradation of DNA molecules, pure and both dirty DNA (±cwd) fractions were digested with endonuclease Alu I after cell lysis (Fig. 4, lanes 1, 2, 3). The electrophoresis run pointed out the extensive DNA degradation that could exceed the sensibility threshold of the electrophoresis detection, of both dirty DNA −cwd and pure DNA (lanes 1, 3), whereas the presence of a smear on lane 2 suggested a protection of cwd on DNA fragments smaller than 400 bp. The smallest bands at the bottom of the gel in both dirty DNAs (lanes 1, 2, 4, 5), were due to RNA, whose presence was verified by cross digestions with DNase and RNase (data not shown). Comparing these data with those reported by Demanèche et al. (2001), we can conclude that DNA was more protect against enzymatic degradation by clay minerals than by coc and cwd.

Conclusions

This is the first experimental study concerning adsorption and binding of dirty DNA molecule (±cwd) on clay minerals and the transformation activities of the relative DNA clay complexes. The investigated conditions were closer to those occurring in situ than those of experiments based on the use of purified DNA molecules. The obtained results suggest that the presence of coc and cwd improved the adsorption of the DNA molecule on clay minerals, whereas only the cwd had a positive effect on the binding of DNA on clays. Moreover, the comparison of F_t of both dirty DNAs adsorbed or bound on clays with those of pure DNA only showed a positive effect of cwd when the dirty DNA +cwd was adsorbed on clays fractions, whereas coc negatively affected the bacterial transformation. The cwd might have also partially protected the DNA molecule against enzymatic degradation. These data suggested that DNA can have higher chances to survive in the soil environment and to be involved in horizontal gene transfer when associated to cwd.

We have proposed several hypothesis to interpret the underlying mechanisms of the observed results, but further research is needed to validate these hypothesis.

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