

## Transformation of *Bacillus subtilis* by DNA Bound on Montmorillonite and Effect of DNase on the Transforming Ability of Bound DNA

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The equilibrium adsorption and binding of DNA from *Bacillus subtilis* on the clay mineral montmorillonite, the ability of bound DNA to transform competent cells, and the resistance of bound DNA to degradation by DNase I are reported. Maximum adsorption of DNA on the clay occurred after 90 min of contact and was followed by a plateau. Adsorption was pH dependent and was greatest at pH 1.0 (19.9  $\mu\text{g}$  of DNA mg of clay<sup>-1</sup>) and least at pH 9.0 (10.7  $\mu\text{g}$  of DNA mg of clay<sup>-1</sup>). The transformation frequency increased as the pH at which the clay-DNA complexes were prepared increased, and there was no transformation by clay-DNA complexes prepared at pH 1. After extensive washing with deionized distilled water (pH 5.5) or DNA buffer (pH 7.5), 21 and 28%, respectively, of the DNA remained bound. Bound DNA was capable of transforming competent cells (as was the desorbed DNA), indicating that adsorption, desorption, and binding did not alter the transforming ability of the DNA. Maximum transformation by bound DNA occurred at 37°C (the other temperatures evaluated were 0, 25, and 45°C). DNA bound on montmorillonite was protected against degradation by DNase, supporting the concept that "cryptic genes" may persist in the environment when bound on particulates. The concentration of DNase required to inhibit transformation by bound DNA was higher than that required to inhibit transformation by comparable amounts of free DNA, and considerably more bound than free DNase was required to inhibit transformation by the same amount of free DNA. Similarly, when DNA and DNase were bound on the same or separate samples of montmorillonite, the bound DNA was protected from the activity of DNase.

Transformation is a process of gene transfer in which homo- or heterospecific "naked" DNA is taken up and expressed by a competent bacterial cell (34). Information on transformation has been obtained primarily during studies performed in the laboratory under conditions that optimized the process (33, 35). The occurrence and significance of transformation in the environment are not known. The current concern about the release into the environment of genetically modified organisms has resulted in an interest in transformation as a possible method for the horizontal dissemination of recombinant DNA molecules in the environment (7, 30, 38, 39).

Transformation has been considered to be a relatively unimportant mechanism for the transfer of genetic information in soil and other natural habitats, mainly because of the presumed susceptibility of naked DNA to microbial degradation. However, the adsorption and binding of DNA on clay minerals and other particulates may protect DNA against degradation and, thus, enhance its persistence in these habitats (38, 39). Such persistence would be undetected (i.e., "cryptic") in the absence of a host susceptible to transformation by the DNA. However, in the presence of a susceptible host, the bound DNA could be taken up and expressed.

There have been few studies on transformation as a mode of gene transfer in soil. Reaney et al. (32), Stewart (34), Stotzky (38), and Stotzky and Babich (39) have reviewed most of these studies. DNA from calf thymus (23) and *Bacillus subtilis* (1, 24) have been shown to adsorb on

analytical-grade sea sand consisting mainly of quartz. A portion of the DNA was retained by the sand, even after extensive elution with different solutions, and the bound DNA was protected against DNase I and retained the capability to transform competent cells (1, 24). Stewart and Sinigalliano (36) showed the transformation of a marine isolate, *Pseudomonas stutzeri* Zobell, when DNA from strain PP100 was mixed with recipient cells of strain PP101 in the presence of both sterile and nonsterile marine sediments. Paul et al. (30), using the high-frequency-of-transformation *Vibrio* strain WJT-1C as the recipient and transforming DNA from the broad-host-range plasmid pQSR50, showed the occurrence of natural transformation in seawater, in both the presence and the absence of the ambient microbial community, and suggested that natural transformation occurs more frequently in the water column than in sediments.

Although DNA from *B. subtilis* has been shown to adsorb on pure sea sand (1, 24), studies on (i) the adsorption of bacterial DNA on clay minerals (37), (ii) the effects on adsorption of various physicochemical environmental factors (e.g., pH and temperature [37]), (iii) the ability of DNA bound on clays to transform competent cells, and (iv) the resistance of clay-bound DNA to microbial degradation are needed to determine whether cryptic genes can persist in soil and other clay-containing habitats (38). The long-term persistence of cryptic genes that could subsequently reappear in multiplying cells, both added and indigenous, must be considered in assessing not only the risks and benefits of the release of genetically modified organisms into the environment, but also the importance of transformation in the evolution of bacteria. This report discusses the equilibrium

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adsorption and binding of DNA from *B. subtilis* on montmorillonite, the ability of bound DNA to transform competent recipient cells, and the protection of bound DNA against inactivation by DNase I.

## MATERIALS AND METHODS

**Growth and maintenance of bacteria.** Strains of *B. subtilis* (donors: BD1512, carrying the *his*, *leu*, and *met* mutations and Cap<sup>r</sup>, and BD170, carrying the *trp* and *thr* mutations; recipients: BD54, carrying the *ile*, *leu*, and *met* mutations, and BD630, carrying the *his*, *leu*, and *met* mutations) obtained from David Dubnau were grown and maintained on slants of tryptose blood agar base (Difco) supplemented with appropriate antibiotics (10 µg ml<sup>-1</sup>), 5 ml of 1 M MgSO<sub>4</sub> · 7H<sub>2</sub>O liter<sup>-1</sup>, and 0.2 ml of 0.1 M MnCl<sub>2</sub> · 4H<sub>2</sub>O liter<sup>-1</sup> (2) at room temperature (24 ± 2°C), at which they maintain their competency better than they do at 4°C (9a). The phenotypes of the strains were verified regularly.

**Preparation of competent recipient cells.** Recipient cultures were grown overnight (18 h) at 37°C in a competence medium (Spizizen II) which consisted of Spizizen salts (SS) [0.6% KH<sub>2</sub>PO<sub>4</sub>, 1.4% K<sub>2</sub>HPO<sub>4</sub>, 0.1% sodium citrate, 0.2% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>], 0.2% Bacto Casamino Acids (Difco), 0.1% yeast extract (Difco), 0.5% glucose (each autoclaved separately), 50 µg of the necessary amino acids ml<sup>-1</sup> (sterilized by filtration), and 2.5 µl of 0.01 M MgCl<sub>2</sub> ml<sup>-1</sup> (which enhances the competence of *B. subtilis*) (2). Recipient cells were made competent by the method of Dubnau and Davidoff-Abelson (10). A 1:100 dilution of recipient cells grown overnight in the competence medium was inoculated into fresh competence medium and shaken (200 rpm) in a water bath at 37°C for 6.5 h, when the culture reached the late log-early stationary phase. The cells were harvested by centrifugation at 9,000 × *g* for 10 min and suspended in the competence medium containing 10% glycerol, and 1-ml aliquots were distributed into Eppendorf tubes (1.5 ml), quick-frozen in a dry ice-ethanol bath, and stored at -80°C.

**Preparation of DNA.** DNA was prepared by using the method of Dubnau and Davidoff-Abelson (10). Cells were grown overnight in VY broth (veal infusion broth [Difco] containing 0.5% yeast extract), and 5 ml was transferred to 95 ml of fresh VY broth and grown to ca. 10<sup>10</sup> cells ml<sup>-1</sup>. The cells were harvested by centrifugation (9,000 × *g*), washed in TES buffer (30 mM Tris, 5 mM EDTA, 50 mM NaCl [pH 7.5]), and resuspended in 10 ml of TES containing 1 mg of lysozyme (Sigma) and 50 µg of pancreatic ribonuclease (Sigma). After shaking for 30 min at 37°C, TES (10 ml) containing 160 mg of Sarkosyl (Sigma) and 0.5 mg of Pronase (Sigma) was added, and the mixture was incubated in a water bath at 37°C for 2 h and then brought to 24°C with continuous shaking. An equal volume (20 ml) of phenol (saturated with TES and adjusted to pH 7.5 with NaOH) was added, the mixture was vortexed and then centrifuged (12,000 × *g* for 10 min), and the aqueous phase was extracted a second time with phenol to remove any remaining protein. Two volumes (40 ml) of ice-cold ethanol (100%) were added to the aqueous phase to precipitate the DNA, which was spooled on a glass rod and dissolved in DNA buffer (10 mM Tris, 0.1 mM EDTA, 4 mM NaCl [pH 7.5]). The purity of the DNA was assessed by the 260/280-nm ratio and by electrophoresis on a 0.7% agarose gel followed by staining with ethidium bromide (26). Only DNA that had a 260/280-nm ratio between 1.8 and 2.0, indicating that its purity was high, was used. Moreover, a single band of DNA was obtained by electrophoresis, which confirmed the purity.

**Transformation procedure.** Freshly prepared or frozen competent cells, rapidly thawed at 45°C, were incubated with 0.5 to 2.0 µg of DNA ml<sup>-1</sup> of DNA buffer containing ca. 10<sup>8</sup> competent cells with shaking (70 rpm) or on a rotating wheel at 37°C for 30 min. Serial decade dilutions of the suspensions diluted with SS were plated on tryptose blood agar base containing 10 µg of chloramphenicol ml<sup>-1</sup> when chloramphenicol-resistant transformants were evaluated. For the enumeration of transformants of auxotrophic cells, the medium consisted of SS, 0.5% glucose, 2.0% agar, and 50 µg of the appropriate amino acids ml<sup>-1</sup>. Competent cells that received no DNA were plated on the various selective media and served as negative controls. The development of equal numbers of colonies on the selective media from both DNA-containing cultures and cultures without DNA (an unlikely event, on the basis of the results of these studies) would indicate that colonies from DNA-containing cultures were mutants rather than transformants. When there was no growth on the selective media from the control cultures without DNA, it was assumed that transformation occurred in the DNA-containing cultures. The total number of viable cells was determined by plating the cultures on tryptose blood agar base. All plates were incubated at 37°C for 18 to 24 h. The transformation frequency was expressed as the number of transformants/number of total viable cells.

To standardize the transformation procedure, the time at which the recipient cells attained maximum competency was determined at 2.0, 2.5, 3.0, and 3.5 h after the end of the log phase. Maximum competency occurred at 2.5 h into the stationary phase. The effect of the time of interaction between DNA and competent cells on the frequency of transformation was studied at 0, 30, 60, 120, and 180 min after the cells attained maximum competency (i.e., 2.5 h after the end of the log phase). The contact time for optimum transformation between DNA and competent cells was 30 min, after which competency decreased. For example, the numbers of isoleucine transformants were 5.8 × 10<sup>4</sup>, 1.6 × 10<sup>4</sup>, 6.2 × 10<sup>2</sup>, and 5.5 × 10<sup>2</sup> ml<sup>-1</sup> after 30, 60, 120, and 180 min, respectively.

**Preparation of homoionic clay.** The <2-µm fraction of montmorillonite (Fisher Scientific Co.) was separated by differential centrifugation and made homoionic to Ca, as described previously by Dashman and Stotzky (8), Harter and Stotzky (18), and Jackson (20).

**Preparation of clay-DNA complexes.** One hundred microliters of a suspension of montmorillonite (in deionized distilled water [ddH<sub>2</sub>O]) containing different concentrations of clay was mixed with 100 µl of a DNA solution (in DNA buffer), the concentration of which varied with the initial concentration of purified DNA. Different concentrations of DNA (4 to 240 µg) were also reacted with a constant concentration (i.e., 2.2 mg) of clay. The reactions took place in 1 ml of DNA buffer in centrifuge tubes (polystyrene tubes, 12 by 75 mm).

The contact time for maximum equilibrium adsorption of DNA on montmorillonite was determined by rotating tubes containing mixtures of DNA and clay on a wheel at 70 rpm at 24 ± 2°C for 0, 30, 60, 90, 120, and 180 min and then centrifuging the contents at 40,000 × *g* for 20 min (all of the clay particles were sedimented at this speed). The supernatants were removed, and their DNA contents were measured by determining the A<sub>260</sub>.

**Desorption of DNA from clay-DNA complexes.** The clay-DNA complexes formed after equilibrium adsorption were washed by resuspending the centrifuged pellets by vortexing them in 1 ml of ddH<sub>2</sub>O (pH 5.5) or DNA buffer (pH 7.5) and centrifuging them at 40,000 × *g* for 20 min, and the super-

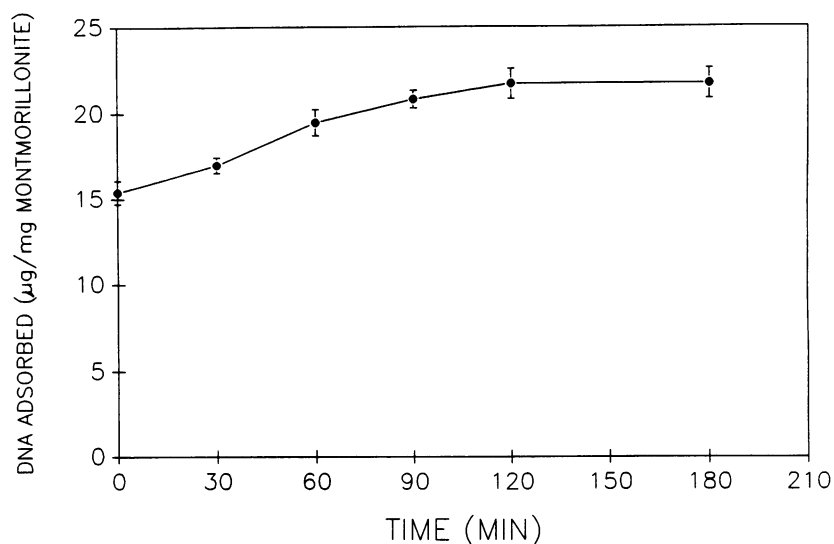


FIG. 1. Effect of time on adsorption of DNA from *B. subtilis* BD170 on Ca-montmorillonite. Data are expressed as means  $\pm$  SEM.

natants were analyzed at 260 nm. This procedure was repeated until no more DNA was detected in the supernatants, and the complexes were then washed four more times ("ultimate washing"). The absence of DNA in the supernatants after the fourth wash was confirmed by the polymerase chain reaction with 14-mer random primers (Promega) and a Coy TempCycler (Ann Arbor, Mich.). The clay pellets containing DNA that was tightly bound were stored at  $-20^{\circ}\text{C}$  until they were used for the transformation of competent cells.

**Equilibrium adsorption isotherms.** Adsorption isotherms were constructed from two types of experiments: a constant concentration of clay versus variable concentrations of DNA and a constant concentration of DNA versus variable concentrations of clay. The amount of DNA adsorbed on montmorillonite at equilibrium was calculated by subtracting the amount of DNA present in the first supernatant from the

amount of DNA initially added to the clay. The amount of DNA bound on the clay was calculated by subtracting the sum of the amounts of DNA in all of the supernatants (i.e., all washings plus the equilibrium adsorption supernatant) from the amount of DNA initially added.

**Transformation by clay-bound DNA.** The pellets of bound clay-DNA complexes (2.2 mg of montmorillonite containing ca.  $12\ \mu\text{g}$  of DNA  $\text{mg}$  of clay $^{-1}$ ) were added to 1 ml of competent cells (either from frozen stocks or freshly prepared) and rotated at 70 rpm at  $37^{\circ}\text{C}$  for 30 min. The cells were plated on the appropriate selective media, transformants and total viable cells were enumerated, and transformation frequencies were calculated.

**Effect of pH on adsorption of DNA on montmorillonite.** DNA ( $62\ \mu\text{g}$  in  $30\ \mu\text{l}$  of DNA buffer) and clay (2.2 mg in  $100\ \mu\text{l}$  of  $\text{ddH}_2\text{O}$ ) were added to 1 ml of DNA buffer adjusted to pH 1.0 to 9.0 with HCl or NaOH and rotated at 70 rpm for 90

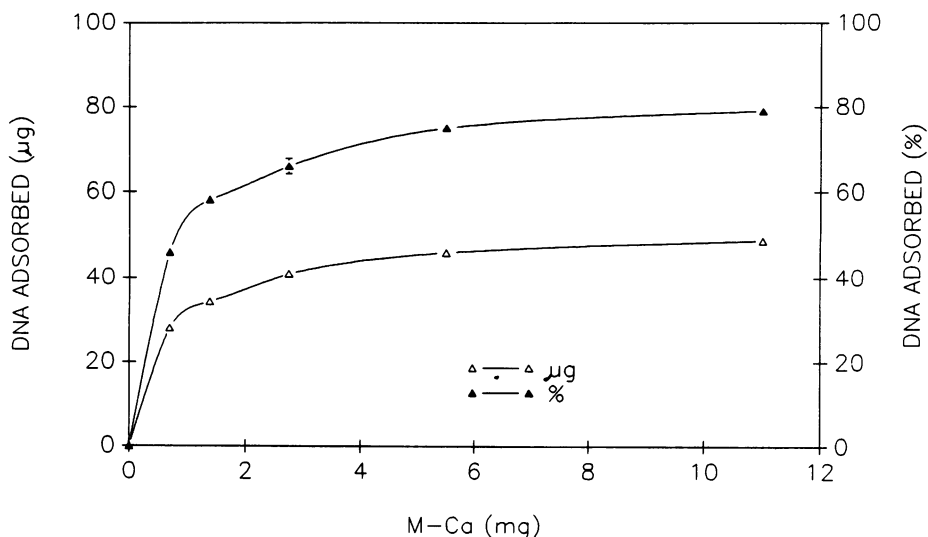


FIG. 2. Equilibrium adsorption isotherm of  $62\ \mu\text{g}$  of DNA from *B. subtilis* BD170 on different concentrations of Ca-montmorillonite. Data are expressed as means  $\pm$  SEM, which are indicated when not within the dimensions of each symbol.

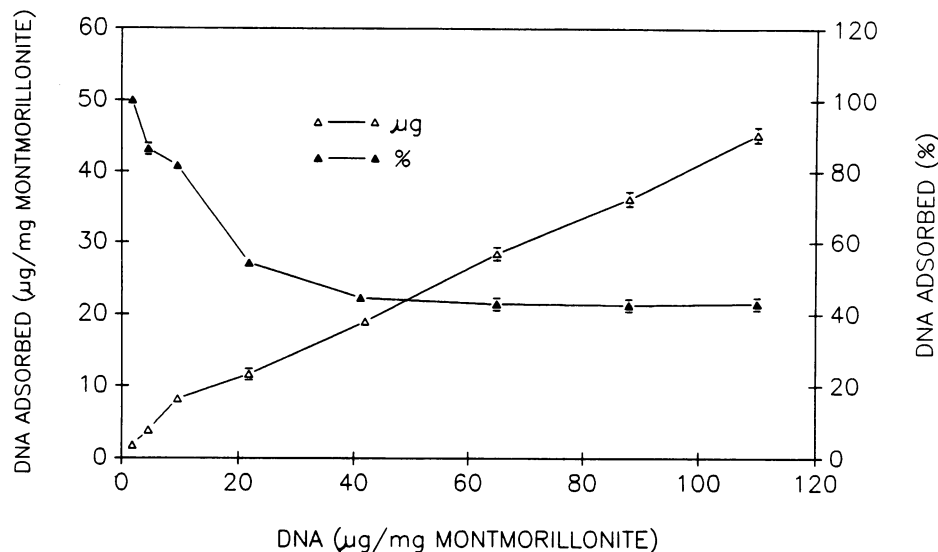


FIG. 3. Equilibrium adsorption isotherm of different concentrations of DNA from *B. subtilis* BD170 on 2.2 mg of Ca-montmorillonite. Data are expressed as means  $\pm$  SEM, which are indicated when not within the dimensions of each symbol.

min at  $24 \pm 2^\circ\text{C}$ . The complexes were then washed with ddH<sub>2</sub>O (pH 5.5) until no more DNA was desorbed, and the DNA in the supernatants was determined as described above.

**Transformation by clay-DNA complexes formed at different pH values.** Transformation by the adsorbed DNA-clay complexes formed at different pH values was determined at pH 7.5 by using the method described above.

**Effects of temperature on transformation.** To determine the effects of temperature on transformation by clay-DNA complexes, the transformation of competent auxotrophic cells by DNA bound on montmorillonite was studied at 0, 25, 37, and 45°C.

**Binding of DNase on montmorillonite.** Different concentra-

tions of DNase I (Sigma) from a stock solution containing 1 mg of DNase dissolved in 1 ml of DNase buffer (50 mM Tris, 10 mM MgCl<sub>2</sub>, 50 µg of bovine serum albumin [Sigma] [pH 7.5]) (28) were bound on montmorillonite as described previously for the clay-DNA complexes except that the supernatants were analyzed for protein at 280 nm (18). Ultimate washing with ddH<sub>2</sub>O (pH 5.5) was done until no more DNase was desorbed from the clay (four washes).

**Effect of montmorillonite on the inhibition of transformation by DNase.** The effects of free and clay-bound DNase on the abilities of free and clay-bound DNA to transform competent cells were studied in the following ways.

(i) **DNA bound on clay and free DNase.** Pellets of bound montmorillonite-DNA complexes (30 µg of DNA 2.2 mg of

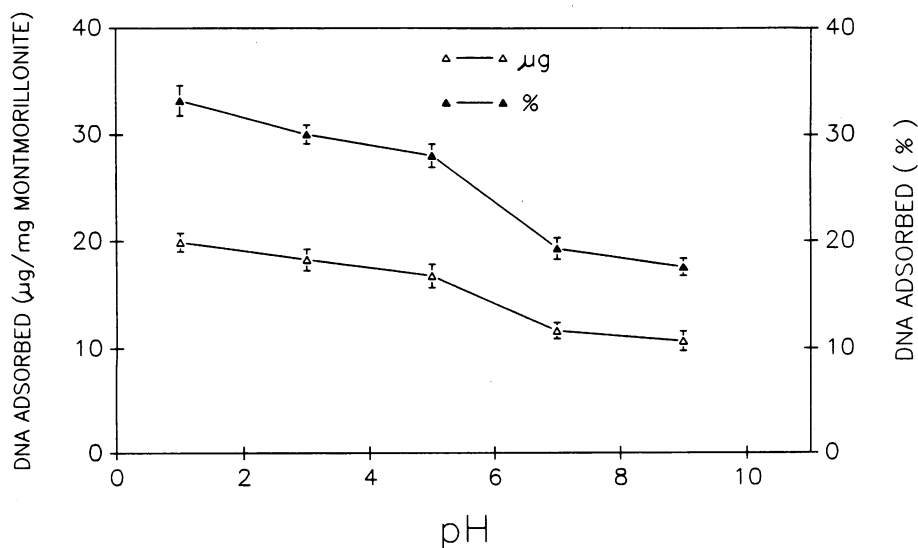


FIG. 4. Effects of pH values on the equilibrium adsorption of 62 µg of DNA from *B. subtilis* BD1512 on 2.2 mg of Ca-montmorillonite. Data are expressed as means  $\pm$  SEM.

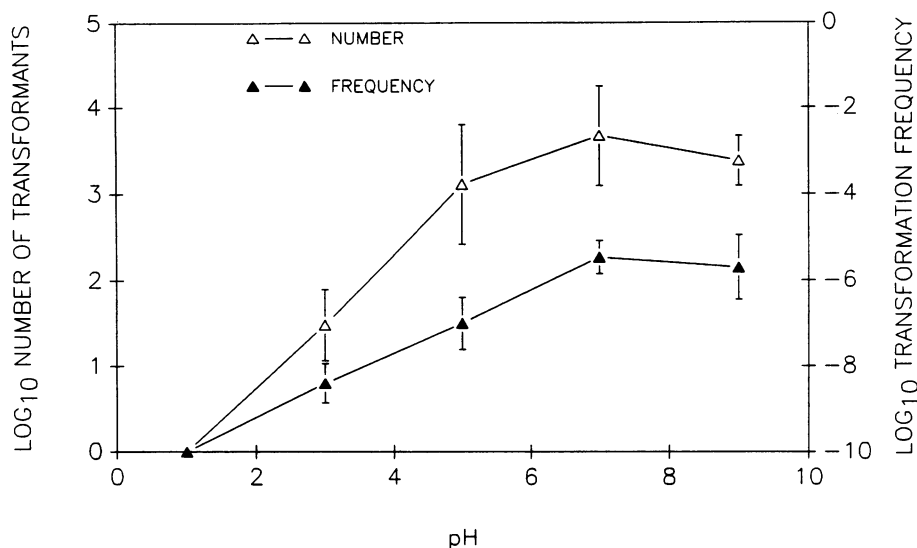


FIG. 5. Effects on transformation of *B. subtilis* BD630 of the pH values at which DNA from *B. subtilis* BD1512 was adsorbed at equilibrium on Ca-montmorillonite (see Fig. 4).

clay<sup>-1</sup>) were mixed with 20  $\mu$ l of DNase buffer containing 0.31 to 5  $\mu$ g of free DNase by vortexing for 1 min at 24  $\pm$  2°C, and 1 ml of competent cells was added after 15 min and incubated at 37°C for 30 min. The reaction was stopped by placing the samples on ice, and the numbers of transformants and total viable cells were enumerated on selective media after 18 to 24 h.

(ii) **DNase bound on clay and free DNA.** Pellets of bound montmorillonite-DNase complexes (27 to 610  $\mu$ g of DNase 2.2 mg of clay<sup>-1</sup>) were mixed with 30  $\mu$ g of free DNA in 20  $\mu$ l of DNA buffer by vortexing for 1 min at 24  $\pm$  2°C, 1 ml of competent cells was added after 15 min and incubated at 37°C for 30 min, and the numbers of transformants and total viable cells were determined as described above.

(iii) **DNase and DNA bound on separate samples of montmorillonite.** DNase and DNA were bound on separate aliquots of clay. The concentration of bound DNase ranged from 0.045 to 15.6  $\mu$ g 2.2 mg of clay<sup>-1</sup>. Pellets of bound clay-DNA (30  $\mu$ g of DNA 2.2 mg of clay<sup>-1</sup>) and bound clay-DNase complexes, resuspended in 1 ml of DNA buffer and 1 ml of DNase buffer, respectively, were mixed in a centrifuge tube and rotated on a wheel at 70 rpm for 15 min. The mixture was centrifuged at 40,000  $\times$  g for 20 min, the resultant combined pellet was added to 1 ml of competent cells and incubated at 37°C for 30 min, and transformants and total viable cells were enumerated after 18 to 24 h as described above.

(iv) **DNase and DNA bound on the same sample of montmorillonite.** DNase, from 14 to 340  $\mu$ g, was bound on 2.2 mg of clay, and 80  $\mu$ g of DNA in 80  $\mu$ l of DNA buffer was added to the clay-DNase complexes, which were rotated at 70 rpm for 90 min at 24  $\pm$  2°C and then washed with ddH<sub>2</sub>O (pH 5.5) until no more DNA was desorbed. Approximately 30  $\mu$ g of DNA was bound on the clay-DNase complexes. Pellets of the clay-DNase-DNA complexes were added to 1 ml of competent cells and incubated at 37°C for 30 min, and transformants and total viable cells were enumerated after 18 to 24 h as described above.

**Statistics.** All experiments were performed in triplicate, and each experiment was repeated at least once. The data

are expressed as the means  $\pm$  the standard error of the means ( $\bar{x} \pm$  SEM). Where appropriate, the data have been normalized to a base of 1 mg of montmorillonite.

## RESULTS

**Equilibrium adsorption of DNA on clay.** Maximum equilibrium adsorption of DNA on montmorillonite occurred after 90 min of contact (Fig. 1). The adsorption isotherms of a constant amount of DNA added to different concentrations of clay showed an increase in the adsorption of DNA with increasing concentrations of clay followed by a plateau (Fig. 2). When an additional 2.2 mg of montmorillonite was added to the equilibrium supernatant containing the ca. 20% DNA that was not adsorbed, only ca. 4% of this DNA was adsorbed. The DNA not adsorbed originally apparently had a lower affinity for the clay.

The percent DNA adsorbed on a constant amount of montmorillonite decreased as the concentration of DNA increased (Fig. 3). At a concentration of 1.8  $\mu$ g of DNA mg of montmorillonite<sup>-1</sup>, 100% of the DNA was adsorbed, whereas only ca. 43% was adsorbed when 40 to 110  $\mu$ g of DNA was added to the equivalent of 1 mg of clay. The amount of DNA adsorbed increased as the concentration of DNA increased, but a plateau was not attained, even when the equivalent of 110  $\mu$ g of DNA mg of clay<sup>-1</sup> was added, indicating a constant partitioning of the DNA between the clay and the solution phase.

**Effect of pH on adsorption of DNA.** Adsorption of DNA on montmorillonite was greatest at low pH values; 19.9 and 10.7  $\mu$ g of DNA mg of montmorillonite<sup>-1</sup> were adsorbed at pH 1.0 and 9.0, respectively (Fig. 4).

**Transformation with clay-DNA complexes formed at different pH values.** The clay-DNA complexes prepared at the different pH values were used to transform competent cells at pH 7.5. No transformation was observed with complexes prepared at pH 1.0, and transformation frequencies increased as the pH at which the complexes were formed increased (Fig. 5).

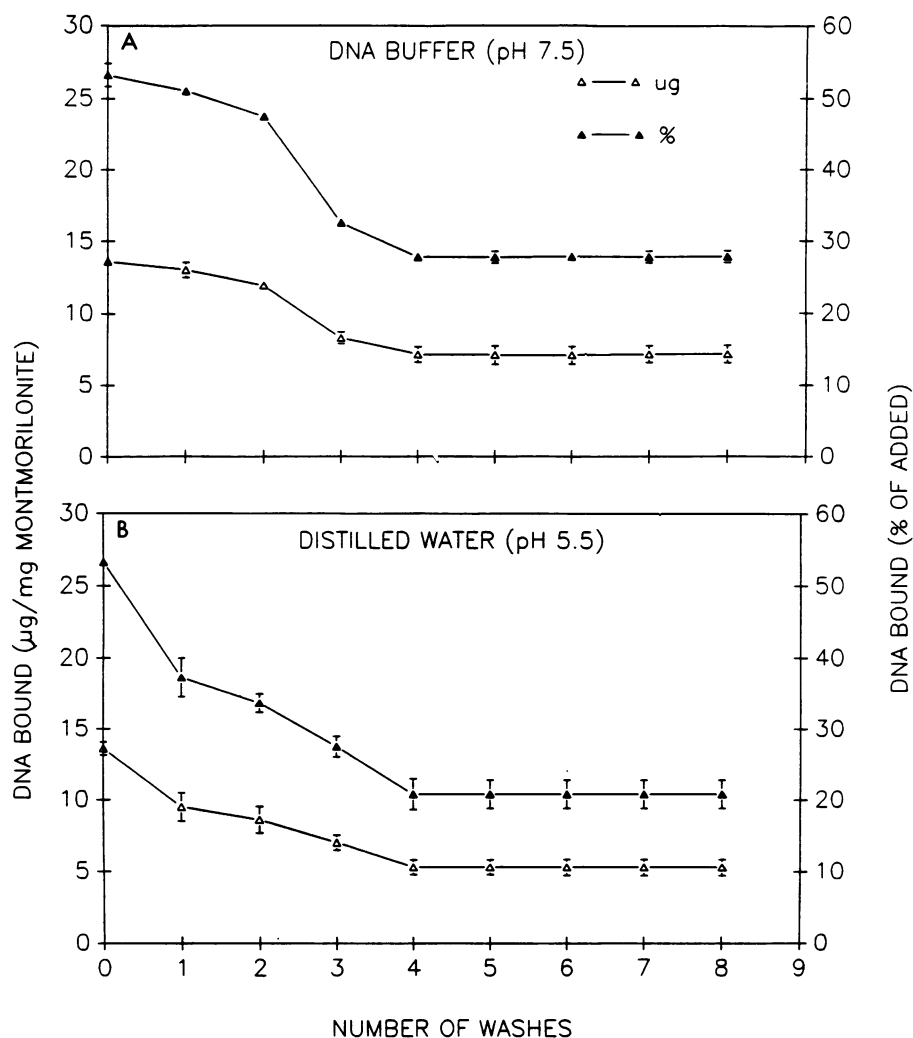


FIG. 6. Elution of ca. 14 µg of DNA from *B. subtilis* BD1512 adsorbed at equilibrium on the equivalent of 1 mg of Ca-montmorillonite with DNA buffer (A) or distilled water (B). Data are expressed as means  $\pm$  SEM, which are indicated when not within the dimensions of each symbol.

**Elution of adsorbed DNA.** The DNA adsorbed at equilibrium on montmorillonite was eluted with either DNA buffer (pH 7.5) or ddH<sub>2</sub>O (pH 5.5). After four washes, no more DNA was eluted, and ca. 20% (Fig. 6B) and 28% (Fig. 6A), respectively, of the DNA added initially was retained (i.e., bound) by the clay. The inability to desorb more bound DNA was confirmed with the polymerase chain reaction, as no DNA was detected in the supernatants from the last five washes after 30 cycles of amplification. The DNA did not appear to intercalate the clay, as determined by X-ray diffraction analysis (data not shown).

**Transformation by bound DNA.** When the clay-DNA complexes were evaluated for their abilities to transform competent cells, transformation was observed with all complexes, even after each of the first six washes with the bound clay-DNA complexes. For example, the number of transformants ranged from  $4.7 \times 10^3$  (after the first wash) to  $1.6 \times 10^3$  (after the sixth wash) (Fig. 7).

**Effect of temperature on transformation.** Transformation with montmorillonite-DNA complexes, except for transformation of isoleucine at 0°C, occurred at all temperatures

evaluated. Maximum transformation of the auxotrophic markers occurred at 37°C. (Fig. 8).

**Effect of montmorillonite on the inhibition of transformation by DNase.** The binding of either DNA or DNase I or both on montmorillonite reduced the inhibition of transformation by DNase. Transformation by 30 µg of free DNA was inhibited ca. 99.8% by 1.2 µg of free DNase, whereas even 5 µg of free DNase resulted in only ca. 90% inhibition of transformation by 30 µg of DNA bound on 2.2 mg of montmorillonite (Fig. 9A). Conversely, even 277 µg of DNase bound on the equivalent of 1 mg of clay did not reduce transformation by 30 µg of free DNA as much as 1.2 µg of free DNase did (Fig. 9B). When DNA and DNase were bound on the same sample of clay, 77.3 µg of bound DNase mg of montmorillonite<sup>-1</sup> was necessary to inhibit transformation by 13.6 µg of bound DNA mg of clay<sup>-1</sup> (Fig. 10A). When DNA and DNase were bound on separate samples of montmorillonite and together added to competent cells, not even 7.1 µg of bound DNase mg of clay<sup>-1</sup> reduced transformation by 13.6 µg of bound DNA mg of clay<sup>-1</sup> as much as

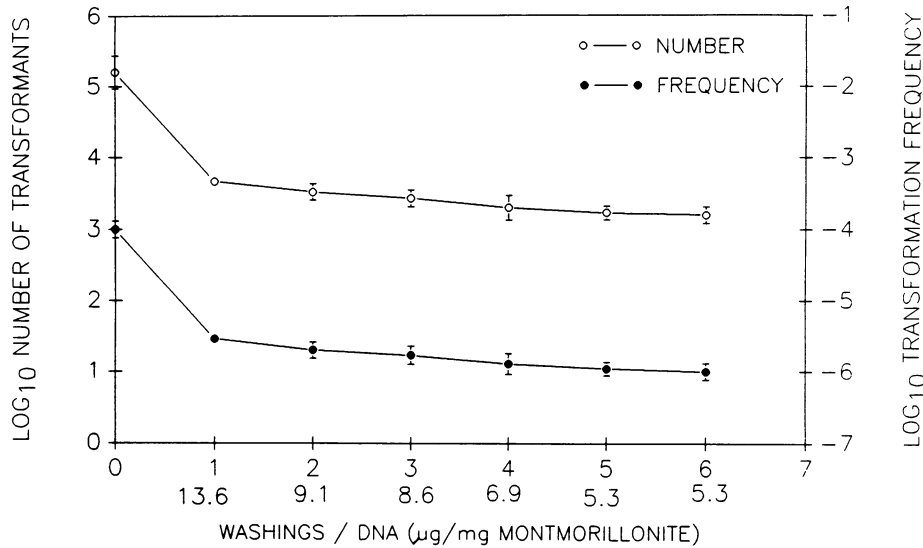


FIG. 7. Transformation of *B. subtilis* BD630 by DNA from *B. subtilis* BD1512 bound on Ca-montmorillonite after successive washes with DNA buffer (see Fig. 6A). The number of washes and the amount of DNA remaining on the clay (1-mg equivalent) after each wash are indicated. Data are expressed as means  $\pm$  SEM, which are indicated when not within the dimensions of each symbol.

1.2 µg of free DNase reduced the transformation by 30 µg of free DNA (Fig. 10B).

DISCUSSION

This study reports the equilibrium adsorption and binding of DNA from *B. subtilis* on the clay mineral montmorillonite, the ability of the bound DNA to transform competent cells, and the protection of the bound DNA against inactivation by DNase. The strains of *B. subtilis* used in this study provide a good model with which to study gene transfer by transformation in natural environments, as these strains normally lack plasmids and transducing phages (9a) and as *B. subtilis* apparently does not conjugate (13). Furthermore, many species of *Bacillus* are indigenous to soil, and transformation

of *B. subtilis* in sterile soil has been demonstrated (12-14, 22).

The adsorption of DNA on montmorillonite was relatively rapid and reached a plateau after 90 min, and ca. 80% of the DNA was adsorbed at equilibrium. The ca. 20% DNA that was not adsorbed apparently had a lower affinity for the clay, as it did not adsorb on additional montmorillonite added to the supernatant. Maximum adsorption occurred at pH 1.0, and minimum adsorption occurred at pH 9.0. DNA and RNA from various organisms have been shown to be adsorbed on clay minerals and soils (3-6, 11, 15, 29, 31). Pinck et al. (31) and Ogram et al. (29) observed that soils with the highest contents of clay minerals adsorbed the largest amounts of nucleic acids. The adsorption of DNA or RNA on montmorillonite and sand was affected by the ionic strength and

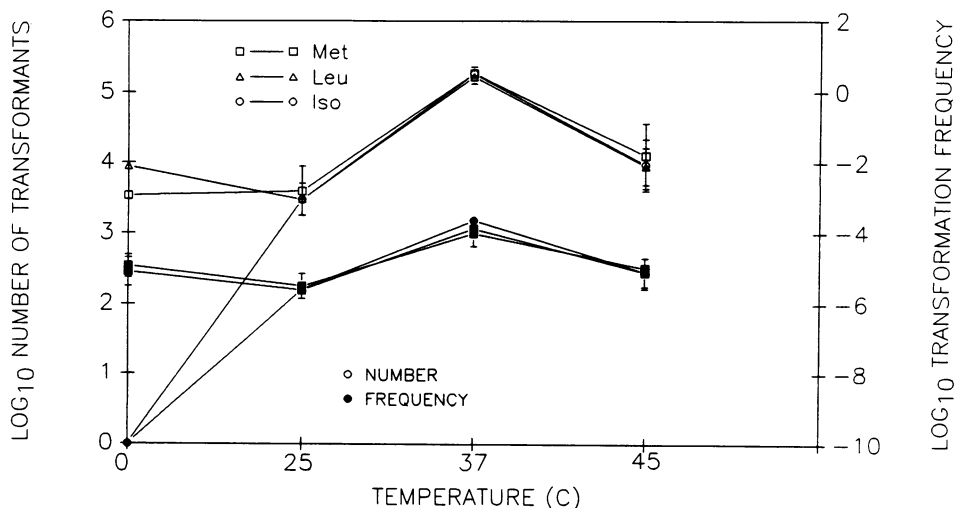


FIG. 8. Effects of temperature on transformation of *B. subtilis* BD54 by ca. 14 µg of DNA from *B. subtilis* BD170 bound on the equivalent of 1 mg of Ca-montmorillonite. Data are expressed as means  $\pm$  SEM, which are indicated when not within the dimensions of each symbol.

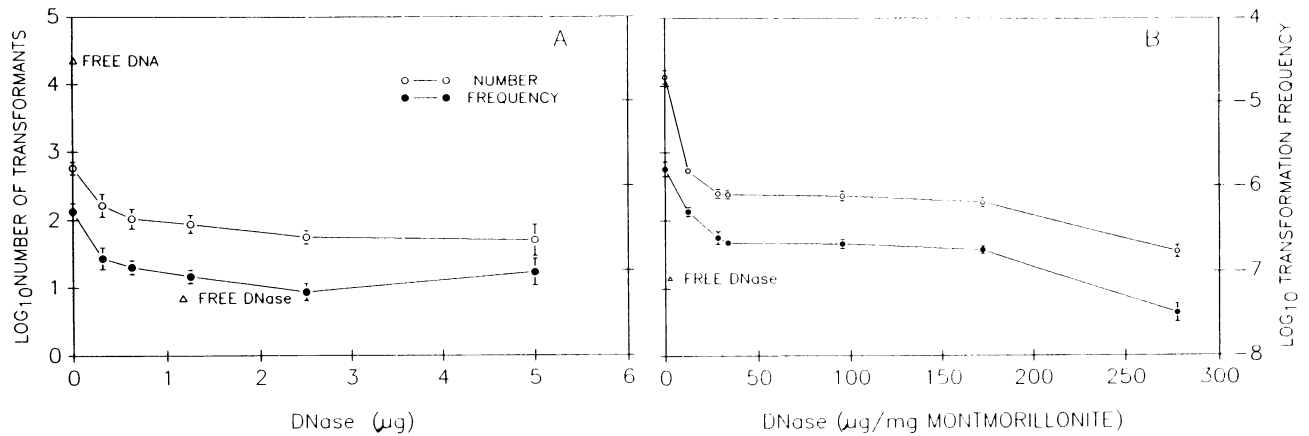


FIG. 9. Inhibition by free DNase I of the transformation of *B. subtilis* BD630 by 13.6 μg of DNA from *B. subtilis* BD1512 bound on the equivalent of 1 mg of Ca-montmorillonite (A); inhibition of transformation by 13.6 μg of free DNA by DNase I bound on the equivalent of 1 mg of Ca-montmorillonite (B). Transformation by free DNA in the absence of DNase and the inhibition of transformation by free DNA in the presence of free DNase are indicated. Data are expressed as means ± SEM.

pH of the buffers used (15, 25). Bower (5) and Wrenshall and Dyer (40) observed that montmorillonite and kaolinite adsorbed nucleic acids to the greatest extent between pH values 0.1 and 4 and that adsorption decreased from pH 4 to 7 and was almost negligible above pH 8. Greaves and Wilson (15) noted an increase in the adsorption of calf thymus DNA and yeast RNA on montmorillonite when the pH was lowered from pH 5.0 to 3.5, and there was only a small amount of adsorption above pH 5.0. Lorenz and Wackernagel (25) also showed that the pH of the solution significantly affected the binding of DNA on analytical-grade sea sand. However, they reported a plateau in adsorption at pH values 5 to 6, followed by minimum adsorption at pH 7 and a steep increase in adsorption from pH 7 to 9, which they attributed to the “charge dependency” of the process of adsorption. Unfortunately, this dependency and an explanation of their results were not discussed.

The greater adsorption of DNA on montmorillonite at the lower pH values may have resulted from protonation of the amino groups on purines and pyrimidines as the pH was decreased below the isoelectric point (pI) of DNA, i.e., ca.

pH 5.0 (21), thereby resulting in a progressive increase in the positive charge on the DNA. As the pH of the clay-DNA system was lowered to pH 5 and below, electrokinetic repulsion between the clay and the DNA was reduced, and increased coulombic attraction occurred between the positively charged groups of DNA and the pH-independent negatively charged sites on montmorillonite. Consequently, DNA was apparently adsorbed in a cationic form, similar to the adsorption of amino acids (8), peptides (9), and proteins (3, 18, 19, 27, 37) on montmorillonite.

When the clay-DNA complexes were extensively washed with ddH<sub>2</sub>O (pH 5.5) or DNA buffer (pH 7.5), about 80 and 70%, respectively, of the DNA was desorbed. The remainder of the added DNA was apparently tightly bound on the clay. The mechanisms responsible for this binding are not known, although hydrogen bonding may be involved, as it appears to be involved in the binding of proteinaceous materials on montmorillonite (37).

The adsorption and binding of DNA on montmorillonite reduced the frequency of transformation (Fig. 9A). Nevertheless, the adsorbed and bound DNA was capable of

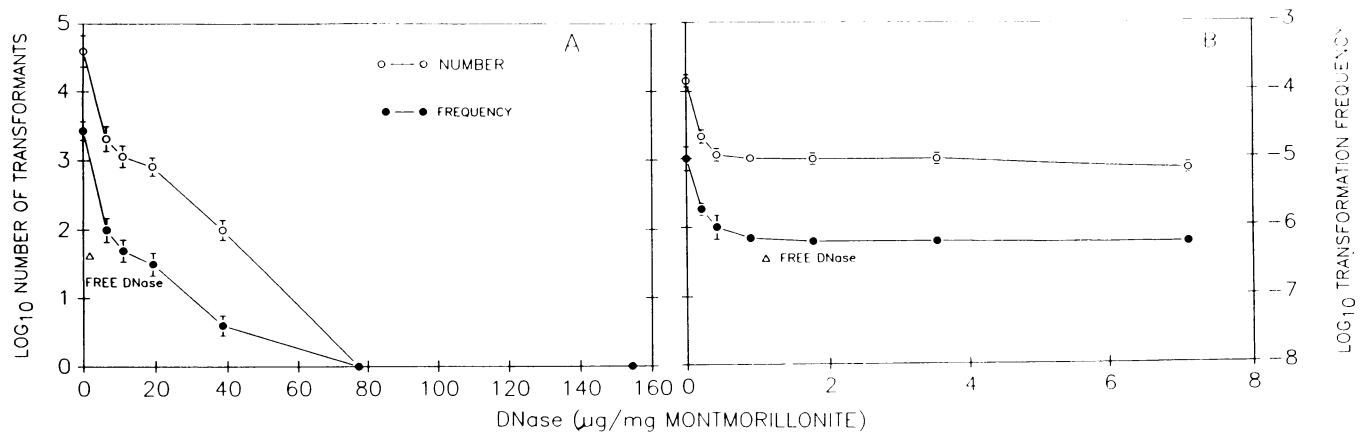


FIG. 10. Inhibition by clay-bound DNase of transformation of *B. subtilis* BD630 by clay-bound DNA (13.6 μg mg of montmorillonite<sup>-1</sup>) from *B. subtilis* BD1512, either bound on the same (A) or bound on separate (B) samples of Ca-montmorillonite. Data are expressed as means ± SEM, which are indicated when not within the dimensions of each symbol.



transforming auxotrophic and antibiotic-sensitive cells (except when adsorption occurred at very acidic conditions, e.g., pH 1.0), indicating that adsorption and desorption did not alter the integrity of the transforming DNA. The lower frequencies of transformation, conducted at pH 7.5, by clay-DNA complexes formed at pH values below 7.0 suggested that the DNA was partially (or entirely, as at pH 1.0) denatured by the high concentration of protons.

The DNA bound on montmorillonite was protected, to some extent, from the activity of DNase, even though the DNA apparently did not intercalate the clay. The concentration of DNase required to inhibit transformation by bound DNA was higher than that required to inhibit transformation by comparable amounts of free DNA, and considerably more bound than free DNase was required to inhibit transformation by the same amount of free DNA. Similarly, when DNA and DNase were bound on the same or separate samples of montmorillonite, the bound DNA was protected from the activity of DNase. The DNA appeared to be more protected when both DNA and DNase were bound on the same sample of clay, as about 10 times more DNase was required to inhibit completely the transformation by bound DNA. Lorenz and Wackernagel (25) and Aardema et al. (1) have also shown the protection of DNA bound on sand against degradation by DNase, and Greaves and Wilson reported that calf thymus DNA, yeast RNA (16), and adenine (17) adsorbed on montmorillonite were more resistant to degradation by microbial nucleases than when they were free, thereby increasing their persistence in soil. Bower (5) suggested that adsorption of nucleic acids by clay minerals might be a factor that reduced their decomposition; e.g., in the presence of montmorillonite and kaolinite, 16 and 60%, respectively, of the nucleic acids were hydrolyzed to release inorganic phosphorus by wheat germ nuclease compared with 90% hydrolysis in the absence of the clay minerals.

The results of the present studies showed that DNA from *B. subtilis* can be adsorbed and bound on montmorillonite, that the bound DNA retains the capability of transforming competent cells, and that the bound DNA is more resistant to degradation by DNase, either free or bound on the same or separate samples of clay, than is free DNA. These results support the concept of the occurrence of cryptic genes bound on particulates in the environment (38). Inasmuch as the binding of DNA on clay minerals protects the DNA from degradation but does not prevent transformation, such protected cryptic genes may persist undetected in soils but be subsequently expressed when a susceptible host comes into contact with the clay-DNA complexes and transformation occurs. These concepts are important in assessing the risks and benefits associated with the release of genetically modified organisms into the environment, and they are also important to theories of the evolution of bacteria.

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