APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1991, p. 1057–1061
0099-2240/91/041057-05$02.00/0

Vol. 57, No. 4

Adsorption of Plasmid DNA to Mineral Surfaces and Protection against DNase I

GERD ROMANOWSKI, MICHAEL G. LORENZ, AND WILFRIED WACKERNAGEL*
Genetik, Fachbereich Biologie, Universität Oldenburg, D-2900 Oldenburg, Germany

Received 30 July 1990/Accepted 2 February 1991

The adsorption of [^{3}H]thymidine-labeled plasmid DNA (pHC314; 2.4 kb) of different conformations to chemically pure sand was studied in a flowthrough microenvironment. The extent of adsorption was affected by the concentration and valency of cations, indicating a charge-dependent process. Bivalent cations (Mg^{2+}, Ca^{2+}) were 100-fold more effective than monovalent cations (Na^+, K^+, NH_4^+). Quantitative adsorption of up to 1 μg of negatively supercoiled or linearized plasmid DNA to 0.7 g of sand was observed in the presence of 5 mM $MgCl_2$ at pH 7. Under these conditions, more than 85% of DNA adsorbed within 60 s. Maximum adsorption was 4 μg of DNA to 0.7 g of sand. Supercoil molecules adsorbed slightly less than linearized or open circular plasmids. An increase of the pH from 5 to 9 decreased adsorption at 0.5 mM $MgCl_2$ about eightfold. It is concluded that adsorption of plasmid DNA to sand depends on the neutralization of negative charges on the DNA molecules and the mineral surfaces by cations. The results are discussed on the grounds of the polyelectrolyte adsorption model. Sand-adsorbed DNA was 100 times more resistant against DNase I than was DNA free in solution. The data support the idea that plasmid DNA can enter the extracellular bacterial gene pool which is located at mineral surfaces in natural bacterial habitats.

The application of genetically engineered microorganisms in agriculture and other environmental techniques involves the deliberate release of bacteria with recombinant DNA into the environment (for a review, see reference 9). Many of these bacteria will carry their specific genetic modification(s) on plasmid DNA. Considerations covering aspects of ecology, safety, and legislation necessitate the monitoring of released microorganisms and their recombinant DNAs in the natural habitats. Recombinant plasmid DNA may be released by lysis or actively excreted in certain growth phases, leading to an uncoupling of the lifetimes of bacteria and plasmid. In aquatic environments, such DNA is subject to rapid degradation (4). In contrast, adsorption to mineral components of sediments and soil renders DNA resistant to nucleolytic inactivation (1, 10, 12). If recombinant plasmids are protected against enzymatic degradation, they may contribute to an extracellular pool of genetic material in soil. This plasmid-encoded genetic information may be accessible to naturally competent gram-positive and gram-negative bacteria by transformation, as has been demonstrated for mineral-adsorbed chromosomal DNA (11, 14).

In this work, we used a flowthrough column system to examine the interaction between plasmid DNA and mineral surfaces. With this model system, we determined several parameters of plasmid adsorption and DNase I resistance. The results are discussed with respect to the persistence of extracellular DNA in natural environments.

MATERIALS AND METHODS

Isolation of labeled plasmids. Plasmid DNA was labeled with [*methyl*-^{3}H]thymidine (Amersham Buchler, Braunschweig, Federal Republic of Germany). The 2.4-kb plasmid pHC314 (3), a high-copy-number derivative of pBR322, was propagated in *Escherichia coli* DH1 (6) grown in Luria-Bertani medium with 10 μg of ampicillin ml^{-1}. At 5×10^7 cells ml^{-1}, deoxyadenosine was added to a final concentration of 250 μg ml^{-1}. At 10^8 cells ml^{-1}, [^{3}H]thymidine (37 MBq) was added to the growing culture. At a cell titer of 8×10^8 cells ml^{-1}, bacteria were harvested (10,000 × *g* for 10 min). Plasmid DNA was isolated by the alkaline extraction procedure (2, 15) and further purified by CsCl-ethidium bromide density gradient centrifugation (15). Alternatively, the Quiagen plasmid purification procedure (Diagen, Düsseldorf, Federal Republic of Germany), applied according to the recommendations of the manufacturer, was used. The preparations consisted of about 90% supercoil and open circular forms. DNA concentrations were determined by UV absorption or by a fluorimetric method (8). The radioactivity was measured by liquid scintillation counting with an Optifluor scintillator (Packard Inc., Downers Grove, Ill.) in a counter equipped with an external standard. The specific activities ranged from 25,000 to 64,000 dpm/μg of DNA.

Preparation of relaxed and linear plasmids. Blunt-end linearized plasmid DNA was obtained by restriction with *Sca*I or *Hae*III (Boehringer, Mannheim, Federal Republic of Germany). Open circular plasmid DNA was generated by random nicking with DNase I in the presence of ethidium bromide (18). After heat inactivation of the enzyme (10 min at 65°C), the different plasmid forms were electrophoretically separated. Gel slices containing the nicked plasmid were cut out, and the DNA was extracted by electroelution in a Biotrap apparatus (Schleicher & Schuell, Dassel, Federal Republic of Germany). Phenol extractions and isopropanol precipitations of linearized and nicked plasmid DNA were done by standard methods (15).

Column system and DNA adsorption. Adsorption and DNase I degradation experiments were performed in a flowthrough column system (12). Chemically pure sea sand (0.7 g; Merck, Darmstadt, Federal Republic of Germany) was used to fill in glass columns (5 by 70 mm) and equilibrated at 22°C for 20 min with the Tris-HCl (10 mM, usually pH 7.0) buffered salt solution used in each adsorption experiment. The sand bed in the column had an interstitial volume of 200 μl. Before the addition of DNA, the column was flushed twice with 1 ml of the salt solution. DNA

* Corresponding author.

(usually 100 ng) in 200 μl of the same salt solution was pipetted on top of the mineral layer and forced into it with the help of a rubber suction nozzle put on top of the glass column, and the column was incubated at room temperature (usually 10 min). Nonadsorbed DNA was removed by continuous-flow elution with the same salt solution by using high-precision peristaltic pumps (LKB 2132 Micro Perpex; LKB, Bromma, Sweden) at a flow rate of 12 ml h^{-1}. An elution volume of 3 ml was sufficient for complete removal of all nonadsorbed radioactivity. Adsorption of plasmid DNA to the column system itself was below 0.5%. The radioactivity in the eluate was measured, and the amount of adsorbed DNA was calculated by subtraction of the eluted from the applied radioactivity. Adsorbed radioactivity was also directly measured in extracts of column contents (see alkaline EDTA extraction procedure, below).

Degradation of plasmid DNA. In the degradation experiments, 200 μl of DNase I solution (in 10 mM Tris-HCl, pH 7.0, with 5 mM $MgCl_2$) was forced into the column containing sand-adsorbed DNA. The reaction was terminated by forcing 200 μl of the same buffer with 0.1% (wt/vol) sodium dodecyl sulfate into the column, immediately followed by an alkaline extraction of the column content. The DNase I degradation of DNA in solution was measured in separate experiments. DNA and DNase I were incubated in 200 μl of 10 mM Tris-HCl, pH 7.0, with 5 mM $MgCl_2$. For the determination of acid-soluble products, 0.5 ml of 12% (wt/vol) ice-cold trichloroacetic acid and 0.2 ml of bovine serum albumin were added to 0.5 ml of the extract. After 5 min of precipitation on ice, the acid-precipitable fraction was sedimented at 15,000 × *g*. Acid-soluble products were determined by liquid scintillation counting of the radioactivity in the supernatant.

Alkaline EDTA extraction procedure. The contents of a column were transferred to an Eppendorf reaction tube containing 0.8 ml of 1.25-fold-concentrated extraction buffer (the basic buffer contained 20 mM EDTA, 10 mM Na_2HPO_4, and 20 mM Tris-HCl, pH 9.0). For extraction of DNA, the tube was vigorously vortexed for 15 s. Mineral particles were allowed to settle down, and the supernatant was transferred to another reaction tube. The extraction of the sand was repeated four times with 0.7 ml of the basic extraction buffer as described above. The extracts were pooled, and the radioactivity was determined, with the interstitial volume after the last extraction step taken into consideration. The efficiency of the recovery was at least 90% of the initially adsorbed radioactivity.

RESULTS

Cation requirement for DNA adsorption. The adsorption of plasmid DNA to the surface of sand particles was rapid. Within 60 s, more than 85% of the maximum adsorption was obtained (Table 1). After 10 min, at least 98% of 100 ng of pHC314 DNA was adsorbed. Therefore, in all subsequent experiments an incubation time of 10 min was chosen. The plasmid conformation (supercoil or linear) did not significantly influence adsorption kinetics (Table 1).

The concentration and valency of cations had a profound influence on the adsorption of plasmid DNA to the sand surface. The adsorption isotherms (Fig. 1) show that with concentrations of $MgCl_2$ higher than 0.1 mM (Fig. 1A) and of NaCl higher than 50 mM (Fig. 1B), the extent of adsorption increased. Complete adsorption of 100 ng of plasmid DNA was achieved when 2 mM $MgCl_2$ or 200 mM NaCl was present. The isotherms together with the data in Table 2 demonstrate that bivalent cations like Mg^{2+} and Ca^{2+} were much more effective than monovalent cations like Na^+, K^+, and NH_4^+. It is concluded that adsorption of plasmid DNA is mediated by cations reducing the electrostatic repulsion forces between negatively charged DNA and sand surfaces. Supercoiled and linear DNA adsorbed equally well. Without addition of cations there is only a marginal binding (less than 6%).

TABLE 1. Kinetics of plasmid DNA adsorption

Incubation time (min)	DNA adsorbed (%)[a]	
	CCC	Linear
1	92.9	85.9
2.5	97.5	93.9
10	98.7	98.7
60	99.1	99.1

[a] Sand was charged with 100 ng of covalently closed circular (CCC) and linearized pHC314-DNA in 10 mM Tris-HCl, pH 7.0, with 5 mM $MgCl_2$ and incubated at 22°C for the times indicated. Nonadsorbed DNA was removed by elution with the same buffer.

Extending the elution time resulted in 82 and 63% of the

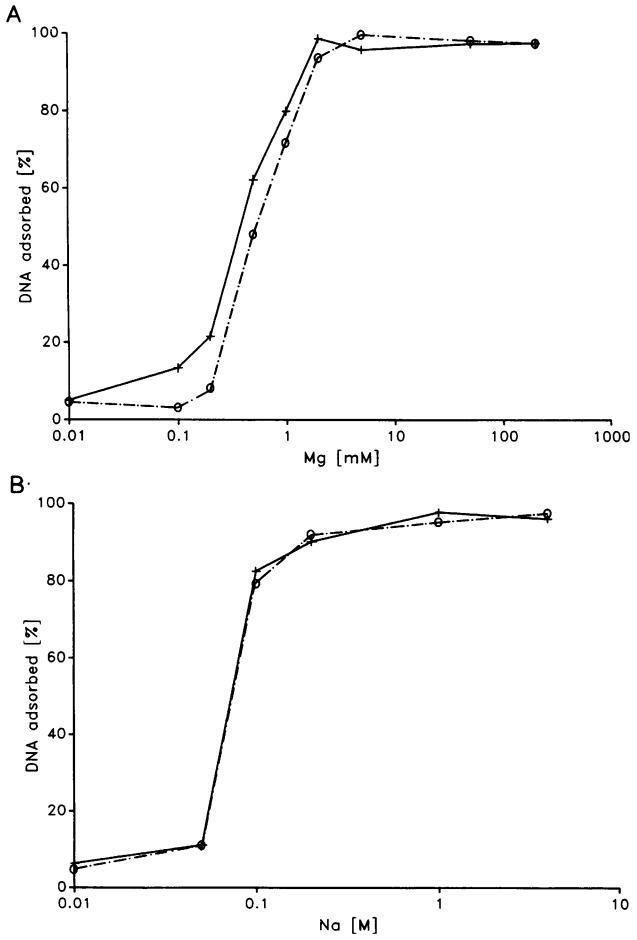

FIG. 1. Adsorption of plasmid DNA to sand in the presence of $MgCl_2$ (A) and NaCl (B). Sand (0.7 g) was incubated at 22°C with 100 ng of covalently closed circular (○) and linearized (+) pHC314 DNA in 200 μl of 10 mM Tris-HCl, pH 7.0, with various cation concentrations as indicated.

TABLE 2. Influence of cation concentration on adsorption of plasmid DNA

Cation	Concn for half-maximum adsorption (mM)[a]
Mg^{2+}	0.5
Ca^{2+}	0.4
Na^{+}	80
K^{+}	30
NH_4^{+}	180

[a] Sand (0.7 g) was incubated at 22°C for 10 min with 100 ng CCC-pHC314-DNA in 10 mM Tris-HCl, pH 7.0, with various concentrations of cations (bivalent cations, 0.1 to 1 mM; monovalent cations, 10 to 500 mM).

applied DNA still adsorbed after 1 and 9 days, respectively. This indicates a stable association between plasmid DNA and sand. If DNA adsorption is charge dependent, the pH of the solution should affect the extent of binding. Figure 2 shows that, at low Mg^{2+} concentrations, the amount of bound DNA decreased with increasing pH. Probably, repulsion forces between negatively charged DNA and sand surfaces are enhanced in the alkaline pH range. In accord with this assumption, less DNA adsorbed at a low Mg^{2+} concentration at any pH than at a higher concentration of bivalent cations (Fig. 2).

Binding capacity of sand and effect of plasmid conformation. Determination of the binding capacity of the sand columns revealed a quantitative binding of up to 1 μg of DNA at 5 mM $MgCl_2$, pH 7, regardless of DNA conformation (Fig. 3, see inset). Under these conditions, no saturation of 0.7 g of sand was achieved with plasmid DNA of any conformation. Above 1 μg of DNA, less supercoil DNA adsorbed compared with the relaxed and linearized forms (Fig. 3). Because open circular plasmid DNA adsorbed to the same extent as linear DNA, an influence of duplex DNA ends seems unlikely. This was supported by the finding (data not shown) that *Hae*III-restricted pHC314 DNA (eight blunt-end fragments) adsorbed in equal amounts as singly cut or relaxed plasmid DNA over the concentration range shown in Fig. 3.

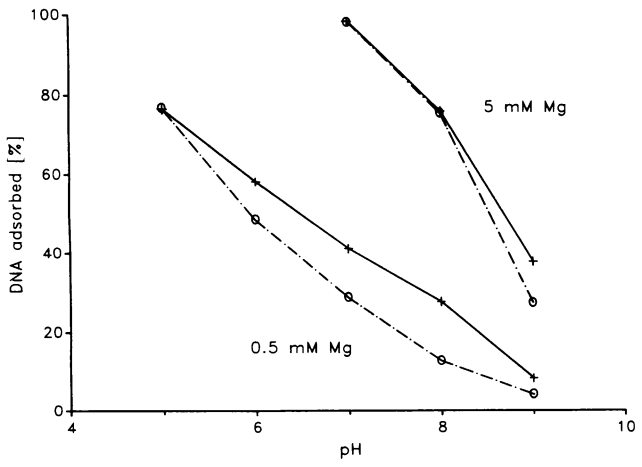

FIG. 2. Influence of pH on the adsorption of plasmid DNA to sand. Sand was incubated at 22°C with 100 ng of covalently closed circular (○) and linearized (+) pHC314 DNA in 10 mM Tris-HCl with 0.5 or 5 mM $MgCl_2$. The pHs of DNA samples and elution buffer were adjusted by HCl as indicated.

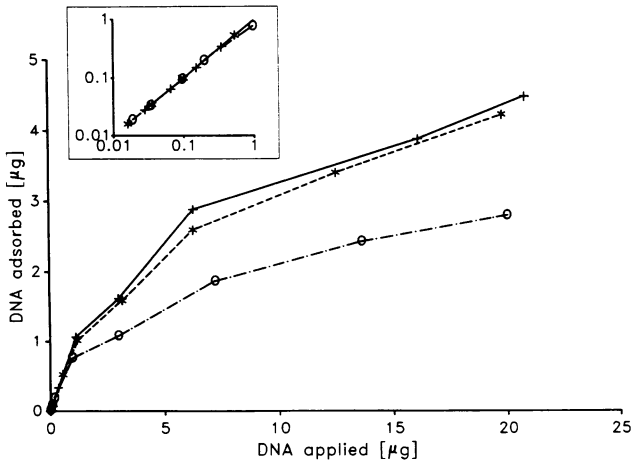

FIG. 3. Capacity of sand for plasmid DNA adsorption. Sand was incubated with various concentrations of covalently closed circular (○), open circular (*), and linearized (+) pHC314 DNA in 10 mM Tris-HCl, pH 7.0, with 5 mM $MgCl_2$. Inset: adsorption of DNA in the range 0.01 to 1 μg.

Resistance against DNase I. Sand-adsorbed plasmid DNA was treated with DNase I. In comparison experiments the same amounts of DNA and enzyme were incubated in 200-μl (corresponding to the interstitial volume of the sand bed) reaction mixtures. Free DNA in solution was degraded by DNase I starting at 50 ng ml^{-1}, whereas adsorbed DNA molecules required a nearly 100-fold-higher concentration of DNase I for acid-soluble product formation (Fig. 4A). In the range between 10 and 1,000 ng of DNase I ml^{-1}, no acid-soluble products from sand-adsorbed DNA were detectable. However, the adsorbed plasmid DNA was still subject to nucleolytic attack, as demonstrated by the gradual conversion of supercoil plasmid DNA to the relaxed and linear forms (Fig. 5). The degradation of free and sand-adsorbed linearized plasmid DNA (Fig. 4A) was comparable to that of the covalently closed DNA, indicating that the conformation of plasmid DNA did not affect its degradability. Treatment of adsorbed plasmid DNA with low concentrations of DNase I over an extended period also indicated an increased stability of the plasmid-DNA-sand complex compared with that of free DNA in solution (Fig. 4B).

DISCUSSION

Using a flowthrough column system, we examined parameters of plasmid DNA interaction with mineral surfaces of sand, which mainly consists of quartz (13). Nucleic acids in aqueous solution resemble a polymeric electrolyte, making applicable Hesselink's model of polyelectrolyte adsorption (7). This model describes the adsorption of a charged polymer to charged adsorbents involving electrostatic interaction. This kind of adsorption is not restricted to oppositely charged components. If the hindrance by the negative electrostatic potential is counteracted by the ionic strength of the solution, adsorption can occur even if polymer and surface of adsorbent are both anionic, as in the case of DNA and quartz. The process of polymer adsorption is accompanied by a transition in molecule conformation resulting in a structure of alternating adsorbed (train) and nonadsorbed (loop) segments between loosely dangling ends.

In fact, plasmid adsorption was found to be a charge-

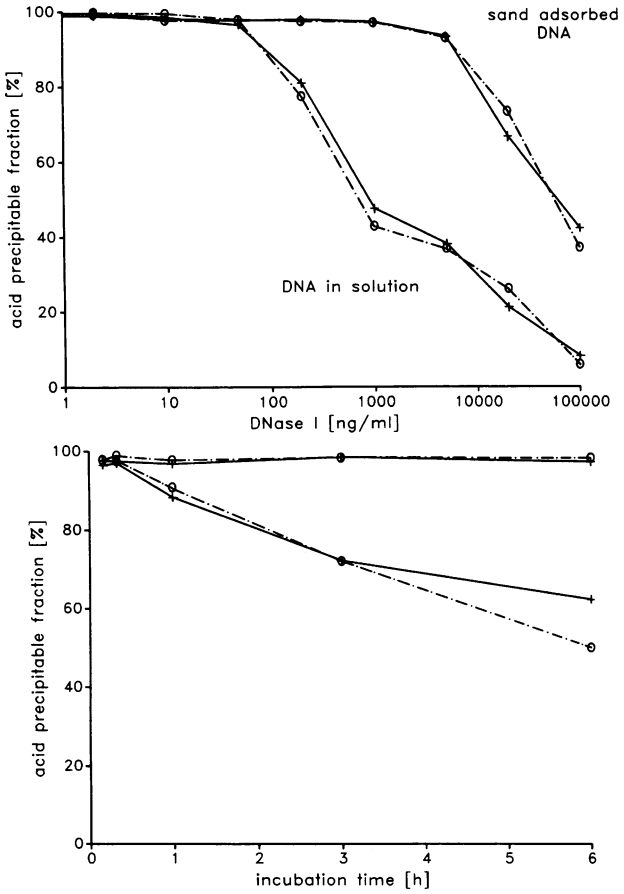

FIG. 4. Resistance of sand-adsorbed plasmid DNA against nucleolytic degradation by DNase I. (A) Sand-adsorbed covalently closed circular (○) and linearized (+) pHC314 DNA (100 ng) was incubated at 22°C for 10 min with various concentrations of DNase I in a buffered salt solution (10 mM Tris-HCl, pH 7.0, with 5 mM $MgCl_2$). (B) Kinetics of plasmid DNA degradation by DNase I. Sand-adsorbed covalently closed circular (○) and linearized (+) pHC314-DNA (100 ng) was incubated at 22°C with 50 ng of DNase I ml^{-1} in a buffered salt solution (see above). Degradation of free DNA in the same buffered solution was determined in separate experiments. For determination of acid-soluble products, see Materials and Methods.

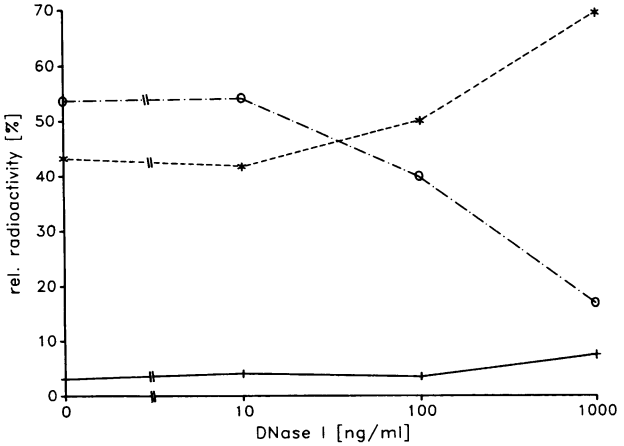

FIG. 5. Conformational transition of sand-adsorbed plasmid DNA by low concentrations of DNase I. Sand-adsorbed supercoiled DNA (500 ng) was incubated for 10 min at 22°C with 0 to 1,000 ng of DNase I ml^{-1} in 10 mM Tris-HCl, pH 7.0, with 5 mM $MgCl_2$. Column contents were extracted (see Materials and Methods), and the DNA was recovered by isopropanol precipitation and separated by gel electrophoresis. Relative radioactivity is expressed as the ratio of supercoiled (○), relaxed (*), and linearized (+) plasmid DNA determined by liquid scintillation counting of the acid-dissolved (12% trichloroacetic acid, 1 h, 95°C) gel slices containing the appropriate bands.

dependent process influenced by cation concentration and pH of the solution. According to Hesselink (7), polymer adsorption is affected by (i) surface charge density and surface area of the adsorbent, (ii) degree of polymerization and dissociation of the polyelectrolyte, (iii) net adsorption energy, and (iv) ionic strength of the solution. Since the surface charge density of the DNA molecule is a constant for a given pH, adsorption should be a function of monovalent cation concentration. Accordingly, increasing Na^+ concentrations caused an increasing extent of plasmid adsorption (Fig. 2). Above 0.1 M NaCl, salting-out effects may additionally enhance polymer adsorption (7).

The effect of bi- and multivalent cations is not fully covered by the polymer adsorption theory, because these ions exert a variety of effects mainly on the surface charge density of the adsorbent and on the dissociation of the polymer (7). Bivalent cations mediated plasmid adsorption very efficiently. The Mg^{2+} concentration (2 mM) promoting quantitative adsorption of 100 ng of pHC314 DNA was two orders of magnitude lower than the Na^+ concentration (200 mM) giving the same amount of adsorbed DNA (Fig. 1A and 1B). With $MgCl_2$, cation bridges were probably involved in plasmid adsorption, as was suggested for adsorption of chromosomal DNA (12). It is interesting that the low bivalent cation concentrations required for adsorption (Fig. 1A, Table 2) were in the range present in natural environments, such as in groundwater (unpublished results). In fact, adsorption of linearized and supercoiled plasmid DNA to mineral particles directly recovered from natural bacterial habitats has recently been observed (16). Because of the charge-dependent mode of DNA binding, the pH of the solution influenced the extent of adsorption. Towards a high pH, adsorption decreased because of increasing repulsion forces (Fig. 2). Yet, significant amounts of plasmid DNA adsorbed over a wide pH range typical of bacterial soil environments. In addition, repulsion forces were counteracted by increasing concentrations of bivalent cations (Fig. 2). The somewhat lower adsorption of supercoiled plasmid DNA is a phenomenon observed only under restrictive conditions: when one of the factors required for adsorption, e.g., bivalent cations, is limited, differences in the degree of adsorption between linearized and covalently closed molecules are detectable (Fig. 1A and 2). The reason for this differential adsorption is probably the lower availability of surface charges and the limited flexibility of supercoiled DNA which hampers the molecular transitions required for the train-loop formation of polyelectrolyte adsorption (7). These structural properties of the molecule may likewise be the reason for the lower capacity of sand for covalently closed plasmid DNA (Fig. 3).

Adsorption of plasmid DNA was a rapid process regardless of conformation. Compared with the times found in earlier experiments (12) with chromosomal DNA fragments (average length about 18 kb), the adsorption time of the

approximately sevenfold-smaller plasmid molecules was significantly shorter. Probably a lower number of charged groups participated in the train-loop formation, thus shortening the time-critical process of train-loop arrangement (7). On the other hand, the size of the plasmid was sufficient for a high-affinity type of adsorption, as indicated by the stability of the DNA-sand complex in long-term elution experiments.

Association of plasmids with sand conferred protection on the DNA molecule against nucleolytic degradation by DNase I (Fig. 4). Treatment of sand-adsorbed covalently closed circular plasmid DNA with low concentrations of the enzyme insufficient to generate acid-soluble products resulted in partial relaxation or linearization of the molecules (Fig. 5). However, such changes of conformation do not necessarily eliminate the biological activity of plasmids, since natural transformation by open circular and linearized plasmids has been observed (5).

The ecological relevance of the data obtained in this experimental model system consists of two points: (i) adsorption of plasmid molecules to mineral surfaces is mediated by cation concentrations occurring in natural bacterial habitats and is rather independent of plasmid configuration, and (ii) adsorption to mineral surfaces increases the resistance of plasmid DNA to nucleolytic degradation by DNase I. Solid-liquid interfaces at such mineral components of soils are places of increased concentrations of other biologically important molecules and living bacteria (17). Therefore, the adsorption of plasmid DNA with a gain in protection against nucleases increases the chance for plasmid-encoded genetic information to participate in the horizontal gene flow by genetic transformation in soil.

ACKNOWLEDGMENT

This work was supported by the Bundesminister für Forschung und Technologie.

REFERENCES

1. **Aardema, B. W., M. G. Lorenz, and W. E. Krumbein.** 1983. Protection of sediment-adsorbed transforming DNA against enzymatic inactivation. Appl. Environ. Microbiol. **46**:417–420.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline DNA extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Boros, I., G. Pósfai, and P. Venetianer.** 1984. High-copy-number derivatives of the plasmid cloning vector pBR322. Gene **30**:257–260.
4. **DeFlaun, M. F., and J. H. Paul.** 1989. Detection of exogenous gene sequences in dissolved DNA from aquatic environments. Microb. Ecol. **18**:21–28.
5. **Doran, J. L., W. H. Bingle, K. L. Roy, K. Hiratsuka, and W. J. Page.** 1987. Plasmid transformation of *Azotobacter vinelandii* OP. J. Gen. Microbiol. **133**:2059–2072.
6. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
7. **Hesselink, F. T.** 1983. Adsorption of polyelectrolytes from dilute solution, p. 377–412. *In* G. D. Parfitt and C. H. Rochester (ed.), Adsorption from solution at the solid/liquid interface. Academic Press, Inc. (London), Ltd., London.
8. **Labarca, C., and K. Paigen.** 1980. A simple, rapid and sensitive DNA assay procedure. Anal. Biochem. **102**:344–352.
9. **Lindow, S. E., N. J. Panopoulos, and B. L. MacFarland.** 1989. Genetic engineering of bacteria from managed and natural habitats. Science **244**:1300–1307.
10. **Lorenz, M. G., B. W. Aardema, and W. E. Krumbein.** 1981. Interaction of marine sediment with DNA and DNA availability to nucleases. Mar. Biol. **64**:225–230.
11. **Lorenz, M. G., B. W. Aardema, and W. Wackernagel.** 1988. Highly efficient genetic transformation of *Bacillus subtilis* attached to sand grains. J. Gen. Microbiol. **134**:107–112.
12. **Lorenz, M. G., and W. Wackernagel.** 1987. Adsorption of DNA to sand and variable degradation rates of adsorbed DNA. Appl. Environ. Microbiol. **53**:2948–2952.
13. **Lorenz, M. G., and W. Wackernagel.** 1988. Impact of mineral surfaces on gene transfer by transformation in natural bacterial environments, p. 110–119. *In* W. Klingmüller (ed.), Risk assessment for deliberate releases. Springer Verlag, Berlin.
14. **Lorenz, M. G., and W. Wackernagel.** 1990. Natural genetic transformation of *Pseudomonas stutzeri* by sand-adsorbed DNA. Arch. Microbiol., **154**:380–385.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Romanowski, G., et al.** Unpublished data.
17. **Stotzky, G.** 1986. Influence of soil mineral colloids on metabolic processes, growth, adhesion, and ecology of microbes and viruses, p. 305–428. *In* Interactions of soil minerals with natural organics and microbes. Specific publication no. 17. Soil Science Society of America, Madison, Wis.
18. **Wallace, R. B., M. Schold, M. J. Johnson, P. Dembek, and K. Itakura.** 1981. Oligonucleotide directed mutagenesis of the human β-globin gene: a general method for producing specific point mutations in cloned DNA. Nucleic Acids Res. **9**:3647–3656.