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Protection of Sediment-Adsorbed Transforming DNA Against Enzymatic Inactivation

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The action of DNase I on transforming DNA, both adsorbed to marine sediment and in solution, was investigated. DNase I reduced the transformation frequencies of free DNA more than of adsorbed DNA. Changes in salt concentration or pH did not have a significant influence on the DNA-sediment complex. Soil components other than organic materials and clay minerals can bind DNA and retard its enzymatic degradation.

Recent studies on gene exchange show that transformation (4) and conjugation (14) can occur in soils. Such genetic processes may play a role in the adaptation of organisms to changes in their habitats. DNA can be released from bacterial populations (7, 10, 13). This raises the question whether nucleic acids survive as naked molecules in the natural environment and whether they are subject to transformation and recombination. Graham and Istock (4) stated that, in soil, DNase cannot stop the transformation of *Bacillus subtilis* by excreted homologous DNA. Studies on the extraction of DNA from marine sediments showed that a considerable amount of DNA is tightly bound to the inorganic fraction (8). Sediment-adsorbed DNA was shown to be more resistant to enzymatic digestion than was free DNA.

In the present study, we examined whether transforming DNA can be protected against enzymatic inactivation by adsorption to sediment particles. We found that DNase I inactivated free DNA more than adsorbed DNA. The DNA-sediment complex remained stable at NaCl concentrations from 19.5 mM to 1.95 M and at pH values from 5 to 9.

MATERIALS AND METHODS

Sediment. Analytical grade seasand was used. It consisted chiefly of quartz (more than 95%), with traces of feldspars and heavy minerals. The sand did not contain any clay minerals or organic materials. The sand did not affect the activity of DNase I.

Preparation and determination of DNA. DNA was extracted from *B. subtilis* (prototrophic, Marburg strain, DSM no. 401) by the method of Marmur (9). The DNA (600 μg/ml) was stored in saline citrate at 4°C, with the addition of a few drops of chloroform. DNA concentration was determined by spectrophotometrically measuring the absorbance at 258 nm. An extinction of 1.0 corresponded to 50 μg/ml.

DNase I reactions. Glass columns (5 by 140 mm) were loaded with 0.7 g of sediment and autoclaved at 121°C for 15 min. After cooling, they were washed with 2 to 3 ml of 19.5 mM NaCl (pH 6.5). The DNA stock solution was diluted to 76 to 78 μg/ml with 19.5 mM NaCl. The columns were charged with this solution. A 0.4-ml sample of the DNA solution was layered on top of the sediment bed and passed through at a rate of 0.2 ml/min until the meniscus came in contact with the sediment surface. The columns were then incubated at 21°C for 2 h. Afterwards, the columns were charged with DNase I the same way they were charged beforehand with DNA. All tests were run in duplicate or triplicate. In the first series of experiments, the influence of varying concentrations of DNase I on sediment-adsorbed DNA was investigated. DNase I concentration ranged from 10 to 8,000 ng/ml (in 5 mM $MgCl_2$). The incubation period was 10 min. In the second series of experiments, the time course of the reaction of 10 ng of DNase I per ml (in 5 mM $MgCl_2$) with sediment-adsorbed DNA was studied. In both cases, the incubation temperature was 21°C. The reactions between DNase I and DNA were terminated by eluting the columns with 2 ml of 10 mM EDTA in 19.5 mM NaCl (pH 7.7). The EDTA was subsequently eluted with 3 ml of 19.5 mM NaCl. In control experiments, columns incubated with DNA not treated with DNase I were eluted with 5 ml of 19.5 mM NaCl.

In experiments on free DNA, DNase I was added as a 100-fold-concentrated solution (in 0.5 M $MgCl_2$). The reactions were carried out in 1.5-ml Eppendorf reaction tubes. The DNA stock solution was diluted to 7.6 to 7.8 μg/ml with 19.5 mM NaCl. The tubes were then incubated with DNase I at 21°C. All tests were done in duplicate or triplicate. The reactions between DNA and various concentrations of DNase I, ranging from 1.6 to 800 ng/ml, were studied. A 2-μl sample of the concentrated enzyme solution was added to 200 μl of DNA solution and incubated for 12 min. This corresponded to the actual reaction period in the sediment experiments, in which loading and eluting the enzyme extended the reaction time by approximately 2 min. The interstitial volume of the sediment bed was 0.15 ml, and the flow rate was 0.2 ml/min. The total volume of the enzyme solution applied was 0.4 ml. Thus, 45 s (partial reaction) passed before the enzyme solution completely filled the interstitial volume of the sediment bed; another 75 s passed (full reaction) before the

meniscus fell to the level of the sediment surface, and 45 s more passed (partial reaction) before all of the enzyme solution had passed through the sediment bed. In experiments in which the time course of the reaction between free DNA and DNase I was investigated, 0.2 ml of DNA was incubated with 2 μl of DNase I (1.0 μg/ml). The enzyme reactions were terminated by adding 0.2 ml of EDTA (20 mM, pH 7.7) and placing the reaction tubes in an ice bath. The DNase I was denatured by adding 0.4 ml of chloroform-isoamyl alcohol (25:1) and swirling gently for 30 s. The mixture was then centrifuged in an Eppendorf centrifuge at 10,000 rpm for 2.5 min. The supernatant was carefully decanted and placed on ice.

Transformation. *B. subtilis* 1G20 (trpC2, kindly provided by G. Venema, University of Groningen, Groningen, The Netherlands) was made competent by the method of Bron and Venema (2). The competent cultures were suspended in 10% glycerol and frozen in liquid nitrogen. After thawing, 1.35 ml of culture was mixed with 0.7 g of sediment, and 0.8 ml of culture was mixed with 0.2 ml of free DNA solution. The maximum end concentration of undigested DNA was between 0.76 and 0.78 μg/ml in both cases. During the transformation, the cultures were shaken in a water bath at 34°C. After 45 min, DNase I was added (end concentration, 100 μg/ml) to stop the further uptake of DNA by the cells. The cultures were then incubated for another 5 min at 34°C. The cultures were then diluted and spread on minimal agar. This medium consisted of minimal salts (12) and 0.5% glucose solidified with 1.5% agar; 1 ml of 0.6% iron(III) ammonium citrate and 1 ml of trace element solution A5 (11) without manganese were added per liter of medium. To determine the viable cell count, tryptophan was added (final concentration, 14 μg/ml). The plates were incubated at 37°C for 40 h. The colonies were then counted.

DNA extraction from sediment. Sediment columns were incubated as mentioned above with 0.2 ml of DNA solution (76 to 78 μg/ml). All procedures were done in duplicate. In the first experiment, the columns were eluted with a NaCl solution of known concentration, and in the second experiment, they were eluted with a 1.95 M NaCl solution of known pH. The concentration of the NaCl solution in the first experiment ranged from 19.5 mM to 1.95 M (pH 7.8). The pH of the 1.95 M NaCl solution in the second experiment ranged from 5 to 9. The pH of all solutions was adjusted with HCl or NaOH. The elution of the DNA from the sediment columns was monitored by measuring the absorbance at 254 nm in a flow-through cuvette (Isco, optical unit type 6). After the sediments were eluted with 5 ml of the test solution, they were extracted with hot perchloric acid and tested for residual DNA by the method of Lorenz et al. (8).

Chemicals. DNase I (grade II) was purchased from Boehringer Mannheim; agar and Casamino Acids were from Difco Laboratories, Detroit, Mich.; and isoamyl alcohol was from J. T. Baker Chemicals B.V., Deventer, Holland. All other chemicals, including analytical grade seasand, were obtained from E. Merck AG, Darmstadt, Germany.

RESULTS

Both free and sediment-adsorbed DNA were incubated with various concentrations of DNase I (Fig. 1). At all concentrations of DNase I, the transformation ability of free DNA was reduced more than that of sediment-bound DNA. In the experiments with free DNA, no transformants were detected when the DNase I concentration was 200 ng/ml. In the experiments with sediment-bound DNA, transformants were still detected when the DNase I concentration was 8,000 ng/ml. In the latter case, the transformation frequency was 0.2% of the frequency observed when sediment-bound DNA was not treated with DNase I.

When the time course of the reaction was examined, it was found that DNase I reduced the transformation ability of free DNA more than that of sediment-adsorbed DNA (Fig. 2). The enzyme brought about a 100% inactivation of the free transforming DNA within 2 h, and of the sediment-adsorbed DNA in more than 6 h.

In the absence of DNase I, the transformation frequency obtained with DNA in sediments was about 60% of that with free DNA (see the legends to Fig. 1 and 2).

To determine the amount of DNA remaining in the sediment after elution, the sediments were extracted with acid. We found that 1.8 μg of DNA per g of sediment resisted elution with 5 ml of 19.5 mM NaCl (pH 7.8). The major portion of the unbound DNA was eluted from the sediment with the first milliliter of NaCl solution passed

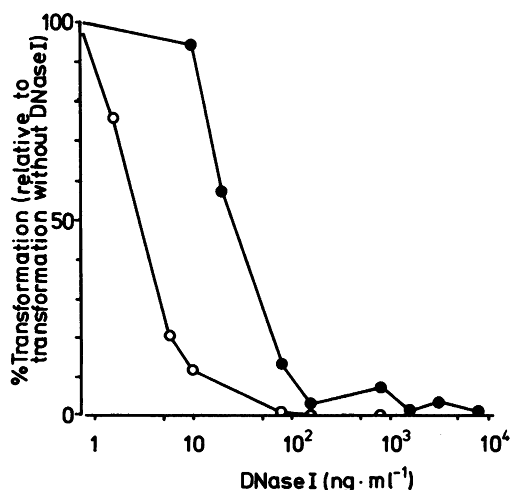

FIG. 1. Influence of DNase I concentration on dissolved (○) and sediment-adsorbed (●) transforming DNA (wild type) during 12 and 10 min of incubation, respectively, at 21°C. After enzyme inactivation, *B. subtilis* 1G20 (trpC2) was incubated with both preparations. The 100% level refers to a transformation frequency of 1.3×10^{-4} to 2.2×10^{-4} in the sediment assay, and 2.3×10^{-4} to 3.9×10^{-4} in the experiments with free DNA.

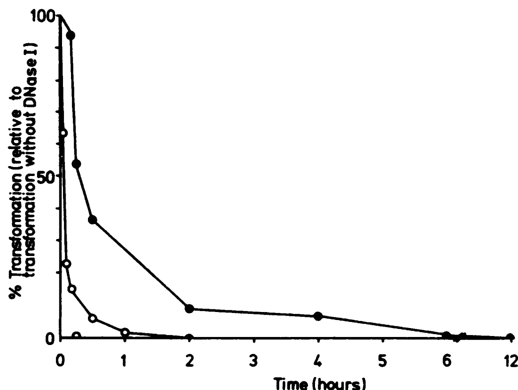

FIG. 2. Inactivation kinetics of transforming DNA (wild type) by DNase I (10 ng/ml). Free (○) and adsorbed (●) DNAs were incubated with DNase I before transformation of *B. subtilis* 1G20 (trpC2). See Fig. 1 for transformation frequencies.

through. With further elution, the absorbance of the effluent fell to zero.

The influence of pH and NaCl concentration on sediment-bound DNA was investigated. After eluting with 5 ml of concentrated NaCl solution, more DNA was bound than with less concentrated NaCl solutions. We found that 2.8 μg of DNA per g of sediment was bound after washing with 1.95 M NaCl (pH 7.8), and 1.8 μg of DNA per g of sediment was bound after washing with 19.5 mM NaCl (pH 7.8).

More DNA remained in the sediment after eluting with solutions of low pH than with solutions of high pH; 4.0 μg of DNA per g of sediment was bound after washing with a solution of pH 5.1 (1.95 M NaCl), and 3.0 μg of DNA per g of sediment was bound after washing with a solution of pH 8.9 (1.95 M NaCl).

DISCUSSION

DNA, or at least a gene, of *B. subtilis* resisted attack by DNase I more when bound to sediment than when free in solution. This agrees well with previous results. We reported (8) that calf thymus DNA bound to marine sediment and resisted attack by a combination of DNases. The enzyme concentrations were higher than in the present experiments, but the detection method was less sensitive. Our present results show that transforming DNA resisted enzymatic attack longer when bound to sediment than when free in solution. However, prolonged protection of the sediment-bound DNA against enzymatic degradation was observed only at low concentrations of DNase I (see Fig. 1 and 2). The sand we used had no interlayer surfaces characteristic of clay minerals which could serve as binding sites for DNA (5). In other long-term studies, part of the DNA resisted enzymatic degradation by binding to the interlayer surfaces of the clay mineral (6). We used sand in our experiments because it did not affect the activity of the DNase I. The mechanism by which the reaction between DNA in sediments and DNase I was inhibited remains unclear. The results shown in Fig. 1 do not provide a conclusive explanation.

Other experiments described here were concerned with the total amount of DNA adsorbed to the sediment and with the influence of pH and NaCl concentration on the DNA-sediment complex. The capacity of the sediment we used to bind DNA was low compared with that of clay minerals. Clay minerals are known to be strong adsorbers of nucleic acids (see references 1 and 3). Greaves and Wilson (5) determined that 1 mg of the clay mineral montmorillonite binds about 250 μg of DNA. We found that substantially less DNA (1.8 μg per g of sediment) remained bound to the sediment after elution. The elution of DNA from the sediment was monitored at 254 nm in a flow-through cuvette. Afterwards, the presence of DNA was demonstrated by chemical and biological methods. Therefore, the residual DNA appears to have been adsorbed to the sediment grains. This agrees with our earlier results (8).

Salt concentration and pH, at least in the range examined in these experiments, did not significantly affect the stability of the DNA-sediment complex. This agrees with the results of Greaves and Wilson (5), who examined the adsorption patterns of DNA to clay minerals.

Graham and Istock (4) showed that, in soil, transformation of *B. subtilis* cannot be interrupted by adding DNase. They assumed that adsorption to organic material protected transforming DNA against enzymatic degradation. We showed that inorganic components of soils, such as quartz, feldspars, and heavy minerals, bound and stabilized transforming DNA. On the basis of these experiments, we could draw no conclusions as to whether *B. subtilis* was transformed in the sediment by DNA released from sediment or by sediment-adsorbed DNA. It should be noted that transformation by DNA in sediment was less efficient than transformation by free DNA (see figure legends). Graham and Istock (4) stated that bound DNA is not available for transformation. Perhaps, in our experiments, only DNA desorbed from sediment was taken up by competent cells. This problem, however, is still to be solved. Whether binding of transforming DNA to sediment and the resulting protection against enzymatic degradation play a role in transformation processes in soils remains to be answered.

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