APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1988, p. 2908–2915
0099-2240/88/122908-08$02.00/0

Vol. 54, No. 12

Recovery of DNA from Soils and Sediments

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Received 9 May 1988/Accepted 2 September 1988

Experiments were performed to evaluate the effectiveness of two different methodological approaches for recovering DNA from soil and sediment bacterial communities: cell extraction followed by lysis and DNA recovery (cell extraction method) versus direct cell lysis and alkaline extraction to recover DNA (direct lysis method). Efficiency of DNA recovery by each method was determined by spectrophotometric absorbance and using a tritiated thymidine tracer. With both procedures, the use of polyvinylpolypyrrolidone was important for the removal of humic compounds to improve the purity of the recovered DNA; without extensive purification, various restriction enzymes failed to cut added target DNA. Milligram quantities of high-purity DNA were recovered from 100-g samples of both soils and sediments by the direct lysis method, which was a >1-order-of-magnitude-higher yield than by the cell extraction method. The ratio of labeled thymidine to total DNA, however, was higher in the DNA recovered by the cell extraction method than by the direct lysis method, suggesting that the DNA recovered by the cell extraction method came primarily from active bacterial cells, whereas that recovered by the direct lysis method may have contained DNA from other sources.

Several newly emerging methods for monitoring specific microbial genotypes in environmental samples and for analyzing microbial community structure at the genetic level, which do not require the culturing of the microorganisms from the samples, depend upon the efficient recovery of DNA as an essential part of the procedures (1, 7; T. Barkay and G. Sayler, *in* American Society of Testing and Materials, ed., *Biotechnology Risk Assessment*, in press; W. E. Holben and J. M. Tiedje, Ecology, in press; R. K. Jain, R. S. Burlage, and G. S. Sayler, Crit. Rev. Biotechnol., in press); these methods are advantageous because they avoid problems associated with enumeration procedures that depend upon culturing of organisms from environmental samples (16). Two quite different approaches have been proposed for the recovery of DNA from environmental samples.

The first approach, which we will refer to as the cell extraction method, was initially reported by Goksøyr and colleagues (6, 19) for recovering bacterial DNA from soil; the methodological approach involves the separation of bacterial cells from the soil particles by differential centrifugation followed by lysis of the recovered cells, recovery of the DNA, and purification of the DNA by hydroxyapatite column chromatography. Holben et al. (10) recently used this basic approach, but modified the procedure by using polyvinylpolypyrrolidone (PVPP) to remove soil organic matter from cell preparations to simplify purification of recovered DNA; by using an extensive lysis procedure to ensure breakage of soil bacterial cells, such as *Bradyrhizobium* spp., that produce surface polysaccharide layers; and by replacing the hydroxyapatite column chromatography with repetitive cesium chloride density gradient ultracentrifugation purification steps. They reported recovery of about 33% of the bacterial cells from soil and that the recovered DNA was of sufficient purity to perform gene probe detection of specific genotypes in the soil bacterial community and to perform restriction enzyme and Southern blot analyses of the recovered DNA.

In contrast to this method, which involves the initial separation of microorganisms from the environmental matrix prior to lytic release of DNA, a different approach, which we will refer to as the direct lysis method, has been developed by Ogram et al. (13) for recovering DNA from sediments. This second method for DNA recovery from environmental samples involves release of DNA from the cells by physical disruption, without separating the cells from the environmental matrix, that is, direct lysis followed by alkaline extraction of the DNA in buffer, and purification of the extracted DNA by ethanol precipitation, cesium chloride density gradient centrifugation, and hydroxyapatite column chromatography. The estimated recovery efficiency for DNA by the direct lysis method was reported to be as high as 90% from sediments containing 19 to 44% clay and 3 to 16.5% organic carbon. The recovered DNA was also reported to be of high purity.

In the current investigation, we compared the two methodological approaches for recovering DNA from environmental samples, including examination of modifications of individual steps within each procedure, to determine the relative efficiencies of each for recovering DNA from soils and sediments. We were particularly concerned with the effects of clay and humic content on the efficiency of DNA recovery, with the quality of the recovered DNA, and with the potential differences in the applicability of the two approaches for soils and sediments.

MATERIALS AND METHODS

Sediment and soil. Surficial sediments were collected from the Ohio River at Louisville, Ky., with an Ekman dredge. Sediment samples were pooled, mixed, and maintained at 5°C. Dry weight analysis was performed by drying sediments to a constant weight at 105°C. Organic content of dried sediments was determined gravimetrically after combustion at 550°C for 1 h. Physical composition (i.e., percent clay, percent silt, and percent sand) was determined as described in Black (4). Soil was collected, characterized, and shipped from Corvallis, Ore., by the U.S. Environmental Protection Agency. Physical composition was determined as for sedi-

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TABLE 1. Characteristics of sediment and soil

Sample	Organic content (%)	Particle distribution			Viable heterotrophs (CFU/g, dry wt)	Total cells (no./g, dry wt)
		Sand (%)	Silt (%)	Clay (%)		
Soil	7.8	58	20	22	2.0×10^7	3.5×10^9
Sediment	6.6	28	44	28	1.3×10^6	6.6×10^9

ments. Sediment and soil characteristics are shown in Table 1.

Enumeration of bacteria. Total bacterial cell numbers were determined by the acridine orange direct count procedure as described by Hobbie et al. (9), using an Olympus Vanox epifluorescence microscope. Enumeration of viable aerobic heterotrophs was performed by standard plate count procedures on 10% strength tryptic soy agar (Difco Laboratories) supplemented with 1.5% Bacto-Agar (Difco). Plates were incubated at 25°C, and CFU were determined at 48 h.

Thymidine labeling. Bacterial cells in the soil and sediment samples were radioisotopically labeled in situ by a modification of the thymidine incorporation method of Thomas et al. (17). Water was added to soil samples (650 g, dry weight) to achieve 100% saturation so that thymidine could be mixed through the soil. Sediment samples (750 g, wet weight), which were fully saturated with water, were used without further hydration. A 1-μCi portion of tritiated thymidine (1 mCi/ml; Dupont, NEN Research Products) per g (dry weight) of soil was added to each sample in 10 ml of sterile sodium phosphate buffer. The samples were then shaken vigorously on a wrist-action shaker at 25°C for 8 h to permit incorporation of the [^{3}H]thymidine into bacterial DNA. Because both DNA extraction procedures used in this study incorporate several washing steps, as well as cesium chloride-ethidium bromide density gradient centrifugation and dialysis of recovered DNA, no extensive washes were performed to remove unincorporated thymidine. Each sample was split into six 100-g (dry weight) subsamples. Soil and sediment samples to be subjected to the direct lysis procedure were frozen for 24 h at −10°C until further processing. Samples to be subjected to the cell extraction procedure were processed immediately.

Recovery of DNA. (i) Cell extraction method. The bacterial fractions of the sediment and soil samples were separated from the inorganic and humic fractions by using a modification of a method previously developed for recovering bacteria from soils for genetic analyses (6, 18, 19). A 100-g (dry wt) amount of sediment or soil was suspended in 300 ml of 0.1 M sodium phosphate buffer (pH 4.5), and 20 g of PVPP was added. The PVPP was prepared by the procedure described by Holben et al. (10). The samples were homogenized with a Waring blender. Samples were blended at medium speed for three 1-min periods, with 1 min of cooling on ice between each blending cycle. A 2-ml portion of 20% sodium dodecyl sulfate (SDS) was added to each sample, and the samples were blended for an additional 5 s. The samples were placed on ice, and the foaming was allowed to settle for 5 min. The samples were then transferred to 250-ml centrifuge bottles. The bottles were shaken by hand for 1 min and centrifuged in a GSA rotor (Sorvall RS-5 centrifuge) for 10 min at 1,000 × *g* and 10°C. The supernatants from each individual replicate were pooled in an Erlenmeyer flask and maintained on ice until further centrifugation. Subsamples were collected from each fraction for cell recovery determination by the acridine orange direct count method.

The sediment or soil pellets were washed back into the blender with 300 ml of 0.1 M sodium phosphate buffer (pH 4.5) and further blended and centrifuged as described above, but without further addition of SDS. This process was repeated for a total of three cycles. The supernatants were combined with the earlier pooled supernatants and maintained on ice. The combined supernatants were centrifuged for 30 min at 10,000 × *g* and 10°C to collect the bacterial cells. The pelleted material containing the combined bacterial cell fraction was suspended in 200 ml of 0.1% sodium hexametaphosphate–0.1% sodium pyrophosphate at 5°C. The samples were shaken by hand for 1 min and then centrifuged for 30 min at 10,000 × *g* and 10°C. The supernatant was discarded and the procedure was repeated. These washings decreased the amount of particulate organic (nonbacterial) material in the cell pellet.

As a final washing procedure, the cell pellet was suspended in 150 ml of Chrombach buffer (0.33 M Tris hydrochloride, 0.001 M EDTA, pH 8.0) and centrifuged as described for previous washings. This final wash appeared to remove a large portion of the remaining humic material in the cell pellet. The final pellet was transferred to a 50-ml centrifuge tube by washing with Chrombach buffer and adjusted to a final volume of 25 ml.

The cell pellets were mixed vigorously on a Vortex mixer to completely suspend any clumps of pelleted material. Lysozyme (Sigma Chemical Co.) was added to final concentrations of 5 mg/ml, and the suspensions were incubated for 2 h at 37°C. The efficiency of this lysis procedure was checked by counting cells after addition of lysozyme; tests were also conducted with isolated bacteria and added clay to ensure the adequacy of the lysis procedure.

The suspensions were then heated to 60°C and SDS was added to a final concentration of 1.0%, after which the suspensions were incubated for 10 min. The suspensions were cooled on ice for 2 h and then centrifuged for 20 min at 12,000 × *g* in a Sorvall SS34 rotor at 5°C. The supernatant (first lysate) was transferred to a sterile centrifuge tube. The pelleted material was washed with 10 ml of Chrombach buffer and centrifuged as described above. The supernatant was collected and combined with the first lysate.

To purify the DNA in the combined cell lysate, solid ammonium acetate was added to the samples to give a final concentration of 2.5 M and the samples were immediately centrifuged for 30 min at 12,000 × *g* and 5°C. This process resulted in the precipitation of a considerable amount of organic debris, but no DNA could be detected in this precipitated material by cesium chloride-ethidium bromide density gradient centrifugation.

DNA in the supernatant fraction was precipitated by addition of 2.5 volumes of ice-cold 95% ethanol and incubation at −20°C for 12 h. The samples were centrifuged for 30 min at 12,000 × *g* and 5°C, the ethanol was decanted, and the pellets were dried under vacuum. The DNA was suspended in 10 ml of TE (10 mM Tris hydrochloride, 1 mM EDTA, pH 8.0) and separated by cesium chloride-ethidium bromide density gradient centrifugation (11). The resultant DNA band was collected, ethidium bromide was removed by successive extractions with water-saturated 1-butanol, and the samples were dialyzed for 24 h against two changes of TE. The DNA was precipitated with ethanol as above and suspended in 100 μl of sterile double-distilled water. Subsamples of the recovered DNA sample were analyzed by agarose gel electrophoresis, and the purity of DNA was checked by spectrophotometric absorbancy measurements at 280 and 260 nm. The remaining suspended DNA was

further purified by hydroxyapatite column chromatography as described below.

(ii) Direct lysis method. DNA was recovered from soils and sediments by directly lysing the cells and then extracting and purifying the DNA by the procedure of Ogram et al. (13). In this procedure, the soil and sediment samples were initially treated with SDS at 70°C for 1 h, after which the cells were physically disrupted with a bead beater (Bio Spec, Bartlesville, Okla.). As a modification to the procedure used by Ogram et al. (13), 20 g of PVPP was added to the lysed cell mixture to remove humic material. The DNA was then recovered by repeated washings with 0.12 M phosphate buffer (pH 8.0). Sodium chloride was added to the DNA suspension to a final concentration of 0.5 M, and the DNA was then precipitated overnight with 0.5 volume of 50% polyethylene glycol (PEG) 8000. The DNA was recovered by centrifugation at 5,000 × *g* for 10 min at 4°C. The supernatants were removed from the loose pellets by aspiration. PEG was removed from the DNA by one extraction with TE-saturated phenol, one extraction with phenol-chloroform-isoamyl alcohol (25:24:1), and two extractions with chloroform-isoamyl alcohol (24:1). Potassium acetate was then added to achieve a final concentration of 0.5 M, and the samples were incubated on ice for 2 h. To further remove humic compounds, 2 g of acid-washed PVPP was added to each sample and the samples were incubated for 15 min at 28°C with intermittent swirling. The samples were then filtered through a 0.5-μm filter (Millipore GS) to remove PVPP and the precipitated humic material. The DNA was then precipitated by adding 2.5 volumes of 95% ethanol and incubating for 12 h at −20°C. The resulting pellets were dried and suspended in TE. The DNA was further purified by CsCl-ethidium bromide density gradient centrifugation by adding 0.8 g of CsCl and 160 μg of ethidium bromide per ml to each sample and centrifuging them in a Ti70 rotor at 50,000 rpm for 18 h at 15°C. The broad fluorescing regions were recovered from the tubes, and the ethidium bromide was removed by extractions with 1-butanol (11). Samples were then dialyzed against two changes of 0.12 M sodium phosphate buffer (pH 6.8).

Hydroxyapatite purification of DNA. Additional purification of DNA was performed by hydroxyapatite column chromatography, using the method described by Ogram et al. (13). High-resolution DNA-grade hydroxyapatite (Calbiochem-Behring) was suspended in 0.01 M sodium phosphate buffer (pH 6.8), boiled for 10 min, and centrifuged for 2 min at 5,000 × *g*, after which the supernatant was removed by decanting. The hydroxyapatite was mixed with a buffer consisting of 8 M urea in 0.12 M sodium phosphate (pH 6.8) and centrifuged again as before. Samples were mixed with 2 g (dry weight) of hydroxyapatite with gentle intermittent swirling at 28°C for 1 h. This mixture was poured into a column made of a 20-ml disposable syringe with siliconized glass wool as a support base and a 16-gauge needle as an outlet. The urea-containing buffer was drained from the columns, and additional urea-containing buffer was passed through the columns to remove some organic contaminants until the effluent absorbance between 320 and 220 nm reached zero. A 50-ml (10-column volume) amount of 0.014 M sodium phosphate (pH 6.8) was then passed through the columns to remove the urea-containing buffer. DNA was then eluted sequentially with 10-ml volumes of 0.20 and 0.25 M sodium phosphate (pH 6.8), and 10-ml fractions were collected; these fractions contained the DNA but not a 230-nm-absorbing substance that elutes shortly thereafter and which was eluted from the columns with 10 ml of 0.30 M sodium phosphate buffer. The phosphate was removed from the recovered fractions by dialysis for 12 h against 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). The DNA was recovered by ethanol precipitation and suspended in 100 μl of 0.1× SSC. DNA purity and concentration were determined by measuring A_{230}, A_{260}, and A_{280}.

Comparison of DNA recovery methods. To evaluate the effectiveness of the two procedures for recovery of DNA from environmental samples, DNA was extracted from replicate soil and sediment samples by each method. DNA extractions were run in triplicate. Samples from both the direct lysis and the cell extraction procedures were collected and analyzed prior to cesium chloride gradient centrifugation, after cesium chloride gradient centrifugation purification and prior to hydroxyapatite column chromatography, and after the final purification by hydroxyapatite column chromatography. Also, comparative extractions were carried out without PVPP. Results were compared by statistical analyses, using paired *t* tests.

Since the DNA in the bacterial cells was radiolabeled by incubation with tritiated thymidine, DNA recovery was assessed by using both liquid scintillation counting to determine recovered counts of tritium in the DNA fraction and spectrometric A_{260} determinations. Standard amounts of pure salmon sperm gave a linear response of A_{260}, with a concentration of 50 μg/ml giving an A_{260} of 1.00. Purity of DNA was assessed by measuring A_{230}, A_{260}, and A_{280}, and determining the $A_{260/280}$ and $A_{260/230}$ ratios. Agarose gel electrophoresis was also performed to compare the DNA size distribution in the recovered DNA. For these analyses, 1-μg amounts of recovered DNA, as estimated by A_{260}, were added to each well. Controls of salmon sperm DNA and size standards of *Hin*dIII-digested lambda phage DNA were also run. Electrophoresis was carried out in 0.7% agarose (Sigma) at 1.3 V/cm^3 for 8 h. The buffer system contained 89 mM Tris hydrochloride, 8.9 mM boric acid, and 2.5 mM EDTA (pH 8.3).

Effects of impurities on DNA dot blot and Southern blot analyses. Because the DNA recovered from soils and sediments may be used for further analysis by dot blot or Southern blot techniques, experiments were conducted to determine how pure the DNA recovered from soil or sediment had to be to minimize interference with such analyses. For these determinations, plasmid pRS19U-5 DNA (4.1 kilobases [kb] containing a 1.3-kb *Sal*I-probe-specific fragment cloned into the multiple cloning site of pTZ19U) was used as the target DNA. Various amounts (1 to 100 ng) of this plasmid DNA were used as controls with no added impurities. Identical amounts of this plasmid DNA were mixed with various impurities: salmon sperm DNA (100 ng to 10 μg); protein (100 ng to 10 μg); humic acid (100 ng to 10 μg); montmorillonite clay (10 ng); and a mixture of clay (10 ng), humic acid (10 μg), protein (10 μg), and salmon sperm DNA (10 μg). Samples were brought to a final volume of 10 μl by the addition of sterile deionized water.

The mixtures of target DNA with and without impurities were spotted onto a nylon membrane, using a dot blot apparatus (Bio-Rad Laboratories). After air drying, the filters were placed onto filter paper pads containing denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 min and then onto pads containing neutralizing solution (3 M sodium acetate, pH 5.5) for 5 min. The filters were then dried for 30 min at room temperature, and the DNA was bonded to the filters by baking at 80°C for 1 h. A ^{32}P-radiolabeled probe, consisting of a 1.3-kb sequence present in pRS19U-5, was made by nick translation; the probe was hybridized against

the dot blots, and autoradiography was performed as described previously (11).

For determining the effects of impurities on Southern blot analysis, 1 μg of target pRS19U-5 DNA was mixed with various amounts of salmon sperm DNA (10 μg), humic acid (100 ng to 10 μg), clay (10 ng), and bovine serum albumin protein (1 μg) in a total reaction volume of 20 μl; the DNA was then digested with 4 U of *Sal*I at 37°C for 2 h in high-salts buffer (11). Also, 0.5 μg of pRS19U-5 DNA was mixed with 1.0 μg of DNA recovered by the two different methods from soils and sediments that had been purified only by cesium chloride density gradient centrifugation or by both cesium chloride density gradient centrifugation and hydroxyapatite column chromatography. The DNA was then digested with 4 U of *Sal*I, 4 U of *Eco*RI, or 4 U of *Pvu*II; all digestions were at 37°C for 2 h in buffer appropriate for that restriction enzyme supplied by the manufacturer (Promega). The DNA fragments were separated by agarose gel electrophoresis and transferred to nylon membranes (11). The DNA was bonded to the membranes by baking at 80°C for 1 h, hybridized with the 1.3-kb probe sequence, and subjected to autoradiography as described for dot blot analyses.

RESULTS AND DISCUSSION

Modification of cell extraction procedure for DNA recovery. The cell extraction method for DNA recovery was originally developed for soils that had relatively high humic and low clay contents (6). The buffer system used for extracting cells (Winogradsky salt solution) was not developed for samples, such as sediments, that have relatively high clay contents. In our examination of buffer systems, we found that an acidic 0.1 M phosphate buffer (pH 4.5) with SDS gave better recoveries of bacterial cells from sediments than other phosphate buffers or Winogradsky salt solution that had been used for soils. The percentages of total cells in 100-g sediment samples, as determined by acridine orange direct count, recovered by a single extraction with various extraction solutions were as follows: distilled water, 7.3%; phosphate buffer (0.1 M, pH 6.8), 9.1%; phosphate buffer plus 0.1% sodium PP_i (pH 6.8), 7.7%; phosphate buffer plus 0.1% SDS (pH 6.8), 9.4%; phosphate buffer plus 0.1% SDS (pH 9.0), 7.3%; phosphate buffer plus 0.1% SDS (pH 4.5), 17.8%; phosphate buffer plus 0.1% SDS and 2% PVPP (pH 4.5), 13.2%. The addition of PVPP with the first cell extraction, a procedure suggested by Holben et al. (10) to remove humic material, slightly lowered the efficiency of cell recovery. The removal of humic material by PVPP treatment, however, greatly improved the quality of the DNA recovered and the ease of purification.

Three repetitive extractions with the acidic phosphate-SDS buffer and PVPP treatment recovered 32.2 and 27.8% of the total bacterial cells from sediment and soil, respectively (Table 2). These cell recoveries are similar to those obtained by Bakken (2), who used four to seven extraction steps and density gradient centrifugation to recover cells from soil with high clay content, and by Holben et al. (10), who reported recovery of 34.9% of the bacterial cells from a soil by using three extraction steps with Winogradsky solution plus sodium ascorbate and PVPP treatment. The use of only three extraction steps represents a compromise between reasonable effort to achieve the greatest yield of DNA and the need to ensure that the DNA is free of contaminating material.

The final cell pellet we recovered had an organic content of 30 to 42%, suggesting that the pellet still contained a significant amount of inorganic clays. This is consistent with findings of Bakken (2) and Balkwill et al. (3), who observed that a large proportion of soil microorganisms remained attached to clay particles even after extensive extraction and density gradient centrifugation procedures. In fact, we have noted that the highest yields of cells are obtained when the rates of centrifugation are adjusted to give larger pellets that contain higher clay contents; higher yields of cells could yield greater amounts of recovered DNA, but problems with removal of additional impurities that occur when slower centrifugation speeds are used may limit actual increased yields of DNA. In some cases we have recovered up to 50% of the bacterial cells in sediments by using three extraction steps and slower spin rates to reduce clay sedimentation; that is, maintaining more clay-associated cells in the supernatant fraction which is collected appears to increase the bacterial cell fraction.

TABLE 2. Recovery of bacteria from 100-g sediment and soil samples

Extract	Cells recovered (cells per g)	Recovery (% of total)	SE (recovery)
Sediment			
Original	3.6×10^9	100	
Extract 1	4.8×10^8	13.2	1.5
Extract 2	3.3×10^8	9.1	3.4
Extract 3	3.6×10^8	10.0	1.6
Total extract	1.2×10^9	32.3	3.5
Soil			
Original	4.4×10^9	100	
Extract 1	3.2×10^8	7.4	2.2
Extract 2	3.6×10^8	8.1	3.2
Extract 3	5.1×10^8	11.7	1.9
Total extract	1.2×10^9	27.2	4.2

The presence of the clay (kaolinite or montmorillonite) in the cell pellet did not appear to affect lysis of sediment bacteria by lysozyme and SDS. Lysis efficiency was >90% as determined by direct-count enumeration after lysis. In control experiments, lysis of *Escherichia coli* was not affected by the addition of 40 mg of kaolinite or montmorillonite clay per ml. Also, lysis efficiency was 97% for a mixture of microorganisms cultured from soil (56% gram negative and 44% gram positive). Lower lysis efficiencies, however, may occur in other situations, such as in soils that have a higher proportion of polysaccharide-producing bacteria which may not be as easily lysed by lysozyme and SDS; thus, there may be situations in which a more exhaustive lysis procedure, such as the one used by Holben et al. (10), is warranted. Examination of lysis efficiency is critical to avoid biased recovery of DNA from selected, easily lysed populations.

Modification of direct lysis DNA extraction procedure. The direct lysis procedure of Ogram et al. (13) relies upon physical disruption to release DNA; such physical disruption techniques are effective at breaking cells but also can shear the DNA macromolecules. Another problem associated with the direct lysis procedure is that a large amount of humic material is extracted along with the DNA. We found that, by using the procedures as described by Ogram et al. (13), extracts from both soil and sediment are a very dark brown. The removal of humic material is difficult, and it tends to persist even with extensive purification by cesium chloride density gradient centrifugation and hydroxyapatite column chromatography. In preliminary experiments, we found that

TABLE 3. Effect of PVPP treatment on recovery and purity of salmon sperm DNA in the presence and absence of humic acid

Sample	PVPP treatment	A_{230}	A_{260}	A_{280}	$A_{260/280}$[a]	$A_{260/230}$[b]	DNA (mg/ml)[c]
Sodium phosphate, 0.012 M	+	0.042	0.010	0.009	1.11	0.24	0.00
DNA, 0.05 mg/ml	−	0.501	1.030	0.584	1.76	2.06	0.05
DNA, 0.05 mg/ml	+	0.486	1.037	0.578	1.79	2.13	0.05
Humic acid, 0.01 mg/ml	−	2.676	2.566	2.415	1.06	0.96	0.13
Humic acid, 0.01 mg/ml	+	1.231	1.008	0.872	1.16	0.82	0.05
DNA, 0.05 mg/ml, + humic acid, 0.01 mg/ml	−	2.715	2.720	2.619	1.04	1.00	0.14
DNA, 0.05 mg/ml, + humic acid, 0.01 mg/ml	+	1.709	2.009	1.468	1.37	1.18	0.10

[a] $A_{260/280}$, Ratio of A_{260} to A_{280} (a high ratio [>1.7] is indicative of pure DNA, whereas a low ratio is indicative of protein contamination).
[b] $A_{260/230}$, Ratio of A_{260} to A_{230} (a high ratio [>2] is indicative of pure DNA, whereas a low ratio is indicative of phenolic [humic] contamination).
[c] Calculated based upon A_{260} against a standard response curve.

if PVPP was not included in the procedure a single run of cesium chloride density gradient centrifugation failed to yield bands of DNA, requiring a second run before DNA which still contained humic material could be recovered for purification by hydroxyapatite column chromatography. We have also found that inconsistencies inherent in hydroxyapatite chromatographic purification of DNA sometimes lead to the recovery of DNA that has a significant 230-nm-absorbing component, that is, the recovery of final DNA that is still contaminated with humic materials. It is, therefore, essential to remove as much humic material as possible before attempting to purify the DNA by hydroxyapatite column chromatography.

Therefore, we examined the applicability of PVPP treatment to the direct lysis method. We began by testing the effect of PVPP treatment on recovery of DNA, both quantitative and qualitative, from solutions with known concentrations of salmon sperm DNA and humic acid. The results clearly show that humic acids mask the detection of DNA and that PVPP reduces the amount of humic compounds without removing DNA (Table 3). Identical quantitative recoveries of DNA were found with and without PVPP treatment in the absence of humic acid; the $A_{260/280}$ and $A_{260/230}$ ratios were approximately 1.8 and 2.1, respectively. Lower $A_{260/280}$ and $A_{260/230}$ ratios were found for humic acid and DNA contaminated with humic acid. While significantly reducing the level of humic acid, PVPP treatment did not completely remove humic compounds, and the $A_{260/280}$ and $A_{260/230}$ ratios for a mixture of humic acid and DNA treated with PVPP were only 1.4 and 1.2, respectively, significantly lower than for pure DNA. Repeated extractions with PVPP did not further reduce concentrations of humic compounds and did not improve the purity of the DNA.

Because PVPP treatment does not remove DNA while lowering the humic acid content, it can be included in the direct lysis DNA extraction method to improve the ease of purification and the quality of DNA recovered. This is clearly shown by comparing the DNA recovered from soil and sediment by this method after both cesium chloride density gradient centrifugation and hydroxyapatite column chromatographic purification with and without PVPP treatment (Table 4). DNA recovered from soil with PVPP treatment had $A_{260/280}$ and $A_{260/230}$ ratios of 1.5 and 2.1, respectively, compared with ratios without PVPP treatment of 1.0 and 0.4. Similarly, DNA recovered from sediment with PVPP treatment had $A_{260/280}$ and $A_{260/230}$ ratios of 1.4 and 2.3, respectively, compared with ratios without PVPP treatment of 1.1 and 0.4. Based on these results, the inclusion of PVPP treatment appears to be both a warranted and a necessary modification to the procedure originally described by Ogram et al. (13).

Besides PVPP treatment, we examined the use of both ethanol and PEG precipitation of DNA from the initial alkaline extract. Because of the large extraction volumes, 2.5 liters of cold ethanol per sample was required to precipitate the DNA. The numerous centrifugation runs needed to recover the precipitated DNA led to inevitable losses, so that not only was this procedure very tedious and costly but also the amounts of DNA recovered were less than with PEG precipitation. Clearly, PEG precipitation of DNA from the initial extract is superior in this method.

Comparison of cell extraction and direct lysis methods for DNA recovery. In considering the merits of the cell extraction versus the direct lysis methods for DNA recovery from soils and sediments, the most important considerations are the relative efforts required, the amount of DNA recovered, and the quality of DNA recovered. The direct lysis method is somewhat more tedious than the cell extraction procedure because of the extraction of humic material and the difficulty of precipitating the DNA from the large volume of extraction solution. The use of PEG instead of ethanol simplifies the precipitation step and the use of PVPP decreases the amount of humic material extracted, thereby simplifying the purification process. With these modifications the direct lysis method is not much more tedious than the cell recovery method.

Both cesium chloride density gradient centrifugation and hydroxyapatite column chromatographic purification appear to be important, even though they cause loss of DNA, because they clearly improved the purity of recovered DNA (Table 5). Also, the purification steps appeared to result in fragmentation of the recovered DNA as evidenced by decreased-size DNA fragments observed following gel electrophoresis. Cesium chloride density centrifugation and hydroxyapatite column chromatography improved the $A_{260/280}$ and $A_{260/230}$ ratios from 1.2 to 1.4 and 0.8 to 1.2, respectively, prior to purification to 1.4 to 1.6 and 0.9 to 2.2 after purification. The highest-purity DNA based upon these absorbance ratios was from the direct lysis procedure, but this may be a reflection of the much higher yield of DNA that

TABLE 4. Effect of PVPP treatment on purity of DNA recovered by direct lysis DNA extraction method

Sample	PVPP treatment	$A_{260/280}$	$A_{260/230}$
Control[a]	+/−	1.78	2.10
Soil	−	0.97	0.41
Soil	+	1.54	2.06
Sediment	−	1.06	0.44
Sediment	+	1.40	2.26

[a] Pure salmon sperm DNA.

TABLE 5. Recovery of bacterial DNA from sediment and soil: comparison of two methods

Method and sample	Thymidine recovered (dpm)	Estimated DNA recovered (mg)	Thymidine/DNA ratio (dpm/μg)	$A_{260/280}$ ratio	$A_{260/230}$ ratio
Prior to CsCl					
Cell extraction					
Soil	14,082	1.67	8.4	1.18	0.75
Sediment	12,960	0.89	5.6	1.17	0.82
Direct extraction					
Soil	46,242	11.64	4.0	1.28	0.98
Sediment	68,078	9.06	8.6	1.42	1.23
Prior to hydroxyapatite column chromatography					
Cell extraction					
Soil	8,600	0.20	43.0	1.25	0.96
Sediment	6,160	0.18	35.0	1.16	0.92
Direct extraction					
Soil	37,760	2.52	13.0	1.26	1.59
Sediment	49,680	3.17	15.7	1.37	2.02
After to hydroxyapatite column chromatography					
Cell extraction					
Soil	480	0.04	33.8	1.61	1.42
Sediment	1,130	0.03	44.3	1.36	0.90
Direct extraction					
Soil	4,780	0.85	5.7	1.54	2.06
Sediment	11,720	1.99	5.9	1.41	2.26

would mask background recovery of non-DNA-absorbing compounds. Gel electrophoresis confirmed that, without extensive purification, the A_{260} determinations were not a true measure of the amount of DNA.

With regard to the amount of DNA recovered, the direct lysis method is clearly superior (Table 5). The direct lysis method yielded 20 to 70 times as much DNA as was recovered by the cell extraction method. With both methods, cesium chloride density gradient centrifugation and hydroxyapatite column chromatography caused loss of DNA (Table 5). Based upon loss of thymidine label, it appears that these purification steps result in losses of about 90%. Assuming a mean DNA content of a bacterial cell of 6 fg (12), the theoretical yield of DNA from the 100-g samples should have been 4.0 mg for soil and 2.1 mg for sediment. The direct lysis procedure recovered 0.9 and 2 mg from soil and sediment, respectively, which would represent efficiencies of 22 and 90%, respectively. The recovery by the cell extraction procedure of 0.04 and 0.03 mg of soil and sediment corrected for efficiency of cell recovery (33%) would be only 3 and 4%, respectively. However, if the 90% loss of labeled thymidine during the purification process is representative of total DNA losses, then the DNA recovered by the cell extraction procedure would be 30% for soil and 45% for sediment of the theoretical yield of bacterial DNA. The recovery efficiencies may actually be higher because some of the loss of thymidine label may represent removal of unincorporated thymidine that was bound to contaminants and removed during purification steps.

Assuming the accuracy of this estimated loss of DNA during recovery, which occurs largely during hydroxyapatite column chromatography and which also appears to be supported by the loss of DNA observed by gel electrophoresis, the direct lysis procedure yields more DNA than could originate from the bacterial cells. It is possible that some of the DNA recovered by the direct lysis procedure could have come from eucaryotic cells or from extracellular DNA. There is no theoretical reason that the direct lysis method, which involves physical disruption of all cells in the sample, should not recover eucaryotic as well as procaryotic DNA. However, probing for eucaryotic DNA with a series of probes, including a 16-base kingdom-specific oligonucleotide that is complementary to a region which codes for 16S rRNA (8), a 1.05-kb *Hin*dIII fragment from a noncoding telomeric region near the end of the extrachromosomal ribosomal DNA of *Tetrahymena thermophila* (5), a 1-kb fragment that codes for actin, and a 1-kb sequence that includes portions of histones 2a and 2b, failed to show evidence (above background signals after autoradiographic detection of dot blot hybridizations) for eucaryotic DNA in the DNA recovered from soil or sediment by either the direct lysis or the cell extraction method. However, each of these probes failed in some cases to hybridize with known eucaryotic DNA (salmon sperm, *Ustilago* sp., or *Saccharomyces* sp.) or showed limited hybridization with *E. coli* DNA or both. Thus, the eucaryotic probe results do not permit us to conclude with certainty that there is no eucaryotic DNA in the extracts. If, though, the results showing no hybridization to gene probes for eucaryotic DNA are correct, the conclusion would be that the direct lysis method recovered extracellular as well as intracellular DNA. Significant amounts of extracellular DNA have been reported for marine and estuarine samples (14, 15) and in freshwater sediments (13).

Effects of impurities on DNA dot blot and Southern blot analyses. To determine the degree of purity required to further utilize the recovered DNA for restriction endonuclease digestion and hybridization analysis, various amounts of contaminants were added to target DNA and the samples were subjected to either dot blot analysis or restriction endonuclease digestion and Southern blot analysis. Dot blot detection of the target DNA was inhibited by the addition of 10 μg of humic acid (Table 6), which produced a colored solution roughly equivalent to the color observed in the DNA extracts from the direct lysis DNA extraction procedures before purification by hydroxyapatite column chromatography. The addition of salmon sperm DNA, montmorillonite clay, bovine serum albumin, or lower amounts of humic acid did not inhibit dot blot hybridization (Table 6).

TABLE 6. Effects of various factors on detection of target DNA by dot blot

Factor[a]	Detection[b] of target DNA at:		
	100 ng	10 ng	1 ng
Control	+++	++	+
SS DNA, 10 μg	+++	++	+
SS DNA, 1 μg	+++	++	+
SS DNA, 0.1 μg	+++	++	+
BSA, 10 μg	+++	++	+
BSA, 1 μg	+++	++	+
BSA, 0.1 μg	+++	++	+
Humic acid, 10 μg	+	−	−
Humic acid, 1 μg	+++	++	−
Humic acid, 0.1 μg	+++	++	+
Clay, 10 ng	+++	++	+
Mix of clay, 10 ng, and BSA, humic acid, SS DNA, 10 μg each	+++	−	−

[a] SS DNA, Salmon sperm DNA; BSA, bovine serum albumin.
[b] +++, Intense signal; ++, moderate signal; +, weak signal; −, no signal.

Also, the DNA recovered by both procedures was of sufficient purity not to interfere with radiolabeled probe detection of target DNA added after recovery of the DNA from soil and sediment by dot blot analyses.

Restriction endonuclease activity was not inhibited by the addition of salmon sperm DNA or bovine serum albumin. However, restriction endonuclease digestion of target DNA with *Sal*I was almost completely inhibited by all concentrations of humic acid tested and was partially inhibited by montmorillonite clay (Fig. 1). This suggests that any humic contamination in the recovered DNA may potentially hinder restriction endonuclease analysis of DNA from environmental samples, but that minor contamination with protein or clay and the presence of significant amounts of background nontarget DNA will not interfere with restriction endonuclease digestion of target DNA.

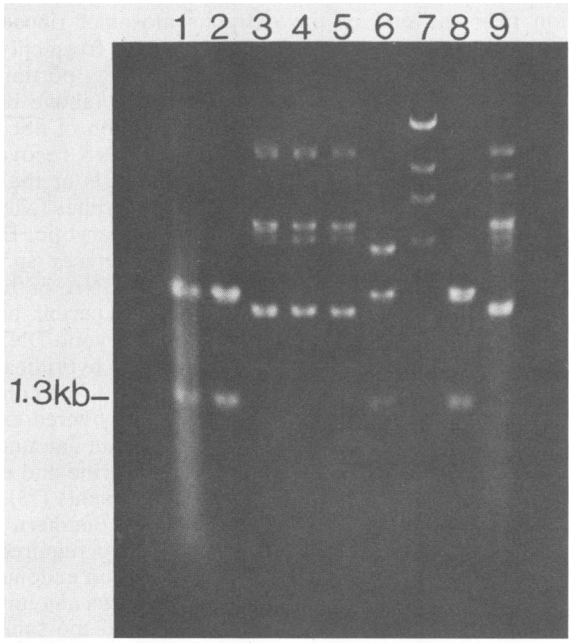

FIG. 1. Agarose gel of *Sal*I-digested DNA. pRS19U-5 DNA (0.5 μg) was digested with 4 U of *Sal*I in the presence of nonspecific DNA, protein, humic acid, clay, and DNA isolated by the direct lysis procedure. The presence of a 1.3-kb band indicates digestion of the added plasmid DNA. Lane 1, 1.0 μg of salmon sperm DNA; lane 2, 1.0 μg of bovine serum albumin; lane 3, 100 ng of humic acid; lane 4, 10 ng of humic acid; lane 5, 1.0 ng of humic acid; lane 6, 10 ng of montmorillonite clay; lane 7, *Hin*dIII-digested lambda DNA size markers; lane 8, no additions (control); lane 9, 1.0 μg of hydroxyapatite-purified DNA isolated from sediment by the direct lysis procedure.

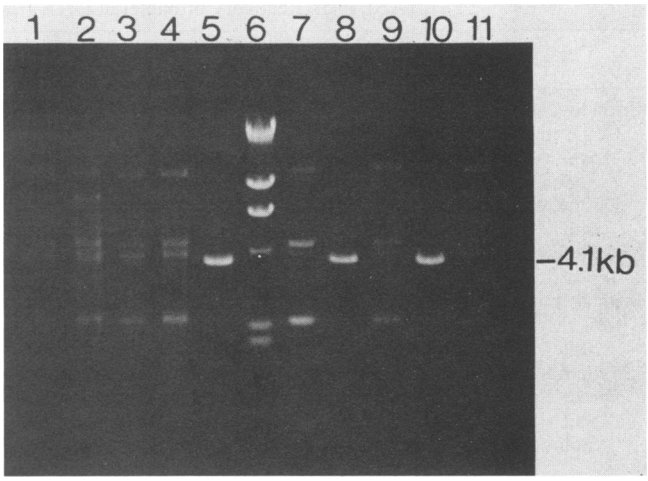

FIG. 2. Agarose gel of *Eco*RI-digested DNA. pRS19U-5 DNA (0.5 μg) was digested with 4 U of *Eco*RI in the presence of DNA isolated from soil and sediment by the direct lysis and cell recovery methods. The presence of a 4.1-kb band represents digestion of the plasmid DNA. Lane 1, DNA recovered from soil by the direct lysis method after cesium chloride density gradient centrifugation (CEC) and hydroxyapatite column chromatographic purification (HAPC); lane 2, DNA recovered from soil by the direct lysis method after CEC without HAPC; lane 3, DNA recovered from sediment by the direct lysis method after CEC and HAPC; lane 4, DNA recovered from sediment by the direct lysis method after CEC without HAPC; lane 5, no addition (control); lane 6, *Hin*dIII-digested lambda DNA size markers; lane 7, undigested control; lane 8, DNA recovered from soil by the cell recovery method after CEC and HAPC; lane 9, DNA recovered from soil by the cell recovery method after CEC without HAPC; lane 10, DNA recovered from sediment by the cell recovery method after CEC and HAPC; lane 11, DNA recovered from sediment by the cell recovery method after CEC without HAPC.

Some endonucleases were inhibited by the DNA recovered from sediments and soils. These results varied with the enzyme used. *Pvu*II cut the target DNA in the presence of DNA recovered from both soil and sediment by both the direct lysis and cell extraction methods. This enzyme was active even when the nontarget DNA was not thoroughly purified, as evidenced by cutting of target in the presence of recovered nontarget DNA before and after hydroxyapatite column chromatographic purification. In contrast, *Sal*I failed to cut the added target DNA in the presence of DNA recovered from soil and sediment by both procedures even after hydroxyapatite column chromatographic purification. *Eco*RI also failed to cut added target DNA in the presence of DNA recovered by the direct lysis DNA recovery procedure even after hydroxyapatite column chromatographic purification; *Eco*RI, however, cut target DNA in the presence of DNA recovered by the cell extraction DNA recovery procedure after, but not before, hydroxyapatite column chromatographic purification (Fig. 2). The results of these experiments, which were performed on pure target DNA that was added to the extracted DNA, suggest that, even though the

extracted DNA appears to be highly purified, as evidenced by the $A_{260/280}$ and $A_{260/230}$ ratios, contaminants may remain that can inhibit some restriction analyses. A second hydroxyapatite column chromatographic purification may be necessary if the recovered DNA is to be used for restriction analyses by one of the more sensitive restriction enzymes. Also, increasing the concentrations of restriction enzymes and increasing the sample volume to dilute contaminants may be advisable to enhance the likelihood of successfully cutting the DNA.

Conclusions. Both the cell extraction and the direct lysis methods permitted the isolation of DNA from soils and sediments. The use of PVPP to remove humic contaminants was essential for adequate purification of the recovered DNA; PVPP did not significantly decrease DNA yields by either the direct lysis or the cell recovery procedure. The direct lysis method yielded at least a 10-fold-greater total DNA recovery than the cell extraction method. Although the $A_{260/280}$ and $A_{260/230}$ ratios indicated that the recovered and purified DNA was of high purity, some restriction enzymes failed to cut target DNA in the presence of DNA recovered by both methods. The recovered DNA did not interfere with dot blot probe analyses even without hydroxyapatite column chromatographic purification. These results suggest that care must be taken in evaluating the extent of purification needed for different uses of the recovered DNA. For dot-blot detection of specific genotypes, hydroxyapatite purification may not be necessary, but for applications requiring enzymatic manipulation of the recovered DNA, both cesium chloride density gradient centrifugation and hydroxyapatite column chromatographic purification may be essential even though some DNA is lost during purification. Similarly, for large quantities of DNA, the direct lysis procedure appears to be the appropriate method to use; when lesser quantities of bacterial DNA are sufficient, or when it is beneficial to ensure exclusion of eucaryotic or extracellular DNA or both, the cell extraction method appears to be the appropriate choice.

ACKNOWLEDGMENTS

This research was supported in part by the U.S. Environmental Protection Agency (EPA) through a cooperative research agreement with the EPA Corvallis, Ore., laboratory.

We thank Kevin Karem and Anne Bronner for technical assistance and Norman Pace, Thomas Geoghegan, and Nancy Martin for supplying probes used for the detection of eucaryotic DNA.

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