APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1988, p. 1426–1429
0099-2240/88/061426-04$02.00/0

Vol. 54, No. 6

Extraction from Natural Planktonic Microorganisms of DNA Suitable for Molecular Biological Studies

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Received 16 October 1987/Accepted 21 March 1988

We developed a simple technique for the high-yield extraction of purified DNA from mixed populations of natural planktonic marine microbes (primarily bacteria). This is a necessary step for several molecular biological approaches to the study of microbial communities in nature. The microorganisms from near-shore marine and brackish water samples, ranging in volume from 8 to 40 liters, were collected on 0.22-μm-pore-size fluorocarbon-based filters, after prefiltration through glass fiber filters, to remove most of the eucaryotes. DNA was extracted directly from the filters in 1% sodium dodecyl sulfate that was heated to 95 to 100°C for 1.5 to 2 min. This procedure lysed essentially all the bacteria and did not significantly denature the DNA. The DNA was purified by phenol extraction, and precautions were taken to minimize shearing. Agarose gel electrophoresis showed that most of the final preparation had a large molecular size (>23 kilobase pairs). The DNA was sufficiently pure to allow complete digestion by the restriction endonuclease *Sau*3AI and ligation to vector DNA. In a sample in which the extracted DNA was quantified by binding to the dye Hoechst H33258, DNA was quantitatively extracted, and 45% of the initially extracted DNA was recovered after purification. Final yields were a few micrograms of DNA per liter of seawater and were roughly 25 to 50% of the total bacterial DNA in the sample. Alternatives to the initial harvest by filtration method, including continuous-flow centrifugation and thin-channel or hollow-fiber concentration followed by centrifugation, were less efficient than filtration in terms of both time and yield, largely because of the difficulty of centrifuging the very small bacteria typical of marine plankton. These methods were judged to be less appropriate for studies of natural populations as they impose a strong selection for the larger bacteria.

The tools of molecular biology offer new and potentially very powerful ways to study organisms in their natural environment and can lead to new insights into natural processes. One area in which a significant application has been developed recently is in the phylogenetic characterization of natural microbial communities by 16S rRNA sequence analysis, as described by Pace and colleagues (8, 9). Conventional identification requires that the organisms be cultured; for reasons that are largely unknown, typically only 1% or less of the naturally occurring individuals grow in culture (2), raising the question of whether the culturable organisms studied to date are even remotely representative of the total community. Studying these organisms at the DNA level avoids the need for culturing; DNA from the natural populations can be extracted and fragmented, and the fragments can be cloned into vectors to form a library. A feature that gives this approach much of its power is that each clone in the library represents a single piece of DNA, and hence a single individual from the natural community; but because the gene is manipulated in *Escherichia coli*, well-established techniques can be used.

We were motivated to perform this study because of a desire to apply 16S rRNA gene cloning and sequencing to the phylogenetic characterization of natural planktonic bacterial communities. A current limitation of this approach is that there are few established procedures for extracting from natural microbial populations DNA suitable for these studies. There are two reports of procedures for extracting and purifying DNA from soil (6, 13); one yielded fragments of only about 0.4 to 1.6 kilobase pairs (kbp) in length (13), which is unsuitable for cloning studies. The second procedure produced high-molecular-weight DNA that is suitable for molecular biological studies (6). While Pace et al. (9) and Olsen et al. (8) have described a procedure that is suitable for attached microbial populations, we are not aware of any published procedure for the extraction and purification of DNA from mixed populations of freshwater or marine planktonic microorganisms. The problem is not a trivial one, because these organisms are very small (mostly <0.6 μm) and dilute (ca. 10^9/liter), making nonselective collection of a sufficient number of cells and quantitative DNA extraction and purification difficult.

In order for the DNA to be suitable for our applications, four criteria for our extraction and purification protocol must be met. (i) The final DNA should be representative of the total DNA within the naturally occurring microbes at the time of sampling. This means that the populations should be sampled quantitatively and that any losses during extraction and purification should be nonselective. Also, the species composition should not change between the time of sampling and the time of extraction. (ii) The final yield should be at least a few micrograms, and preferably >25 μg. A genomic library should consist of millions or 10s of millions of clones. About 0.2 to 0.3% of the clones should code for 16S rRNA (8), and it is desirable to have representatives from species that make up only a few percent or less of the natural populations. A library of this size can be produced from 1 μg or less of DNA, but more should be collected for ease of handling and for auxiliary studies such as hybridization analysis (9). (iii) The DNA should be of very large molecular size (a minimum of 10 kbp, but preferably 50 kbp or larger), to keep the size of the genomic library manageable (smaller inserts require larger libraries) and to increase the likelihood that clones contain an intact copy of the gene(s) of interest.

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The original DNA should be much larger than the insert size to allow for restriction cutting to create compatible ends. (iv) The DNA should be of sufficient purity for enzymatic manipulations, such as restriction endonuclease digestion and ligation.

Taken together, the first two criteria were expected to be the most difficult to fulfill. Because of the typically low natural bacterial abundances, it is necessary to concentrate large volumes (several liters) of the sample in order to end up with several micrograms of DNA, given typical abundances of 10^9/liter and the known amount of DNA per cell. (Note that DNA as a percentage of biomass is very high in planktonic bacteria compared with that in cultured forms [3].) It is also desirable to concentrate and fix the bacteria within a few hours of sampling, because it has been shown that containment artifacts may cause the species composition to change drastically within 16 h or less of sampling (2). Therefore, in order to collect bacteria from large volumes quickly and nonselectively, we chose filtration.

There was also no established method for quantitatively lysing mixed species of bacteria on a filter without damaging the DNA. We found that a freeze-thaw cycle followed by brief hot detergent treatment quantitatively lysed the cells without the need to use enzymes that complicate purification. Overall, the protocol we developed met all the criteria and should be generally applicable in most aquatic and marine environments. It is probably suitable for a variety of different applications, in addition to our own, including searching for genes from genetically engineered microorganisms that have been released into the environment.

MATERIALS AND METHODS

This study was performed in two near-shore environments: Long Island Sound in New York and the Bothnian Gulf of the Baltic Sea in northern Sweden. Long Island Sound has plankton communities typical of the northeast Atlantic coast of the United States (1), and the Bothnian Gulf is a boreal brackish water environment. The sampling sites were a beach at Crane Neck, Long Island Sound (40°55.3′ N, 73°09.3′ W; June 1987 to January 1988; salinity, ca. 28‰; samples were obtained with a bucket), and Station Systrarna, Bothnian Gulf (63.5° N, 19.8° E; July 1987; salinity, ca. 5‰; samples were obtained with a pump from a small boat). Samples were brought back to the laboratory in plastic, 20-liter carboys and processed within a few hours of sampling.

The water was prefiltered through glass fiber filters (type A-E; Gelman Sciences, Inc., Ann Arbor, Mich.) to remove most eucaryotic microorganisms (mostly phytoplankton) and to prevent the clogging of the final filter. A filter with a diameter of 47 mm was used with a gentle vacuum in Long Island Sound, and one with a diameter of 142 mm under 0.75 to 1 atm of pressure was used in the Bothnian Gulf. The bacteria were collected by pressure filtration (0.75 to 1 atm) through 0.22-μm-pore-size filters (Durapore; Millipore Corp., Bedford, Mass.). A filter with a diameter of 90 mm was used in a thin-channel concentrator (Amicon Corp., Lexington, Mass.) at Long Island Sound, and one with a diameter of 142 mm was used in a stainless steel in-line filter holder (Millipore) at the Bothnian Gulf. Filters were stored at −20°C until extraction (a few days later). Bacterial abundance in the unfiltered water and filtrates was monitored by epifluorescence microscopy with acridine orange or ethidium bromide staining (5).

For extraction of DNA, the frozen filters were thawed, cut with a clean razor blade into small strips (roughly 2 mm by 1 cm), and vortexed briefly in STE buffer (10 mM Tris hydrochloride [pH 8], 1 mM EDTA, 100 mM NaCl). The 90-mm-diameter filters were suspended in 4 ml and the 142-mm-diameter filters were suspended in 9 ml of STE buffer in 50-ml, conical bottom polypropylene centrifuge tubes. A 0.1 volume of 10% sodium dodecyl sulfate (SDS) was added dropwise with swirling. The tubes were placed into a boiling water bath (>1 liter) for 1.25 to 2 min. In all subsequent steps, care was taken to avoid shearing of the DNA; liquids were never vortexed or vigorously shaken and poured whenever possible. In the pipetting procedures, 1-ml pipet tips cut obliquely to produce a wide hole were used. The liquid was poured into a clean, 15-ml centrifuge tube (Corex), and the filter pieces were rinsed with an additional 1 ml of STE, which was then poured into the centrifuge tube. Cellular debris suspended in the liquid was pelleted by centrifugation (10 min at 10,000 × *g*) (SS-34; Ivan Sorvall, Inc., Norwalk, Conn.) at 12 to 15°C (centrifugation at 4°C was found to precipitate the SDS, trapping much of the DNA).

For purification of DNA from this crude extract, the volume was first reduced by ethanol precipitation to minimize the loss of DNA during the subsequent extraction; for this volume reduction, the supernatant fluid was poured into a 50-ml centrifuge tube, and 3 ml of 10.5 M ammonium acetate plus 28 ml of ice-cold 95 to 100% ethanol were added to precipitate the DNA (for 10-ml extracts; half these amounts were used for 5-ml extracts). Precipitation proceeded at −20°C for at least 2 h, and the DNA was pelleted by centrifugation (20 min, 14,000 × *g*, 4°C) in a swinging bucket rotor (HB4) (angle rotors left the DNA smeared over too large an area of the tube for easy suspension without shearing). The supernatant fluid was poured off and the pellet was air dried and suspended in 0.5 ml of TE buffer (10 mM Tris hydrochloride [pH 8], 1 mM EDTA). The liquid was transferred into a 1.5-ml centrifuge tube (Eppendorf), and 0.5 ml of phenol (equilibrated with TE [pH 8]) was added. The contents were mixed gently by inversion and then centrifuged in a microcentrifuge (2 min, 15,000 × *g*, room temperature). The lower organic phase was removed carefully, 0.1 ml at a time, and the interface plus top aqueous phase were left in the tube. This was reextracted as described above, but with 0.6 ml of phenol-chloroform (3:1). The final extraction was with 0.5 ml of chloroform, and this time the interface was removed. The DNA in the final aqueous extract was precipitated with 0.12 ml of 10.5 M ammonium acetate plus 1 ml of ice-cold ethanol for at least 1 h at −20°C. The DNA was pelleted (10 min, 15,000 × *g*, 4°C), and the supernatant was poured off. The DNA in the tube was dried under vacuum and then gently suspended in 0.3 ml of TE (pH 7.4; at 37°C for >2 h or at room temperature overnight). Note that the DNA was never transferred from the original 1.5-ml tube, minimizing shearing and loss.

Lysozyme, as used by previous investigators (6, 8, 13), was found to be unnecessary for lysis and made purification more difficult because of the additional protein. Similarly, we did manipulations at slightly alkaline pHs and took no precautions to avoid RNase activity since we were not interested in recovering the RNA. As a result, we found that RNA was absent or was, at most, a minor component of our preparations, so it was not necessary to treat the preparations with DNase-free RNase; the elimination of this step improved the recovery of DNA.

The molecular weight distribution of the DNA was analyzed by 0.5 and 0.7% agarose gel electrophoresis (7). DNA

in subsamples from the extracts was quantified either by visual comparison with the molecular weight markers in gels (rough estimate) or by the Hoechst H33258 (Sigma Chemical Co., St. Louis, Mo.) fluorescent dye assay (12). For digestion of the DNA with the restriction endonuclease *Sau*3AI (Bethesda Research Laboratories, Gaithersburg, Md.), 1 μg of DNA (in 10 μl) was digested for 30 min with 5 U of enzyme.

RESULTS AND DISCUSSION

Traditional methods for extracting DNA start with a pellet of packed cells from culture. However, marine microbiologists working with natural populations have generally avoided centrifugation because of the difficulty in getting a visible and representative pellet from the small, sparsely distributed bacteria. Our preliminary attempts at concentrating the bacteria and producing a pellet were generally unsatisfactory, usually because the procedure was too time-consuming or many of the bacteria were missed. These attempts were done by the following methods. The first was by concentration by thin-channel filtration (model TCF10; Amicon) over a filter (Durapore), from a volume of 8 liters to one of 10 to 25 ml, and centrifugation (in a microcentrifuge at 13,000 × *g*) of the concentrate. This method took only about 2 to 3 h, but it yielded only about 10 to 25% of the total bacteria because the other 75 to 90% apparently adhered to the filter. The second method was continuous-flow centrifugation with an SS-34 rotor (Sorvall) and a continuous-flow adapter at approximately 2.5 liters/h and 10,000 rpm. This method was slow and yielded only about 50% of the bacteria, largely because the smaller cells flowed through the system. The third method was hollow-fiber concentration (model H5MP01-43; Amicon) from a volume of 150 liters to one of 3 liters, for subsequent centrifugation (GSA rotor; Sorvall). The initial concentration step yielded only 38% of the bacteria.

While some of these problems might be overcome, DNA could be easily extracted from the organisms on a filter. Given the advantages of speed, simplicity, and cost of filtration, the other methods were not developed further.

Bacterial abundance in the samples ranged from 1.3×10^9 cells per liter in the sample from the Bothnian Gulf to 8×10^9 cells per liter in the sample from Long Island Sound. Direct counts showed that prefiltration through the glass fiber filters removed 5 to 10% of the bacteria, including the largest bacteria and bacteria that were attached to particles. Direct counts of the filtrates from the Durapore filter were indistinguishable from those of the blanks ($<10^7$ cells per liter, or <1% of the total), indicating that the number of bacteria passing through the Durapore filters was negligible. Therefore, >90% of the total bacteria in the samples were collected on the Durapore filters. Without the prefiltration step, virtually all of the bacteria would have been collected, in addition to every other organism in the sample, but this would have significantly slowed the filtration and would have limited the volume that could have been filtered. Although the glass fiber filters are rated by the manufacturer to collect 99.9% of particles greater than 0.3 μm in diameter, in our experience most bacteria (typically, 0.4 to 0.6 μm in diameter) were observed to pass through these filters. Some small eucaryotes also passed through the filters, although they contributed at most a few percent to the total DNA in our near-shore samples (10). These small eucaryotes may contribute more DNA in offshore and subsurface samples (4).

The extraction efficiency was very high. Epifluorescence microscopic examination of suspended bacteria from both sampling sites showed that exposure to 1% SDS for 1 to 2 min at 95 to 100°C left samples that were indistinguishable from blanks ($<10^7$ cells per liter), indicating that >99% of the bacteria lysed (in fact, usually >80% lysed at room temperature). The same procedure also quantitatively lysed a culture of the marine cyanobacterium *Synechococcus* sp. strain WH7803. In preliminary experiments we tried pretreatment with lysozyme and SDS at lower temperatures, as has been done by others (6, 8, 13). However, we found that given the very low initial biomass we were working with, addition of the amount of lysozyme recommended for lysing bacteria increased the total protein and therefore the size of the interface. This led to significant DNA recovery problems during the phenol extractions. Therefore, we substituted one freeze-thaw cycle followed by extraction in hot SDS. We cannot be certain that the hot SDS treatment is adequate for all bacteria from all other environments; lysis efficiency should be determined in each new environment.

The observation of lysis of suspended bacteria strongly suggests, but does not necessarily prove, that all the bacteria on the filter also lysed. Therefore, we made a separate estimate of the extraction efficiency by calculating the expected amount of DNA (from cell counts and literature data on DNA per cell) and comparing it with the amount of DNA measured in the extract by the Hoechst H33258 dye binding assay. In the sample from the Bothnian Gulf, we calculated from direct counts that 3.9×10^{10} bacteria were collected on the Durapore filter from 32 liters of seawater. To obtain the total amount of DNA on the filter, this should be multiplied by 2.6×10^{-15} g, which is the weight of DNA per bacterium (average for marine bacterioplankton [3; F. Azam, personal communication]), so there should have been 101 μg of DNA on the filter. By the dye binding assay we measured 108 μg of DNA in the primary SDS extract (supernatant from the first centrifugation), for a calculated extraction efficiency of 107%. If we used the largest recent estimate of the amount of DNA per marine bacterium, 5.7 fg per cell (11), the estimated efficiency was 49%. The dye binding assay showed that the final purified extract had 49 μg of total DNA, or in other words, 55% of the original extract was lost during the precipitations and solvent extractions. Therefore, the final recovery of purified DNA was calculated to be 23 to 45% of the total bacterial DNA in the original samples. It should be noted that our purification protocol gave priority to the quality of the DNA (high molecular weight and freedom from impurities) rather than yield.

With such a high recovery, it is very likely that the DNA is representative of the total microbial population. Essentially all the bacteria lysed, and any significant selection could only have occurred during the purification steps. Since we have no reason to assume significant variation in the purification efficiency of DNA from the various species, it is probable that all of the losses during purification were random, leaving a wholly representative DNA sample. However, it is also possible that some of the losses were selective, such as by removal of DNA more tightly bound to protein during the phenol extraction. To minimize losses of this sort, we retained the interface during two extractions with different organic solvents.

Electrophoresis in 0.5% agarose showed that a significant portion (visually estimated to be >50%) of the DNA migrated in a single band slightly above the 23-kbp marker band, with the rest being in a broad smear mostly smaller than 2 to 4 kbp (Fig. 1). The lower-molecular-size material was probably sheared DNA; it could not be digested significantly with RNase. The large-molecular-size material was

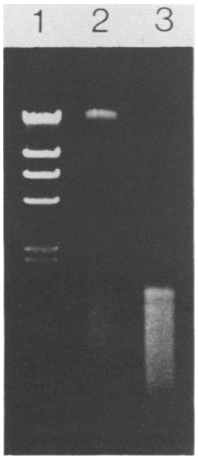

FIG. 1. Ethidium bromide-stained 0.5% agarose gel of total DNA extracted from microorganisms collected from Long Island Sound. Lanes: 1, *Hin*dIII-digested lambda DNA (molecular sizes: 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kbp, from top to bottom, respectively); 2, undigested microbial DNA; 3, microbial DNA digested with *Sau*3AI.

larger than 23 kbp and probably included much larger molecules; such material does not separate significantly from the 23-kbp marker under the electrophoresis conditions we used (7). The large-molecular-size DNA of this extract was significantly larger than the ca. 1 kbp of DNA obtained from soil by Torsvik (13) and was similar to that described by Olsen et al. (8) and Holben et al. (6).

Digestion with the restriction endonuclease *Sau*3AI (which recognizes the 4-base sequence GATC and therefore cuts, on average, every 256 base pairs) resulted in the complete elimination of the larger-molecular-size DNA and an increase in the DNA smaller than 2 kbp (Fig. 1). Thus, the large-molecular-size DNA was completely digested by the enzyme. This suggests that this DNA was not denatured, because the restriction enzyme works only with intact, double-stranded DNA. Also, it shows that the DNA extract did not have impurities that inhibited the enzyme, nor was the DNA modified or otherwise resistant to digestion by this enzyme. The DNA was also pure enough for ligation, an enzymatic reaction that is much more sensitive to impurities.

In conclusion, the procedure described here is simple to perform and yields an excellent recovery of high-purity DNA from natural mixed populations of planktonic microorganisms. We were able to successfully develop a genomic library from this DNA in a modified lambda vector, by subsequent treatment of *Sau*3AI-digested DNA by partially filling it in and ligating it to vector DNA. Thus, the DNA was of sufficient quantity and quality for our own applications and should be equally suitable for other molecular biological manipulations.

ACKNOWLEDGMENTS

We thank Ann-Sofi Rehnstam and SangHoon Lee for help with laboratory procedures, N. Pace for helpful discussions, C. Lee for lending us the thin-channel concentrator, and S. G. Horrigan for critically reviewing the manuscript.

This study was supported by grant OCE 8406712 from the National Science Foundation and by a grant from the Swedish Research Council.

LITERATURE CITED

1. **Deevey, G. B.** 1956. Oceanography of Long Island Sound, 1952–1954. V. Zooplankton. Bull. Bingham Oceanogr. Colloq. **15**:113–155.
2. **Ferguson, R. L., E. N. Buckley, and A. V. Palumbo.** 1984. Response of marine bacterioplankton to differential filtration and confinement. Appl. Environ. Microbiol. **47**:49–55.
3. **Fuhrman, J. A., and F. Azam.** 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. Mar. Biol. **66**:109–120.
4. **Glover, H. E., A. E. Smith, and L. Shapiro.** 1985. Diurnal variations in photosynthetic rates: comparisons of ultraphytoplankton with a larger phytoplankton size fraction. J. Plankton Res. **7**:519–535.
5. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**:1225–1228.
6. **Holben, W. E., J. K. Jansson, B. K. Chelm, and J. M. Tiedje.** 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. **54**:703–711.
7. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Olsen, G. J., D. J. Lane, S. J. Giovannoni, and N. R. Pace.** 1986. Microbial ecology and evolution: a ribosomal RNA approach. Annu. Rev. Microbiol. **40**:337–365.
9. **Pace, N. R., D. A. Stahl, D. J. Lane, and G. J. Olsen.** 1986. The analysis of natural microbial populations by ribosomal RNA sequences. Adv. Microb. Ecol. **9**:1–55.
10. **Paul, J. H., and D. J. Carlson.** 1984. Genetic material in the marine environment: implications for bacterial DNA. Limnol. Oceanogr. **29**:1091–1097.
11. **Paul, J. H., W. H. Jeffrey, and M. DeFlaun.** 1985. Particulate DNA in subtropical oceanic and estuarine planktonic environments. Mar. Biol. **90**:95–101.
12. **Paul, J. H., and B. Myers.** 1982. Fluorometric determination of DNA in aquatic microorganisms by use of Hoechst 33258. Appl. Environ. Microbiol. **43**:1393–1399.
13. **Torsvik, V. L.** 1980. Isolation of bacterial DNA from soil. Soil. Biol. Biochem. **12**:15–21.