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Phylogenetic Analysis of the Bacterial Communities in Marine Sediments

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For the phylogenetic analysis of microbial communities present in environmental samples microbial DNA can be extracted from the sample, 16S rDNA can be amplified with suitable primers and the PCR, and clonal libraries can be constructed. We report a protocol that can be used for efficient cell lysis and recovery of DNA from marine sediments. Key steps in this procedure include the use of a bead mill homogenizer for matrix disruption and uniform cell lysis and then purification of the released DNA by agarose gel electrophoresis. For sediments collected from two sites in Puget Sound, over 96% of the cells present were lysed. Our method yields high-molecular-weight DNA that is suitable for molecular studies, including amplification of 16S rRNA genes. The DNA yield was 47 μg per g (dry weight) for sediments collected from creosote-contaminated Eagle Harbor, Wash. Primers were selected for the PCR amplification of (eu)bacterial 16S rDNA that contained linkers with unique 8-base restriction sites for directional cloning. Examination of 22 16S rDNA clones showed that the surficial sediments in Eagle Harbor contained a phylogenetically diverse population of organisms from the *Bacteria* domain (G. J. Olsen, C. R. Woese, and R. Overbeek, J. Bacteriol. 176:1–6, 1994) with members of six major lineages represented: α, δ, and γ *Proteobacteria*; the gram-positive high G+C content subdivision; clostridia and related organisms; and planctomyces and related organisms. None of the clones were identical to any representatives in the Ribosomal Database Project small subunit RNA database. The analysis of clonal representatives is the first report using molecular techniques to determine the phylogenetic composition of the (eu)bacterial community present in coastal marine sediments.

In recent years molecular phylogenetic analysis has been used to characterize microbial subpopulations and communities in a variety of environments (1). Since only 0.001 to 1% of existing bacteria are cultivable (14, 59), investigators have turned to modern molecular tools based on the PCR and phylogenetics of the 16S rRNA gene (9, 44, 60, 62). Phylogenetic procedures provide information that may complement or augment the data that is derived from culture-based procedures.

In analyzing environmental samples extraction and purification of DNA can be problematic due to a variety of factors (33). To solve these problems, some investigators have attempted to remove the microbial community from the environmental matrix (2, 23, 51, 56), while others have chosen to lyse the cells in situ (2, 5, 7, 29, 35, 37, 40, 51, 55). The primary concerns of either approach are the efficiency of cell lysis as well as integrity and purity of the extracted DNA. In general the in situ approach produces more quantitative results; the lysis efficiencies can be more than 1 order of magnitude superior compared to cell removal techniques (33). Several investigations have focused on these concerns as they apply to lysis procedures based on bead mill homogenization (27, 35, 40, 51). Moré et al. (35) found that a combination of sodium dodecyl sulfate (SDS) and 5 min of bead mill homogenization produced lysis efficiencies of 98% in freshwater sediments. Ogram et al. (40) found that incubation with SDS at 70°C for 1 h followed by 5 min of bead mill homogenization produced lysis efficiencies of greater than 90% in marine sediments.

The purity and quality of the extracted DNA is an important concern since it must be suitable for PCR amplification. There seem to be nearly as many purification schemes published as there are lysis techniques. Because agarose gel electrophoresis effectively removes humic and other enzyme inhibitory compounds from indigenous soil bacterial DNA (32), we pursued this approach for marine sediments. We also wanted intact, high-molecular-weight chromosomal DNA because it has been reported that chimeric PCR product formation increases substantially with increased template fragmentation (25, 26, 54). High-molecular-weight DNA can be isolated by gel electrophoresis.

Only a few investigators have reported methods for the extraction of DNA from marine sediments for phylogenetic studies (11, 28, 49); where reported, the resulting analyses have been selective for very specific types of microorganisms (10, 28). Our report is the first to describe the phylogenies of the (eu)bacterial community within marine nearshore sediments. We collected surficial sediments from Eagle Harbor (EH) and Blakely Harbor (BH), Wash., and describe a procedure for lysis and extraction of DNA from marine sediments. The protocol provides nearly complete cell lysis with resulting high-molecular-weight DNA of purity sufficient for molecular studies. Total community DNA was amplified in a PCR using universal bacterial primers for the 16S rRNA gene, and the amplified DNA was ligated into a plasmid sequencing vector. Twenty-two representative clones were partially sequenced. From a phylogenetic analysis of the environmental clones, we were able to perform a preliminary examination of the diversity of the bacterial community in the EH sediments.

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MATERIALS AND METHODS

Sample site background and sample collection. EH and BH are located off Bainbridge Island in Puget Sound, Wash. (Fig. 1). The sediments of EH are heavily contaminated with coal-tar creosote that is thought to have been released

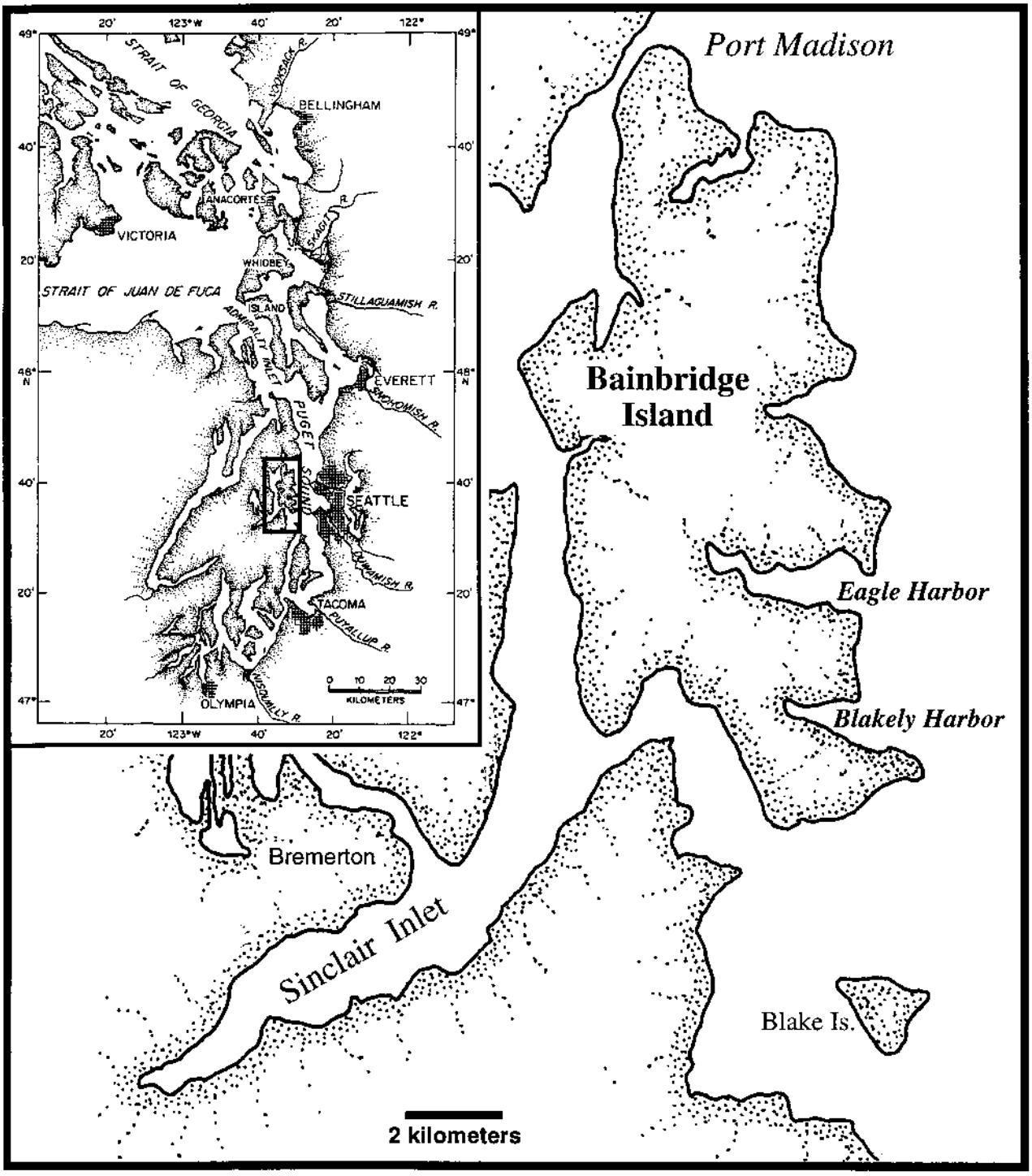

FIG. 1. Location of EH and BH sediment sampling sites in Puget Sound, Wash. The area is boxed in the insert.

by a wood treatment facility located along the southeastern shore. Creosote is composed largely of polycyclic aromatic hydrocarbons (PAHs) (39), compounds that may persist in the environment. EH was designated a national Superfund site by the Environmental Protection Agency (EPA); the distribution and ecological impact of the chemical contaminants were characterized in previous reports (58). During the fall of 1994, the EPA with the assistance of the U.S. Army Corps of Engineers began a containment cap project, in which approximately 1 m of uncontaminated dredge material from another Puget Sound site was placed over the highly contaminated sediments within the central portion of EH (22).

For the development of the cell lysis and DNA purification procedure, we collected sediment samples from EH and BH during sampling trips made by the University of Washington's R.V. *Clifford Barnes* in 1994. Sediment used for creating the 16S rDNA clone libraries was collected on 14 April 1995 with a ship-board box core sampler (box dimensions, 21.5 by 30.5 by 60 cm). This oceanographic sampler allowed for the collection of large volumes of relatively undisturbed sediment. The 1995 EH sampling site was located immediately east of the containment cap at the coordinates 47°37.209′N, 122°30.051′W in 13 m of overlying water. The BH site was located at 47°35.759′N, 122°30.590′W in 12 m of overlying water. The sediment temperatures at both sites were measured with a thermometer and recorded at 12°C. For both sites the top 1 cm of sediment was removed with a sterile spoon, placed into a sterile plastic 4-liter beaker, and mixed by hand. The beaker containing the mixed surficial sediment was held on

ice, and the sediment was distributed into sterile 15-ml conical plastic tubes (Sardstedt). These tubes were maintained on ice for 6 h (EH) or 3 h (BH) and then frozen at −80°C for 15 days. The tubes were then thawed on ice for 1.5 h before DNA extraction. Sediment dry weights were determined by drying sediment samples at 104°C for 24 h.

Total microbial counts. The total number of microorganisms was determined for the sediment samples used for extracting DNA by enumerating cells stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) and using a Zeiss Axioskop 20 epifluorescence microscope. Sediment samples were fixed with 2% glutaraldehyde during the development of our cell lysis procedure. Sediment was diluted in filter-sterilized Marine Diluent (NaCl, 22.79 g; Na_2SO_4, 3.98 g; $MgCl_2 \cdot 6H_2O$, 11.18 g; $CaCl_2 \cdot 2H_2O$, 1.46 g; distilled water, 1,000 ml) and gently vacuum filtered (200 mTorr) onto 25-mm-diameter, 0.2-μm Nuclepore black membrane filters. The filtered cells were rinsed with 95% ethanol and placed on a glass slide. A drop of mounting fluid [50% glycerol, 10 mM *N*-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), thimerosal (100 mg/ml) (pH 8.2)] was placed on the filter and covered with a glass coverslip.

DNA extraction and purification. Total community DNA was extracted directly from marine sediment. The lysis buffer contained a high salt concentration based on the results of Moyer et al. (37), who reported that high concentrations of salt (700 mM) help prevent DNA shearing during mechanical disruption. EDTA was added to inhibit nuclease activity. The NaCl was maintained at 500 mM, while the SDS concentration was increased to a maximum soluble level. Polyvinylpolypyrrolidone (Sigma) was added to the extraction mix to adsorb the humic compounds (2, 49). The DNA was purified by using a modification of the method described by Moré et al. (35). Unless otherwise described below, the formulas for all buffers and molecular procedures are detailed by Ausubel (3).

Four hundred milligrams of sediment was placed into a 2-ml Biostor vial (Island Scientific, Bainbridge Island, Wash.) containing 2 g of 0.1-mm zirconia/silica beads (BioSpec Products, Bartlesville, Okla.) and 20 mg polyvinylpolypyrrolidone (Sigma). Nine hundred microliters of preheated (1 h at 55°C) lysis buffer (500 mM NaCl, 50 mM EDTA, 4% SDS, and 50 mM Tris [pH 8.0]) was added. A tube containing no sediment served as the negative control. The tubes were homogenized for 1 min on a Mini-Beadbeater-8 Cell Disrupter (BioSpec Products) at maximum setting and then incubated in a 70°C water bath for 1 h. Next, they were centrifuged at 16,000 × *g* for 2 min in a Micro Centrifuge (Eppendorf), and the supernatant was transferred to a 1.7-ml microcentrifuge tube. Supernatants were incubated on ice for 15 min and then centrifuged at 16,000 × *g* for 5 min at 4°C. The supernatants were transferred to 1.7-ml microcentrifuge tubes. Two volumes of 100% ethanol at −20°C were added to each tube, which was gently inverted 20 times, incubated at −20°C for 15 min, and centrifuged at 16,000 × *g* for 5 min at 4°C. Supernatant was gently poured off, and the pellets were washed with 1 ml of 70% ethanol at −20°C. The tubes were inverted, allowed to air dry, and resuspended in 50 μl of Tris-EDTA (TE) (pH 7.5). The crude DNA suspensions were purified with SpinBind cartridges (FMC BioProducts, Rockland, Maine) by following FMC instructions (15) with elution into 50 μl of TE (pH 7.5). The volumes of these eluates were reduced to 20 μl with a SpeedVac (Savant). Then, they were electrophoresed on a 1% (wt/vol) agarose gel (Sigma type 1-A) in 1× TAE containing 1 μg of ethidium bromide (Sigma) per ml at 4.8 V/cm for 3 h. A 1-kb DNA ladder (Gibco BRL, Gaithersburg, Md.) was run with each gel. Gels were photographed (Fig. 2) with a Foto/Eclipse (Fotodyne, New Berlin, Wis.) and Video Image 1200 software (Scion Corporation, Frederick, Md.) running on a Macintosh IIci personal computer. High-molecular-weight regions were excised with a razor blade and purified with SpinBind cartridges (FMC BioProducts) by following FMC instructions (15) with elution into 50 μl of 10 mM Tris (pH 8). The DNA quantity and purity were determined with a GeneQuant II (Pharmacia Biotech), which automatically determined the absorbancies at 260 and 280 nm.

PCR amplification. Primers for PCR amplification of bacteria were based on the fD1 and rP1 primers described by Weisburg et al. (60) and called 8FB (5′-GAGGCGCGCCGAGTTTGATCCTGGCTCAG-3′) and 1492RU (5′-TTTTAATTAAGGTTACCTTGTTACGACTT-3′) in our study. These primers corresponded to bases 9 to 27 and 1510 to 1491 of the *Escherichia coli* 16S rRNA gene sequence (6). Primers for amplification of 16S rDNA from an archaeal strain, *Methanococcus maripaludis*, were 23mFA (5′-GAGGCGCGCCATTCYGGTTGATCCYGCC-3′) slightly modified from the 23FPL primer described by Barns et al. (4) and 1492RU. An *Asc*I (New England Biolabs [NEB], Beverly, Mass.) restriction site linker was incorporated into 8FB and 23mFA; a *Pac*I (NEB) site linker was incorporated into 1492RU at the 5′ ends. For amplification reactions we used 200 ng of DNA template in a 25-μl volume and performed the PCR in 0.2-ml thin-wall tubes on a Minicycler-25 (MJ Research) using the conditions described by Dyksterhouse et al. (12). The PCR products were purified by using Qiaquick columns (Qiagen, Chatsworth, Calif.) with elution into 45 μl of TE.

Cloning and sequencing. Forty-three-microliter aliquots of the Qiaquick-purified PCR products were placed into 1.7-ml microcentrifuge tubes. To each tube we added 5 μl of NEB buffer 4, 1 μl of *Asc*I, and 1 μl of *Pac*I. These were allowed to incubate at 37°C for 14 h. The volume (50 μl) was reduced to 20 μl with a SpeedVac. The product was electrophoresed on a 1% agarose gel (Sigma) in 1× TAE containing 1 μg of ethidium bromide per ml at 4.8 V/cm for 2 h (Fig. 2C). The 1.5-kb bands were excised with a razor blade. DNA was isolated from the gel by passing the excised bands through SpinBind cartridges according to an FMC protocol (16). DNA was eluted into 50 μl of TE (pH 7.5) and concentrated to a 20-μl volume with a SpeedVac. To 5 μl of this was added 250 ng of a *Pac*I- and *Asc*I-digested pNoTA/T7 vector (5 Prime→3 Prime, Boulder, Colo.), 1 μl of 10× ligase buffer, 1 μl of 5 mM ATP, 1 μl of 100 mM dithiothreitol, and 1 U of T4 DNA ligase (Gibco BRL). The ligation mixtures were incubated for 14 h at 22°C

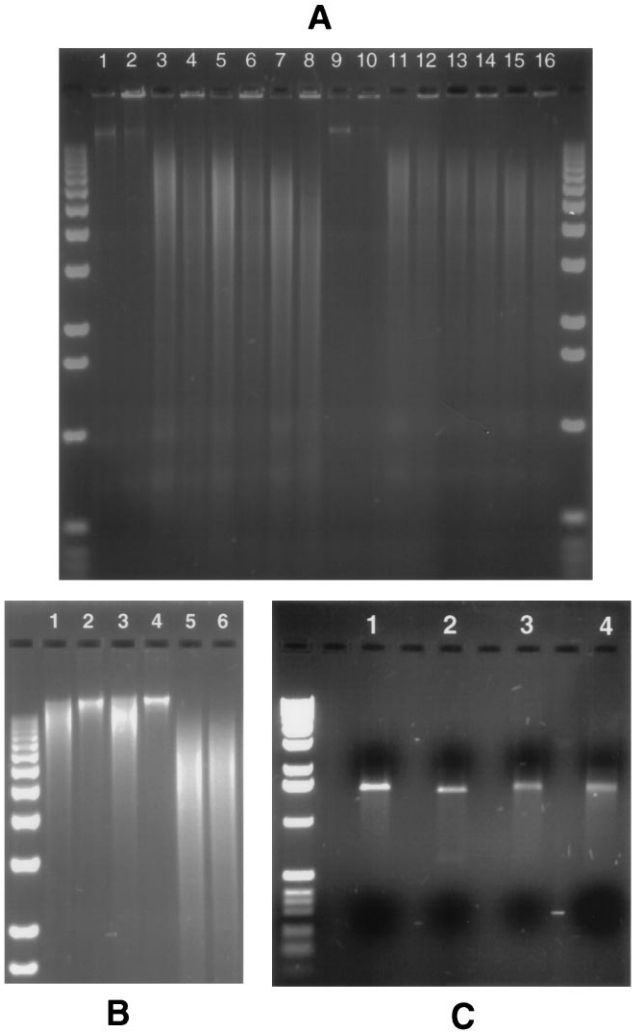

FIG. 2. Gel electrophoresis data from the development of lysis and 16S rDNA amplification procedures for microorganisms in marine sediments. One-kilobase DNA ladders (Gibco BRL) are included on the gels. (A) Effect of 2-min boiling and length of time for bead beating on extractable DNA. Lanes 1 to 8, EH samples; lanes 9 to 16, BH samples. Lanes 1 and 9, 0-min bead beating; lanes 2 and 10, 2.0-min boiling followed by 0-min bead beating; lanes 3 and 11, 5.0-min bead beating; lanes 4 and 12, 2.0-min boiling followed by 5.0-min bead beating; lanes 5 and 13, 7.5-min bead beating; lane 6 and 14, 2.0-min boiling followed by 7.5-min bead beating; lanes 7 and 15, 10.0-min bead beating; lanes 8 and 16, 2.0-min boiling followed by 10.0-min bead beating. Note the degradation of DNA with increased bead beating and that the 2.0-min boiling step caused the DNA to stay in the gel wells. (B) Effect of 70°C incubation on the DNA following bead beating. Lane 1, 1.0-min bead beating followed by 70°C for 1.0 h; lane 2, no bead beating and 70°C incubation for 1.0 h; lane 3, 1.0 min bead beating followed by 70°C incubation for 15.0 min; lane 4, no bead beating and 70°C incubation for 15.0 min; lanes 5 and 6, 7.5-min bead beating only. Note that the bead beating step caused shearing of the DNA and that increasing the time of bead beating caused the DNA size to decrease. The total nucleic acid yields from 400 mg of starting sediment were quantified by measuring the absorbancy at 260 nm and were 102, 51, 66, 43, and 64 μg (lanes 1 to 5, respectively). (C) PCR amplification of 16S rDNA from control strains *E. coli* (lane 1) and *Methanococcus maripaludis*, a marine methanogen (lane 2), and from the extracted sediments of EH (lane 3) and BH (lane 4). The DNA from *E. coli* and the sediment samples were amplified with primers designed for the *Bacteria*, while the *M. maripaludis* DNA was amplified with primers for the amplification of *Archaea*. Note that the bands from the sediment DNA are somewhat fuzzy compared to bands from the pure cultures.

in the dark. One microliter of each ligation mixture was transformed into 40 μl of electrocompetent *E. coli* DH5α with a Gene Pulser (Bio-Rad) set to 1.5 kV, 200 Ω, and 25 μF. Three-tenths of each transformation product was plated onto Luria broth agar (LBA) (3) plus 100 μg of ampicillin per ml, 1 mM IPTG, and 0.015% X-Gal. After an 18-h incubation at 37°C representative transformants showing the Lac^- phenotype (white colonies) were picked with sterile toothpicks and transferred twice on LBA containing 100 μg of ampicillin per ml. Twenty-five transformants were picked; the recombinant plasmids were isolated from the individual transformants by using a boiling lysis miniprep (52). The plasmids were screened for insertions by performing digestions with *Bam*HI and electrophoresis on a 1% agarose gel. A total of 22 16S rDNA clones were sequenced on a 373A automated DNA sequencer (Applied Biosystems) using the −20 Sequencing Primer (5 Prime→3 Prime) and Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems).

Phylogenetic analysis. For each of the 16S rDNA clonal sequences a query was made to the Ribosomal Database Project (RDP) by using the Similarity_Rank analysis service (34) and to GenCANS (63) to suggest the closest relatives within the RDP small subunit prokaryote rRNA database. Data retrieved from the RDP were from release 5.0 (17 May 1995). The primary sequences for the EH clones were manually aligned to 16S rRNA secondary structures of organisms representative of six different phylogenetic groups (20, 21). This method allowed for the best approximation where secondary structure folds occur in the hypervariable regions of the clones of the EH 16S rDNA. References to sequenced microorganisms or environmental clones and to prealigned gapped sequences were electronically retrieved from RDP by electronic mail or by making inquiries to the World Wide Web site on the Internet (34). Sequence and gap editing was performed with the Macintosh program SeqApp (18); the phylogenetic relationships for the various clones were determined by using the Unix version of the maximum-likelihood-based phylogenetic program fastDNAml (42). This program was run on a University of Washington computer (IBM AIX version 3 for RISC System/6000).

Before beginning the analysis with the EH clones, category rates for each position of the 16S rRNA molecule needed to be determined for the six phylogenetic groups. Thirty to thirty-five randomly selected 16S rRNA sequences from each of the six different phylogenetic groups were retrieved from the RDP and used for approximating the rates for each position along the 16S rRNA molecule by running the program DNAml_rates (41) and fastDNAml. For each phylogenetic group nine category rates were separately determined. The rates determined after running DNAml_rates along with the primary sequence information from the representative organisms or environmental clones were used by fastDNAml to construct a phylogenetic tree. The process of tree construction using fastDNAml, followed by determination of the category rates using DNAml rates, was repeated three times; the category rates calculated after the third repeat were used in the final phylogenetic analysis of the EH clones.

In addition to the category rates option the following fastDNAml options were also selected: transition/transversion rate was set to 1.5, global rearrangement was used, and for a given input file the sequence order was jumbled a maximum of 15 times until the best tree was found three times. The 16S rRNA region relative to *E. coli* bases 15 to 365 was utilized for phylogenetic characterization of 22 EH clones. This region spans two hypervariable regions of the 16S rRNA molecule (20, 21). We examined the RDP database to find organisms or environmental clones that were most closely related to the EH clones or located on nearby tree branches. In addition an outgroup organism was selected for each phylogenetic tree. Tree files that were calculated by fastDNAml were plotted with the Macintosh program NJplot (19). We used the RDP program called Check_Chimera to examine for chimeric sequences among the EH clones.

The 16S rRNA gene sequences for the EH clones and representative organisms were also compared by calculating similarity matrices. The aligned sequences used in the phylogenetic analysis were examined by using a Unix version of the Genetics Computer Group (GCG) program Distances with no distance correction (17). This program was available on a University of Washington computer running the Unix AIX version 3.2.5.1 system. The substitution matrix calculated by this GCG program was converted to a similarity percentage in the matrices by subtracting the substitution values from 100.

Sequence of organisms used for constructing phylogenetic trees. Listed below are the representative organisms or environmental clones (34) within the six different phylogenetic groups that were used to construct phylogenetic trees. If known, strain and culture collection numbers are also listed. Except for the environmental clones, nearly full-length 16S rRNA sequences for the closely related representatives and outgroup organisms were used in our phylogenetic analyses.

(i) α *Proteobacteria*. The following α proteobacteria were used: *Agrobacterium tumefaciens* (ATCC 4720), *Azospirillum brasilense* (ATCC 29145), *Beijerinckia indica* subsp. *indica* (ATCC 9039), *Bradyrhizobium japonicum* (LMG 6138), *Caulobacter* sp. strain FWC14, *Caulobacter* sp. strain FWC38, *Methylosinus sporium*, *Methylosinus trichosporium*, *Rhizobium huakuii*, *Rhizobium leguminosarum*, *Rhodobium orientis* (*Rhodohalobium orientum*) MB312, *Rhodomicrobium vannielii* EY33 (ATCC 51194), *Rhodoplanes elegans* AS130, *Rhodospirillum rubrum* ATH 1.1.1; S.1 (ATCC 11170), *Rhodospirillum salexigens* (ATCC 35888), *Rickettsia prowazekii* Breinl (ATCC VR 142), *Sphingomonas capsulata* (ATCC 14666), and *Hyphomicrobium*-like strain US-353.

(ii) δ *Proteobacteria*. The following δ proteobacteria were used: *Bdellovibrio stolpii* UKi2 (ATCC 27052), *Chondromyces apiculatus* Cm a2, *Chondromyces crocatus* Cm c6, *Desulfobacter postgatei* 2 ac 9 (DSM 2034), *Desulfobulbus propionicus* 1 pr 3, Lindhorst (ATCC 33891), *Desulfococcus multivorans* 1 be 1, Goettingen (ATCC 33890), *Desulfomonile tiedjei* DCB-1 (ATCC 49306), *Desulfosarcina variabilis* 3 be 13, Montpellier (DSM 2060), *Desulfovibrio desulfuricans* (ATCC 27774), *Desulfuromonas acetoxidans* (DSM 684; strain 11070), *Myxococcus xanthus* DK1622, *Nannocystis exedens* Na e1 (ATCC 25963), *Stigmatella aurantiaca* (ATCC 25190), and paddy field clones PAD12, PAD15, and PAD25.

(iii) γ *Proteobacteria*. The following γ proteobacteria were used: *Burkholderia pickettii* (ATCC 27511), *Chromatium vinosum* (ATCC 17899), *Cycloclasticus pugetii* (ATCC 51442), *E. coli* K-12, *Ectothiorhodospira halochloris* A (ATCC 35916), *Legionella jamestowniensis* (ATCC 35298), *Legionella oakridgenesis* (ATCC 33761), *Marinobacter hydrocarbonoclasticus* SP 17 (ATCC 49840), *Marinomonas vaga* (ATCC 27119), *Methylomonas methanica* S1 (ATCC 35067), *Methylomonas rubra* VKM-15m (NCIMB 11913), *Oceanospirillum commune* (ATCC 27118), *Oceanospirillum linum*, *Oceanospirillum multiglobuliferum* (ATCC 33336), *Pseudomonas aeruginosa* (ATCC 10145), *Rhodocyclus purpureus* 6770, *Xanthomonas maltophila* (ATCC 13637), protobacterial strain SCB11, a symbiont from a *Codakia costata* gill, a symbiont from a *Solemya reidi* gill, and a symbiont from a *Thyasira flexuosa* gill.

(iv) Gram-positive, high-G+C-content. The following gram-positive, high-G+C-content bacteria were used: *Arthrobacter globiformis* (DSM 20124); *Atopobium parvulum* 1246 (ATCC 33793); *Bifidobacterium bifidum* (ATCC 29521); *Corynebacterium mediolanum* (DSM 20152); *Corynebacterium xerosis* (ATCC 373); *Streptomyces ambofaciens*; *Streptomyces coelicolor* A3(2); Mount Coot-tha region (Brisbane, Australia) 5- to 10-cm-depth soil DNA clones MC 19, MC 58, and MC 87; and paddy field clone PAD13.

(v) Clostridia and related organisms. The following clostridia and related organisms were used: *Acetogenium kivui* (ATCC 33488), *Clostridium barkeri* (ATCC 25849), *Clostridium butyricum* E.VI.3.6.1 (ATCC 860), *Clostridium fervidus* RT4. B1 (ATCC 43204), *Clostridium leptum* (ATCC 29065), *Clostridium pasteurianum* (ATCC 6013), *Clostridium quercicolum* (ATCC 25974), *Clostridium symbiosum* (ATCC 14940), *Eubacterium eligens* (ATCC 27750), *Eubacterium fissicatena* (DSM 3598), *Heliobacterium chlorum* (ATCC 35205), and *Ruminococcus torques* (ATCC 27756).

(vi) Planctomyces and related organisms. The following planctomyces and related organisms were used: *Chlamydia pneumoniae* TW183, *Chlamydia psittaci* 6 BC (ATCC VR 125), *Gemmata ovscuriglobus* UQM 2246, *Pirellula marina* (ATCC 49069), *Planctomyces limnophilus* (ATCC 43296), *Planctomyces maris* (ATCC 29201), *Planctomyces (Pirellula) staleyi* (ATCC 27377), *Verrucomicrobium spinosum* (IFAM 1439), marine snow-associated clone aggregates AGG27 and AGG8, Mount Coot-tha region soil clone MC 18, Pacific Ocean station 25 100-m-depth bacterioplankton DNA clone NH25-19, and paddy field clone PAD41.

Accession numbers for 16S rDNA clones. Partial sequences of 22 16S rRNA genes cloned from EH sediments were submitted to GenBank and have the following accession numbers: U43630, U43631, U43632, U43633, U43634, U43635, U43636, U43637, U43638, U43639, U43640, U43641, U43642, U43643, U43644, U43645, U43646, U43647, U43648, U43649, U43650, and U43651.

RESULTS

Development of cell lysis and DNA extraction procedure. Initially, we wanted to determine the minimum amount of time required to allow the bead mill homogenization to completely lyse cells in the sediment. A time course experiment was performed with 0- to 10-min bead mill homogenization with sediments from EH and BH (Fig. 2A). This experiment also included a 2-min boiling step prior to bead beating to observe what effect this might have on nuclease deactivation. We found that the boiling step caused cell lysis and denaturation of genomic DNA based on the observed lack of electrophoretic mobility in these preparations (Fig. 2A, lanes 2, 4, 6, and 8 for EH sediment and lanes 10, 12, 14, and 16 for BH sediment). We found that, as the time of bead beating increased, the size of the DNA tended to decrease. The DNA yield only slightly increased with extended bead beating (data not shown). Exposure to warm (55°C) lysis buffer alone resulted in a significant release of intact DNA (Fig. 2A, lane 1 for EH sediment and lane 9 for BH sediment). The step of heating the lysis buffer to 55°C was initially performed to help dissolve the SDS but was later found to be an important step for obtaining reproducible results (data not shown).

Based on these preliminary results we decided to reverse the order of the disruption and lysis scheme and lower the incu-

bation temperature. We used a 1-min bead beat followed by a 70°C incubation, both steps in the presence of the lysis buffer. The rationale was that the bead beat would homogenize and disperse the sediment sample and partially lyse the cells present and then the lysis would be completed by the 70°C SDS incubation. An experiment was performed to determine the effect of bead beating and incubation at 70°C on the total nucleic acid yield (Fig. 2B) and the total direct counts of microorganisms. With DAPI-stained preparations and epifluorescence microscopy, we observed that the 1-min bead beating period disrupted the sediment matrix, dislodged attached bacteria, and disintegrated clumps of cells. This step followed by incubation at 70°C produced DNA of comparable yield and higher molecular weight relative to that of extended bead disruption. The bead beating step followed by a 1-h 70°C incubation also produced the largest amount of DNA as determined by measuring the absorbance at 260 nm. For the data shown in Fig. 2B, 102 μg of nucleic acid was recovered with 400 mg of starting sediment for the combined bead beat and 70°C treatment (lane 1), nearly twice as much as either bead beating (lane 5) or the 70°C treatment (lane 2) performed alone. The high DNA yield, the recovery of high-molecular-weight DNA, and the efficient cell lysis (see below) showed that starting with 400 mg of wet marine sediment, using a high-salt SDS buffer, and a 1-min bead homogenization followed by 1 h at 70°C was a very effective method.

Sediment DNA extraction and 16S rDNA amplification. One extraction tube per site was made with EH and BH sediments. Observations of DAPI-stained samples prepared before and after the cell lysis procedure indicated that over 96% of the microorganisms were lysed. Following the bead beating and 70°C incubation steps described above, the extractions produced pellets that were brown. The DNA at this stage was still unsuitable for molecular studies; it did not cut with restriction enzymes nor could the 16S rDNA be amplified with the PCR. These crude preparations were passed through SpinBind columns which removed most of the brown color from them. Following electrophoresis on a 1% agarose gel, the high-molecular-weight region (DNA greater than 12 kb) of the genomic smear was excised. The DNA was isolated from the agarose slice by using SpinBind columns.

Our amplification primers contained eight-cutter restriction enzyme sites, *Pac*I and *Asc*I, at their 5′ ends. We determined using the RDP Check_Probe analysis service (34) that these restriction sites were found in only 25 and 23, respectively, of the 2,849 sequences present in the small-subunit-prokaryotes database. The purified DNA from the sediments was amplified by the PCR and digested with the *Pac*I and *Asc*I restriction enzymes; the products were purified and sized by electrophoresis on a 1% agarose gel (Fig. 2C). The 1.5-kb bands derived from the EH and BH sediment extractions (Fig. 2C, lanes 3 and 4) were slightly "fuzzy" compared to the PCR 16S rDNA products from pure cultures of bacteria (Fig. 2C, *E. coli* in lane 1 and *Methanococcus maripaludis* in lane 2). This suggested that the EH and BH amplified DNA contained a mixture of differently sized 16S rDNA, an interpretation later supported in screening the 16S rDNA clones on agarose gels (data not shown).

16S rDNA sequencing and examination for chimeric sequences. The average length of the EH sequences examined by the fastDNAml program and other analyses was 338 bases but ranged from 312 to 358 bases. Using the RDP analysis service Check_Chimera, we concluded that 21 of the EH clones are probably not chimeric because their Check_Chimera histogram values did not steadily rise and fall and their maximum "oligo-gain values" were less than 28. One clone, EH-5, was closest to appearing chimeric because it had a maximum "oligo-gain" value at position 140 and a symmetrical rise and fall of values from this position. The highest S_ab scores for each fragment, however, belonged to members of the γ *Proteobacteria.*

Phylogenetic and similarity analyses. The 22 clones that we sequenced fell into six major lineages of the *Bacteria* domain (43): the α, δ, and γ *Proteobacteria*; gram-positive organisms with high G+C content; the clostridia and related organisms; and the planctomyces and related organisms. None of the clones were identical to any of the known 16S rRNA sequences from cultured organisms or environmental clones. Of the 22 clones, five pairs were either duplicate or nearly identical clones, so for the 22 sequenced in our study, 17 distinctively different clones were found. The phylogenetic placement of the EH clones within the six major bacterial groups is shown in Fig. 3 and 4. The largest number, eight of the clones (36%), were members of the γ *Proteobacteria* (Fig. 4B). Within this phylogenetic group, two clones (EH-9 and EH-26) were identical to each other. Two different lineages contained four clones (18% each): the δ *Proteobacteria* (Fig. 4A) and the clostridia and related organisms (Fig. 3C). The δ *Proteobacteria* contained four different clones, and the clostridial group contained one pair of identical clones (EH-19 and EH-21). The other lineages—the α *Proteobacteria* (Fig. 3A), planctomyces and related organisms (Fig. 3B), and the gram-positive high-G+C-content subdivision (Fig. 3D)—each contained a single pair of clones that were identical or nearly identical to each other.

Tables 1 through 6 present a set of similarity matrices for the EH clones and their closest phylogenetic relatives among six major phylogenetic groups. Organisms having the highest S_ab values for the EH clones using the RDP's Similarity_Rank program, were also included in our similarity analysis. Organisms having the highest S_ab values were not always the same organisms having the nearest phylogenetic affinity as determined by the maximum likelihood analysis. The EH clones had the greatest similarities to other EH clones, and two pairs of clones were identical to each other (EH-20 and EH-23, and EH-4 and EH-13) and three other EH clone pairs had a similarity greater than 97% (EH-7 and EH-14, EH-19 and EH-21, and EH-8 and EH-25). For the α *Proteobacteria*, the EH clones had a sequence similarity of 92.9% to *M. sporium*, 90.7% to *Caulobacter* sp. strain FWC14, and 90.0% to *R. orientum*. An 87.8% similarity value between EH-2 and *D. multivorans* was the highest seen among the δ *Proteobacteria* representatives. The highest values in the γ *Proteobacteria* were found for EH-11 with *M. hydrocarbonoclasticus* (92.2%) and *C. pugetii* (88.9%) and for EH-3 and EH-15 with a gill symbiont of *C. costata* (90.3 and 89.4%, respectively). The EH clones that were members of the gram-positive high-G+C and clostridium clusters had some of the lowest similarity values to RDP database sequences. EH-4 and EH-13 were most similar, approximately 82%, to other uncultured environmental clones (Mount Coot-tha clones MC 19 and MC 87). EH-19 and EH-21 had the highest similarity values, 87%, with *E. eligens* and *E. fissicatena*; EH-6 and EH-22 had values less than 80% with others in the clostridial lineage. The planctomyces-related clones EH-8 and EH-25 could not be compared to the closest phylogenetic relative, environmental clone PAD41 (Fig. 3B), since only partial, nonoverlapping sequences were available for these clones. The EH planctomyces-related clones had the highest similarity to *P. maris*, although this species was phylogenetically more distant than *P. marina* or the environmental clone AGG8 (Fig. 3B).

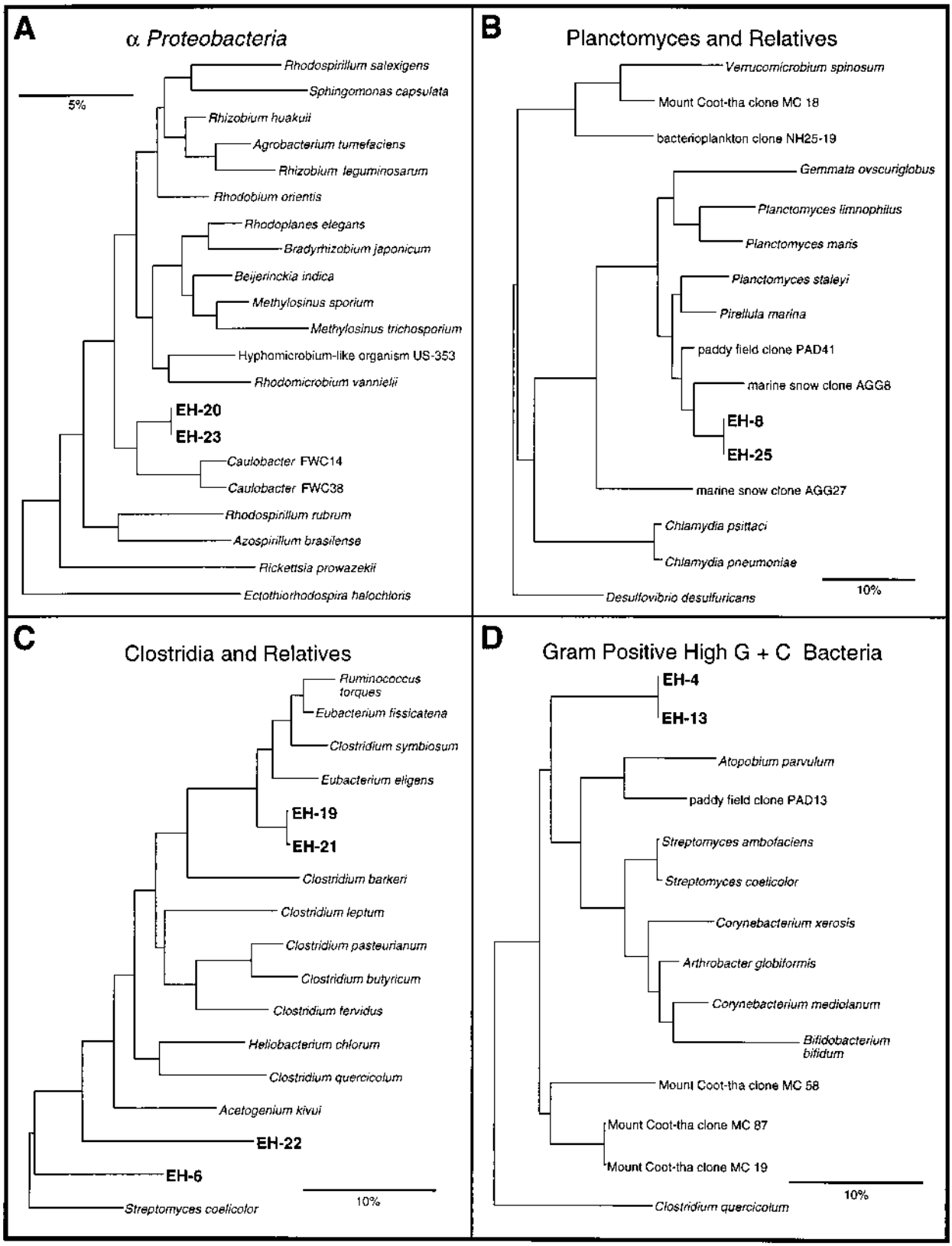

FIG. 3. (A) Phylogenetic tree of the EH 16S rDNA clones that are members of the α *Proteobacteria*. Bar, 5% nucleotide change per 16S rRNA position. *Ectothiorhodospira halochloris*, a member of the γ *Proteobacteria*, served as the outgroup. (B) Phylogenetic tree of the EH 16S rDNA clones that are members of the planctomyces-and-relatives lineage of the *Bacteria*. Bar, 10% nucleotide change per 16S rRNA position. *Desulfovibrio desulfuricans*, a member of the δ *Proteobacteria*, served as the outgroup. (C) Phylogenetic tree of the EH 16S rDNA clones that are members of the clostridia-and-related-organisms lineage of the *Bacteria*. Bar, 10% nucleotide change per 16S rRNA position. *Streptomyces coelicolor*, a member of the gram-positive high-G+C-content group, served as the outgroup. (D) Phylogenetic tree of the EH 16S rDNA clones that are members of the gram-positive high-G+C-content subdivision of the *Bacteria*. Bar, 10% nucleotide change per 16S rRNA position. *Clostridium quercicolum*, a member of the gram-positive low-G+C-content group, served as the outgroup.

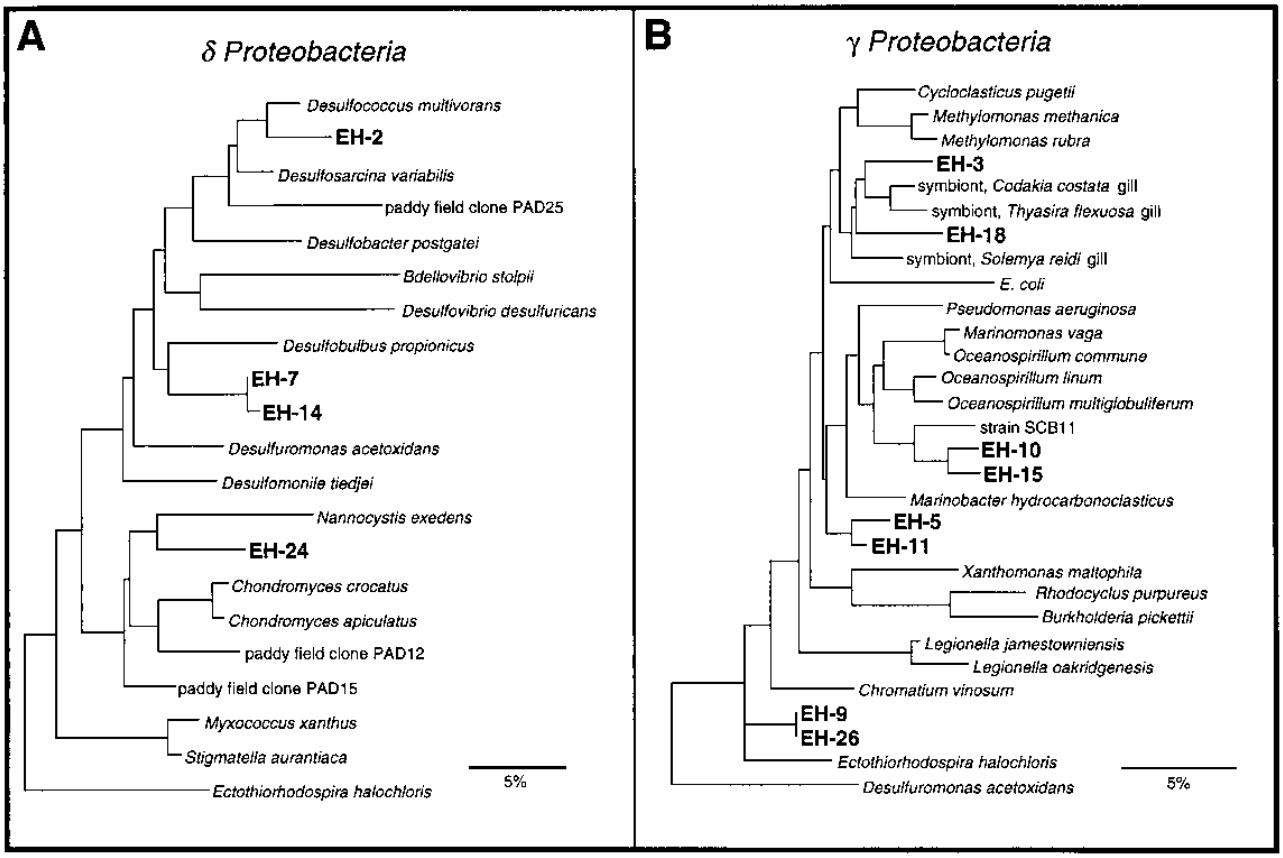

FIG. 4. (A) Phylogenetic tree of the EH 16S rDNA clones that are members of the δ *Proteobacteria*. Bar, 5% nucleotide change per 16S rRNA position. *Ectothiorhodospira halochloris* is a member of the γ *Proteobacteria* and served as the outgroup. (B) Phylogenetic tree of the EH 16S rDNA clones that are members of the γ *Proteobacteria*. Bar, 5% nucleotide change per 16S rRNA position. *Desulfuromonas acetoxidans*, a member of the δ *Proteobacteria*, served as the outgroup.

DISCUSSION

The protocol described here was effective for characterizing bacterial populations in marine sediments. We successfully extracted and amplified the 16S rDNA using sediment from two different marine locations in Puget Sound and also with material from an anaerobic fluidized bed bioreactor that degrades perchloroethylene (data not shown). Results were described from the phylogenetic analysis of a limited number of EH environmental clones. The procedures may be applicable to a variety of bacterial communities; we are particularly encouraged that we were able to amplify the 16S rRNA genes from such a diverse group of microorganisms in marine sediments. We estimate that our extraction procedure would succeed with only 50 mg of sediment, so our protocols should be applicable to fine-resolution vertical profiling of marine sediments. From the analysis of 22 clones retrieved from EH, the sediments in this nearshore marine environment were found to be phylogenetically diverse, with representatives from at least six different groups of the *Bacteria* (43).

Our goal was to develop cell lysis and DNA extraction methods that were simple to perform with minimal biases (1, 26, 47, 48). There are obvious limitations in attempts to remove cells from sediment by washing prior to lysis since the deeply imbedded and firmly attached cells could avoid detachment and be overlooked in a subsequent phylogenetic analysis. To remove these cells, a vigorous procedure would have to be used,

TABLE 1. 16S rRNA gene sequence similarity matrix for α *Proteobacteria*

Organism	% Similarity to:					
	EH-20	EH-23	FWC14	FWC38	*M. sporium*	*R. huakuii*
EH-20	100.0					
EH-23	100.0	100.0				
FWC14	90.7	90.7	100.0			
FWC38	89.0	89.0	91.3	100.0		
M. sporium	92.9	92.9	87.1	83.2	100.0	
R. huakuii	86.3	86.3	86.6	83.1	89.1	100.0
R. orientum	90.0	90.0	87.1	89.0	88.9	93.5

TABLE 2. 16S rRNA gene sequence similarity matrix for δ *Proteobacteria*

Organism	% Similarity to:						
	EH-2	EH-7	EH-14	EH-24	*D. multivorans*	*D. propionicus*	*N. exedens*
EH-2	100.0						
EH-7	81.9	100.0					
EH-14	81.0	97.7	100.0				
EH-24	79.6	76.4	74.9	100.0			
D. multivorans	87.8	81.5	81.5	78.9	100.0		
D. propionicus	76.8	78.4	77.5	74.9	78.2	100.0	
N. exedens	73.4	69.8	69.2	78.7	77.7	74.9	100.0
C. apiculatus	76.3	72.7	71.2	83.0	78.6	77.8	81.8

TABLE 3. 16S rRNA gene sequence similarity matrix for γ *Proteobacteria*

Organism	% Similarity to:												
	EH-3	EH-18	EH-10	EH-15	EH-5	EH-11	EH-9	EH-26	*C. pugetii*	Symbiont, *C. costata*	Symbiont, *S. reidi*	SCB11	*M. hydrocarbonoclasticus*
EH-3	100.0												
EH-18	87.9	100.0											
EH-10	85.2	82.8	100.0										
EH-15	86.0	82.9	94.6	100.0									
EH-5	87.1	85.2	84.4	86.7	100.0								
EH-11	87.7	86.5	83.1	86.7	93.7	100.0							
EH-9	86.3	82.4	82.1	82.5	82.1	83.9	100.0						
EH-26	86.0	82.4	82.1	82.4	82.4	83.9	100.0	100.0					
C. pugetii	90.0	87.3	84.0	85.7	87.7	88.9	82.9	82.6	100.0				
Symbiont, *C. costata*	90.3	85.5	88.3	89.4	87.7	88.3	86.0	85.7	90.0	100.0			
Symbiont, *S. reidi*	87.1	86.2	82.7	85.2	88.8	88.8	85.8	85.4	90.3	91.4	100.0		
SCB11	82.9	79.4	90.5	88.4	83.6	82.7	80.9	80.9	86.7	87.3	88.0	100.0	
M. hydrocarbonoclasticus	86.1	86.3	84.2	86.3	88.2	91.2	84.1	84.1	87.0	87.2	87.7	86.4	100.0
E. halochloris	78.6	77.6	77.5	78.0	77.2	77.8	86.3	86.0	83.3	84.9	86.0	82.9	82.5

probably resulting in cell lysis. These limitations may outweigh the advantage of increased purity in the resultant DNA. We decided on the in situ approach; the problem of template purity was handled in postlysis purification steps. Some investigators have included a sediment washing step prior to in situ lysis (55), a step intended to remove soluble inhibitory compounds and free extracellular DNA. We skipped this step so as not to eliminate the loosely associated members of the sediment community. Our priorities were to lyse the maximum number of cells, thereby releasing all of the genomic DNA, and to purify the DNA through a minimum number of steps to produce template that could be used in a PCR. This first step had to be nonselective and rigorous enough to maximize cell lysis, yet gentle enough to maintain the integrity of the released DNA. Lysis of bacterial cells is typically approached in either of two ways: chemical disruption (using compounds and conditions such as SDS plus heat, lysozyme, osmotic shock, NaOH, proteinase K, or peptidase) or physical disruption (e.g., bead mill homogenization, French press, boiling, microwave treatment, freeze-thaw cycles, or sonication). The benefits of chemical disruption are that it is relatively gentle and produces limited shearing of DNA. The disadvantages are that it typically discriminates against particular cell types and does not completely penetrate sediment samples. The advantage of physical disruption is that it may result in more uniform cell disruption and effectively disperses sediment samples to allow good penetration of the lysis buffer. The disadvantage is that it tends to shear the resulting DNA. Physical (bead mill homogenization) combined with chemical (SDS plus heat) disruption provided the best compromise.

The efficiency of disruption was high with lysis occurring in a large percentage of the indigenous cells. The chromosomal DNA yield reflected a high correlation to our calculated theoretical yield, albeit it was an approximation. This theoretical yield was based on the assumption that most chromosomal DNA is contributed by the prokaryotic and protozoan populations of the sediment. If the average prokaryotic genome size is equivalent to that of *E. coli*, the average genome would weigh 5×10^{-15} g [(649 Da/base) × (4.7×10^6 bases/cell) × (1.650×10^{-24} g/Da)] (3). The total prokaryotic cell count as determined by epifluorescence microscopy after the surficial EH sediments were sonicated was calculated to be 3.5×10^9 to 6.5×10^9 cells/g (dry weight) (16). The total protozoan cell count is reported to be 5×10^4 cells/g (wet weight) with an average genomic size of 5×10^{-6} to 200×10^{-6} μg/cell (30). The theoretical DNA yield would therefore be (5×10^{-9} μg/cell) × (3.5×10^9 to 6.5×10^9 cells/g) + (5×10^{-6} to 200×10^{-6} μg/cell) × (1×10^5 cells/g) ≅ 18 to 53 μg/g of sediment (dry weight). Our yield was 47 μg/g of sediment (dry weight). Based on similar calculations the BH theoretical yield would be (5×10^{-9} μg/cell) × (1.2×10^9 to 7.9×10^9 cells/g) + (5×10^{-6} to 200×10^{-6} μg/cell) × (7×10^4 cells/g) ≅ 6 to 54 μg/g of sediment (dry weight). Our yield was 42 μg/g of sediment (dry weight). Our actual percent recovery may be lower than that suggested by the above calculations due to the assumptions involved. Our calculated and observed yields of DNA in EH reported here are similar to those reported for freshwater sediments and terrestrial soils by other investigators who also based their theoretical yield on a direct total count multiplied by the quantity of DNA per typical *E. coli* cell. Moré et al. (35) and Leung et al. (31) calculated that they recovered 62 and 82% of the theoretical yield, respectively. The results of Steffan et al. (51) suggest that DNA may be easier to recover from freshwater sediments than soil, as they calculated that they recovered 90% of the DNA from freshwater sediments in contrast to only 22% from soil.

We used a cloning strategy that provided directional insertions of the amplified 16S rDNA into the pNOT/T7 vector; we could thereby screen the clonal library using a single vector-specific sequencing primer. After a review of commercially available sequencing vectors, we found only a few that have two or more rare-cutting restriction enzyme sites (greater than or equal to 8 bases) incorporated into their polylinker region. The pNoTA/T7 vector from 5 Prime→3 Prime has sites for both *Pac*I and *Asc*I. These enzymes also have no significant

TABLE 4. 16S rRNA gene sequence similarity matrix for gram-positive high-G+C-content bacteria

Organism	% Similarity to:			
	EH-4	EH-13	*S. ambofaciens*	MC 19
EH-4	100.0			
EH-13	100.0	100.0		
S. ambofaciens	76.9	76.7	100.0	
MC 19	82.2	81.9	83.0	100.0
MC 87	81.9	81.6	80.0	98.7

TABLE 5. 16S rRNA gene sequence similarity matrix for clostridia and related organisms

Organism	% Similarity to:						
	EH-19	EH-21	EH-22	EH-6	*E. eligens*	*E. fissicatena*	*A. kivui*
EH-19	100.0						
EH-21	98.5	100.0					
EH-22	70.5	69.5	100.0				
EH-6	72.6	72.3	74.7	100.0			
E. eligens	87.2	86.5	72.6	75.6	100.0		
E. fissicatena	87.9	87.3	74.1	73.5	89.0	100.0	
A. kivui	73.0	72.6	75.2	74.0	78.2	80.5	100.0
S. coelicolor	75.1	74.5	75.9	79.0	79.1	79.6	81.7

performance limitations in regard to their proximity to the end of DNA fragments. This allows for the placement of relatively short linker sequences at the ends of the 16S rDNA primers and therefore a limited decrease in their affinity to the target sequence.

Suggestions of the possible phenotypes that the EH clones represent among the six major lineages of the *Bacteria* must be made with caution. None of the clones contained 16S rRNA sequence that was identical to data at the RDP, so we can examine only the phenotypes of the cultured organisms that surround our EH clones on the phylogenetic trees. Within the α *Proteobacteria* (Fig. 3A) two identical clones, EH-20 and EH-23, were near a cluster of *Caulobacter* species. Only two strains, *Caulobacter* FWC14 and *Caulobacter* FWC38, were represented in Fig. 3A, but several other *Caulobacter* strains have also been sequenced. Caulobacters are prosthecate (stalked) heterotrophic bacteria, found attached to solid surfaces in aquatic environments and exhibiting a motile free living stage (24). Results of EH clones that are δ *Proteobacteria* (Fig. 4A) suggest that three clones may represent sulfate-reducing bacteria. EH-2 was on a branch near *Desulfococcus multivorans*, while EH-7 and EH-14 were closest to *Desulfobulbus propionicus*. Finding sulfate reducers in marine sediments is not a surprise, since sulfate is a favored terminal electron acceptor in this environment. Widdel (61) has isolated and characterized metabolically diverse species of sulfate reducers from marine sediments. The third EH clone, EH-24, within the δ *Proteobacteria* was associated with fruiting myxobacteria, organisms noted for their gliding motility and production of fruiting bodies (24).

The largest number, eight EH clones, were found within the γ *Proteobacteria* (Fig. 4B). In general, this phylogenetic group contains organisms that appear to be phylogenetically closely related but are phenotypically very different from each other, so suggestions about possible phenotypes for environmental clones that are in the γ *Proteobacteria* may be considered weak. For example, two clones, EH-3 and EH-18, are within a cluster containing sulfur-oxidizing gill symbionts, methanotrophs, and *Cycloclasticus pugetii. C. pugetii* neither oxidizes sulfur nor grows on methane but is capable of degrading toluene, biphenyl, and polycyclic aromatic hydrocarbons (12), contaminants that are found in high concentration in EH sediments (58). EH-10 and EH-15 cluster with a group of organisms belonging to marine genera, the *Oceanospirillum* and *Marinomonas* spp. (24). EH-5 and EH-11 may represent a new lineage within the γ *Proteobacteria*. The identical clones, EH-9 and EH-26, are deeply branched within the γ *Proteobacteria* most closely related to anoxygenic photosynthetic bacteria. Although a relatively large distance away, their closest relative is *Ectothiorhodospira halochloris*. The genus *Ectothiorhospira* occurs in marine and extremely saline environments containing sulfide; some species are known to grow under microaerobic or aerobic conditions in the dark (24).

Two clones, EH-4 and EH-13, were associated with the gram-positive high-G+C-content subdivision of the *Bacteria* (Fig. 3D) and may be representatives of a new branch. Cultured genera within this group include species of *Streptomyces*, *Corynebacterium*, and *Arthrobacter*. The nearest relatives to EH-4 and EH-13 were other environmental clones isolated from very different nonmarine environments including soil samples collected near Brisbane, Australia (Mount Coot-tha clones MC 87 and MC 19) (50) and from a rice field paddy (PAD13) (57).

Two clones, EH-8 and EH-25, were found with the planctomyces and related organisms (Fig. 3B). The closest neighbors to these EH clones were two other environmental clones, isolated from Pacific Ocean marine snow (AGG8) (8) and from a rice field paddy (PAD41) (57). Cultured and characterized organisms that are near the EH clones are heterotrophic budding bacteria that may produce stalks: *Planctomyces* sp., *Pirellula marina*, and *Gemmata ovscuriglobus* (24).

The last four clones, EH-6, EH-22, EH-19, and EH-21, were associated with the clostridia and related organisms (Fig. 3C), a group of obligately anaerobic gram-positive bacteria also having a low G+C content. Two clones, EH-19 and EH-21, were closely related to *Eubacterium eligens* and *Clostridum symbiosum. Eubacterium* and *Clostridium* are genera that are generally chemoorganotrophic, and their metabolism is fermentative, although some *Clostridium* species are chemoautotrophic or chemolithotrophic as well (24). EH-6 and EH-22, although related to the clostridia and relatives, may actually represent members of a new lineage because of their relatively large phylogenetic distance from other members of the clostridia-and-relatives group and their deep branches represented in the tree. No other sequences outside the clostridium cluster of the *Bacteria* were found to be more closely related, however. In future work, we will sequence the remaining portion of the 16S rDNA for these two clones since they could represent newly discovered phylogenetic groups.

An important aspect to realize is that the molecular phylogenetic analysis of environmental clone libraries is reflective of the number of 16S rRNA gene copies present in the sample and not directly of the numbers of different kinds of organisms. The rRNA gene copy number may be 1 to 14 copies per cell and varies from species to species and even from strain to strain (13).

Recently, some investigators have raised questions about possible problems associated with the PCR of 16S rDNA for the phylogenetic analysis of microbial communities. Strong biases may be introduced by the differential PCR amplification of DNA from heterogeneous templates. Studies have focused on differ-

TABLE 6. 16S rRNA gene sequence similarity matrix for planctomyces and related organisms

Organism	% Similarity to:				
	EH-8	EH-25	AGG8	*P. maris*	*P. marina*
EH-8	100.0				
EH-25	99.7	100.0			
AGG8	84.5	84.2	100.0		
P. maris	90.0	88.8	83.9	100.0	
P. marina	82.3	82.0	82.9	84.1	100.0
P. staleyi	80.5	80.2	82.1	83.8	85.1

ent aspects of the PCR, including biases resulting from product plateau (36), selective amplification (13, 47), and accumulation kinetics (38, 45, 46, 53). Product plateau is also referred to as nonexponential phase or saturation phase. These terms refer to the stage of PCR amplification in which the exponential accumulation of product ceases. Once a quantity of product reliant on the reaction composition and possibly other factors has been achieved, the accumulation slows and proportionality between the different products becomes unpredictable.

Morrison and Gannon (36) examined the effect of the plateau phase on quantitative PCR. They suggest that the quantitative use of PCR is compromised when a reaction enters the plateau phase of amplification. They illustrated the plateau effect in their experiments by showing that amplification reactions using serial 10-fold dilutions of a template gave nearly identical yields. To determine if our amplification methods reached the plateau phase, an analogous dilution experiment was conducted with the EH DNA. Although product concentrations were estimated by ethidium bromide staining of an agarose gel, the ratio of product to template appeared consistent with the dilution (data not shown). We consider this to be evidence that the amplification reaction was still in the exponential phase and therefore should not be subject to plateau phase effects.

The PCR of mixtures of homologous genes may result in a "leveling effect" in which a 1:1 ratio of products is formed regardless of the initial ratios. Different primer sets used for the same genes may cause different quantitative results. For example, Suzuki and Giovannoni (53) reported that their 519F and 1406R primers resulted in good product proportionality compared to template, while the 27F and 338R primers yielded a 1:1 ratio in the products. The 27F and 338R primers also were more efficient in amplification during the initial cycles, giving the same product molarity in fewer than 10 cycles, comparable to the amount observed after 35 cycles with the 519F and 1406R primers. They believe that the 1:1 product ratio, or the observed leveling effect, results from primer-template and template-template competition during the annealing step of the PCR. Based upon the highly conserved nature of the 16S rRNA gene, interactions between DNA templates from different microbial species may be important reactions that also need to be addressed. Suzuki and Giovannoni (53) found that with the 27F-338R primer pair the bias was highly dependent on cycle number. The leveling effect began at about the eighth PCR amplification cycle. Interestingly, this would have been approximately the same time that this reaction achieved the same product molarity found for the 519F-1406R primer pair reaction at cycle 35.

The result from these possible effects is that the proportions found for particular 16S rDNA sequences in the amplified products may not represent the 16S rDNA proportions found in the original sample. The recent results of Farrelly et al. (13) are particularly troubling since their results suggest that preferential amplification is dependent upon the microbial species present in a mixture. They prepared mixtures containing pairs of four bacterial species and found that *Bacillus subtilis* 16S rDNA amplified at a preference to that of "*Thermus thermophilus*." In contrast, they did not see preferential amplification with mixtures of "*T. thermophilus*" with *E. coli* or *Pseudomonas aeruginosa*. Since their results were presented as relative molar ratios, it was not clear whether the preference described resulted from factors such as the higher affinity of their amplification primers for the *B. subtilis* DNA, amplification inhibition of the "*T. thermophilus*" DNA, or an enhancement of the *B. subtilis* DNA amplification in the presence of "*T. thermophilus*." The explanation for these results needs to be elucidated.

Although additional research is required to understand the possible biases associated with the amplification of 16S rDNA from environmental sources, molecular phylogenetic methods using the PCR and cloning of 16S rDNA are the most sensitive techniques available for describing the composition of complex microbial communities. Some questions will probably remain, however, about possible differences and comparability between controlled laboratory experiments with a limited number of species and environmental samples containing large numbers of different microbial species. We found six major phylogenetic groups represented by the 22 EH clones but do not conclude that the proportions found in our study are representative of the original sediment sample. Additional steps could be taken to estimate the abundance and proportion of individual species that are detected in a clonal library. For example, fluorescently tagged oligonucleotide probes could be developed (1), and the number of organisms enumerated by this method could be compared to the total number present.

Our methods were able to amplify and clone the 16S rDNA from phylogenetically diverse members of the *Bacteria*. For our future work, we will compare the composition of the microbial communities in the surficial sediments of EH, a site contaminated with creosote PAHs, to that in BH, a control and uncontaminated site in Puget Sound (Fig. 1). Using the protocol that we have outlined here, microbial ecologists will be able to have a better understanding of both the structure and dynamics of the microbial community within marine sediments. The long-term goal for future microbial ecology studies in the marine environment will be to tie the phylogenetic data together with data about specific microbial activities, sediment geochemistry, and the other facets of benthic oceanography.

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