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Population Structure and Phylogenetic Characterization of Marine Benthic Archaea in Deep-Sea Sediments

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During the past few years *Archaea* have been recognized as a widespread and significant component of marine picoplankton assemblages and, more recently, the presence of novel archaeal phylogenetic lineages has been reported in coastal marine benthic environments. We investigated the relative abundance, vertical distribution, phylogenetic composition, and spatial variability of *Archaea* in deep-sea sediments collected from several stations in the Atlantic Ocean. Quantitative oligonucleotide hybridization experiments indicated that the relative abundance of archaeal 16S rRNA in deep-sea sediments (1500 m deep) ranged from about 2.5 to 8% of the total prokaryotic rRNA. Clone libraries of PCR-amplified archaeal rRNA genes (rDNA) were constructed from 10 depth intervals obtained from sediment cores collected at depths of 1,500, 2,600, and 4,500 m. Phylogenetic analysis of rDNA sequences revealed the presence of a complex archaeal population structure, whose members could be grouped into discrete phylogenetic lineages within the two kingdoms, *Crenarchaeota* and *Euryarchaeota*. Comparative denaturing gradient gel electrophoresis profile analysis of archaeal 16S rDNA V3 fragments revealed a significant depth-related variability in the composition of the archaeal population.

More than 50% of the earth's surface is covered by deep-sea sediments that are primarily formed through the continual deposition of particles from the productive ocean surface. Much of the organic input into the oceanic sediments is recycled by the benthic microbial communities (1). Although several recent studies have focused on the characterization of microbial communities involved in carbon and sulfur cycling in coastal benthic environments (13, 24, 34, 42, 54), microbial populations in deep-sea sediments remain poorly studied. This is particularly true for the *Archaea*, whose population structure, global distribution, and possible contribution to postdepositional diagenesis in deep-sea sediments are virtually unknown. Molecular studies based on the phylogenetic analysis of environmentally derived 16S rRNA genes (rDNA) can bypass the limitations of culture-dependent approaches and offer an increasingly comprehensive picture of diversity and distribution of microbial populations (57).

The *Archaea* are divided into two kingdoms: the *Euryarchaeota* and the *Crenarchaeota*. The *Euryarchaeota* was traditionally considered the more physiologically diverse group. This kingdom includes the methanogens, which inhabit strictly anaerobic niches; the extreme halophiles, which are limited to highly saline, land-locked water bodies; and some of the thermophiles, usually found in close proximity to terrestrial and shallow-water hot springs and at deep-sea hydrothermal vents. Until recently, the *Crenarchaeota* were thought to include an evolutionarily closely related group of organisms, characterized by an extremely thermophilic, sulfur-metabolizing phenotype. Recently, a third kingdom, the *Korarchaeota*, has been proposed to describe a group of as-yet-uncultivated organisms whose 16S rRNA sequences have been retrieved from a hot spring in Yellowstone National Park (5). As a whole, the *Archaea* were considered to be confined to specialized environments, including those at high temperature, high salinity, and extremes of pH and in strictly anaerobic niches that permit methanogenesis. Recently, several studies based on the comparison of 16S rRNA genes have radically changed our view of the *Archaea*, revealing the ubiquitous character of these microorganisms, which also appear to thrive in aquatic and terrestrial temperate environments. Crenarchaeal phylotypes have been found among marine picoplankton (9, 10, 19, 39), in the gut of a deep-sea holothurian (38), in freshwater sediments (26, 35, 50), in soil (6, 7, 30), in deep subsurface sediments (8), in continental shelf anoxic sediments (56), and in moderate-temperature (15 to 30°C) hydrothermal vent microbial mats (41). Actively dividing cells of *Cenarchaeum symbiosum*, a crenarchaeote inhabiting the tissues of a temperate water marine sponge, have been recently identified, demonstrating growth of this organism at temperatures of 10°C (46). Moreover, the biochemical characterization of a heat-labile DNA polymerase from *C. symbiosum* is consistent with the postulated nonthermophilic phenotype of this crenarchaeote (51). New lineages of the *Euryarchaeota* have also been found among marine picoplankton (9, 10, 20), in salt marsh sediments (42), in continental shelf anoxic sediments (56), associated with the digestive tracts of marine fishes (55), and in hydrothermal vent microbial mats (41). Detailed studies on the distribution of the planktonic *Crenarchaeota* and *Euryarchaeota* illustrated that the *Euryarchaeota* were most abundant in surface waters, whereas the *Crenarchaeota* dominated at depth (36, 37). Additional evidence demonstrating the wide distribution of *Archaea* in oxic and anoxic marine sediments and in the water column has been obtained by using either lipids as biological markers for the detection of these microorganisms (11, 25, 27). Moreover, quantitative probe hybridizations of total RNA

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TABLE 1. Locations of sampling stations and sample distribution

Station	Cruise, dive no.	Latitude	Longitude	Depth (m)	Sediment core	Depth interval of sediment subcore (cm)[a]
CR, LEO 2500	R/V *Atlantis* II cruise 133, DSV *Alvin* dive 3076	39°19.92′N	70°39.80′W	2,616	CR-1	0–2 11–13 25–27
CR, LEO 2500	R/V *Atlantis* II Cruise 133, DSV *Alvin* dive 3080	38°49.15′N	72°08.25′W	2,605	CR-2	0–2 21–23
AP	R/V *Oceanus* cruise 282	37°23.23′N	68°49.95′W	4,500	AP	0–2 11–13 15–17
AC	R/V *Oceanus* cruise 282	39°50.05′N	70°13.95′W	1,500	AC[b]	0–2 7–9

[a] An rDNA library was constructed for each depth interval.
[b] AC sediment subcores for RNA extraction were collected at each of the following depth intervals: 0, 4, 6, 8, and 16 cm.

have been used to quantify *Archaea* in marine sediments from the Arctic Ocean (49).

Based on our previous report of novel *Archaea* in continental shelf sediments (56), we assessed the vertical distribution and phylogenetic diversity of *Archaea* in deep-sea sediments collected from several locations in the northwestern Atlantic Ocean. Phylogenetic analyses of archaeal rDNA sequences were used in combination with rRNA-targeted probe hybridization to nucleic acid extracts and denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified rDNA fragments. We report here a high diversity of novel crenarchaeal and euryarchaeal phylotypes associated with deep-sea sediments, whose relative abundance ranged from about 2.5 to 8% of the total prokaryotic rRNA. These results may reflect a rich physiological diversity associated with *Archaea* in deep-sea sediments.

MATERIALS AND METHODS

Sample collection. Deep-sea sediments were collected from different locations in the northwestern Atlantic Ocean (Table 1). Acrylic tube cores were collected on the continental rise (CR) at the Long-term Ecosystem Observatory 2500 (LEO 2500), from the DSV *Alvin* (dives 3075 to 3084) at an average depth of 2,600 m. Surface-deployed box corers operated from the R/V *Oceanus* were collected on the Atlantis Canyon (AC; 1,500 m) and on the abyssal plain (AP; 4,500 m). The top 6 to 10 cm of the sediment were soft and showed signs of bioturbation, whereas below 10 cm the sediment appeared to be thicker and undisturbed. In situ measurements taken from the DSV *Alvin* at LEO 2500 (CR) indicated that oxygen was completely depleted within 5 cm in the sediment (23a), whereas laboratory measurements indicated that the depth of oxygen depletion in the AC sediment was 1.0 cm (53a). In situ temperatures of ca. 2°C were recorded at the CR sites. Subcores were taken at different depth intervals by using sterile syringes modified by the removal of end flanges. The subcores were frozen on board and kept at −80°C until they were processed in the laboratory.

RNA extraction and quantitative oligonucleotide hybridization. Total RNA was extracted from 3.5 g of each sediment subcore. Aliquots (0.3 g) of sediment were transferred on ice to screw-cap Eppendorf tubes containing 350 μl of low-pH buffer (250 mM $NaC_2H_3O_2$, 50 mM EDTA; pH 5.1), 500 μl of phenol (pH 5.1), 35 μl of sodium dodecyl sulfate (SDS), and 0.5 g of zirconium beads (Biospec, Inc., Bartlesville, Okla.). The RNA was extracted by two bead-beating treatments and two extractions with phenol (pH 5.1), phenol-chloroform-isoamyl alcohol (50:49:1), and chloroform-isoamyl alcohol (24:1), respectively. The RNA was precipitated with 2 volumes of ethanol and was collected by centrifugation, washed in 80% ethanol, dried, and resuspended in RNase-free sterile distilled water. A plasmid containing clone CRA7-0 cm was linearized with *Bam*HI and in vitro transcribed with T7 RNA polymerase (Stratagene Cloning Systems, La Jolla, Calif.) to generate archaeal reference rRNA, and the integrity of the transcript was checked on a denaturing agarose gel. The sediment RNAs and the RNA standards were serially diluted and denatured in a 0.5% glutaraldehyde solution containing polyriboadenylic acid (0.5 mg/ml), applied in duplicate or triplicate to nylon membranes (Microcon Separations, Inc., Westboro, Mass.) with a slot-blotting apparatus, and then immobilized by baking at 80°C for 2 h. Membranes were preincubated in prehybridization buffer (0.9 M NaCl, 50 mM NaH_2PO_4 [pH 7.2], 5.0 mM EDTA, 0.5% SDS, 10× Denhardt's solution) at 40°C for 1 h and then hybridized in the presence of ^{32}P-end-labeled oligonucleotide probes at 40°C overnight. Membranes were washed twice in wash solution (1× SSC [0.15 M NaCl, 0.015 M sodium citrate {pH 7.0}], 1% SDS) at 40°C for 1 h, followed by two 15-min washes at 44°C (S-*-Univ-1390-a-A-18) (62), 54°C (S-D-Bact-0338-a-A-18) (3), or 56°C (S-D-Arch-0915-a-A-20) (2). The intensity of the hybridization signal was measured with a PhosphorImager (model 400S; Molecular Dynamics, Inc., Sunnyvale, Calif.) and quantified relative to *Escherichia coli* RNA and in vitro-transcribed archaeal reference rRNA (CRA7-0 cm). Calculations for total prokaryotic rRNA were based on the sum of detected bacterial and archaeal rRNA.

DNA extraction, amplification, library construction, and screening. Genomic DNA was extracted from 3.5 g of each deep-sea sediment subcore. Each sample was thawed on ice, resuspended in 10 ml of extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM EDTA [pH 8.0], 100 mM Na_2HPO_4 [pH 8.0], 1.5 M NaCl), and incubated at 37°C for 30 min with vigorous shaking. Then, 100 mg of lysozyme (100 mg/ml), 100 μl of pronase (20 mg/ml), and 80 μl of mutanolysin (5,000 U/ml) were added to the sample, and the mixture was incubated at 37°C for 1 h with gentle shaking. Next, 50 μl of proteinase K was added to the mix followed by incubation at 37°C for 30 min and at 55°C for 30 min and then by the addition of 1.5 ml of 20% SDS and 1 ml of 20% *N*-laurylsarcosine. The sample was incubated at 65°C for 2 h and slowly rotated. The sample was extracted twice with an equal volume of phenol, twice with an equal volume of phenol-chloroform-isoamyl alcohol (50:49:1), and twice with an equal volume of chloroform-isoamyl alcohol (24:1). The aqueous phase was precipitated with 0.6 volumes of isopropanol at room temperature for 1 h. The DNA was collected by centrifugation, washed in cold 70% ethanol, dried, and resuspended in sterile distilled water, and the DNA concentration was measured spectrophotometrically. The 16S rRNA gene sequences were selectively amplified from the genomic DNA by PCR by using primers designed to anneal to the conserved positions of the 5′ and 3′ regions of 16S rRNA genes. Primers used to selectively amplify archaeal 16S rRNA genes were as follows: S-D-Arch-0025-a-S-17 (5′-CTGGTTGATCCTGCCAG-3′) (48) or S-D-Arch-0344-a-S-20 (5′-ACGGGGCGCAGCAGGCGCGA-3′) (47) with S-*-Univ-1517-a-A-21 (5′-ACGGCTACCTTGTTACGACTT-3′) (58) to yield full-length or 1,120-bp PCR products, respectively. Primers S-D-Arch-0025-a-S-17 and S-*-Univ-0907-a-A-20 (5′-CCGTCAATTCMTTTRAGTTT-3′) (4) were also used to amplify PCR products of ca. 900 bp. Serial dilutions of template DNA were incubated in a thermal cycler in the presence of *Taq* DNA polymerase for 40 cycles under the following conditions: 94°C for 30 s, 48°C for 30 s, and 72°C for 30 s. PCR products were gel purified by using QIAquick spin columns (Qiagen, Inc., Chatsworth, Calif.) and resuspended in sterile distilled water. Amplified 16S rRNA gene fragments were cloned in either pCRII plasmid vector (Invitrogen, Inc., Carlsbad, Calif.) or pMOS Blue T-vector (Amersham International, Little Chalfont, United Kingdom), and the resulting ligation products were used to transform competent *E. coli* INVαF′ cells. Ten 16S rDNA environmental libraries were constructed from different sediment samples (Table 1), and a total of 209 randomly chosen colonies were analyzed for insert-containing plasmids by direct PCR followed by gel electrophoresis of the amplified products.

RFLP, sequence, and phylogenetic analyses. Insert 16S rDNA fragments were digested with the tandem tetrameric restriction endonuclease pairs, *Hae*III and *Hpa*II (Promega, Inc., Madison, Wis.). The reaction products were visualized by electrophoresis on a 2.5% (wt/vol) agarose gel containing ethidium bromide (0.5 mg/liter). Representative clones for each library showing unique restriction fragment length polymorphism (RFLP) patterns were selected, and their sequences were determined for both strands on an ABI 373 Automated Sequencer (Applied Biosystems, Foster City, Calif.). The average numbers of nucleotides of sequence determined were 1,300 for the CR and AP clones and 820 for the AC clones. Sequences were submitted to the CHECK_CHIMERA program at the Ribosomal Database Project (RDP) (33) to detect the presence of chimeric artifacts and were manually aligned to 16S rRNA sequence data from the RDP

and recent GenBank releases by using the Genetic Data Environment multiple sequence editor. Environmental and cultivated members of the domains *Archaea*, *Bacteria*, and *Eucarya* were included in the alignment. The conserved sequence regions and the established secondary structure of the 16S rRNA were used as guides to ensure that only homologous nucleotides were compared. Approximately 1,204 homologous nucleotides were used to infer the phylogenetic position of the benthic *Archaea*. Shorter environmental sequences available from the database were later added to the alignment and the phylogenetic analyses were repeated. The addition of shorter sequences did not alter the topology of the tree. Evolutionary distances were computed from pairwise similarities by using the correction of Jukes and Cantor (29). Distance trees were constructed by the least-squares algorithm of DeSoete (12) from a normal evolutionary distance matrix. Maximum likelihood trees were constructed by using fastDNAml (15, 17), which uses the generalized two-parameter model of evolution (32), and by using jumbled orders for the addition of taxa to avoid potential bias introduced by the order of sequence addition. The transition/transversion ratio was optimized, and bootstrap analysis was used to provide confidence estimates for phylogenetic tree topologies (16).

DGGE. A nested-PCR approach was used to amplify the variable region 3 (V3) of the archaeal 16S rRNA gene. The full-length archaeal 16S rDNA was amplified from the genomic DNA as described above. The PCR products were gel purified and used as a template to amplify the V3 region by using the GC-clamp primer 344F(GC) (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCG CCGCCCCCGCCACGGGGCGCAGCAGGCGCGA-3′) and S-*-Univ-0518-a-A-17 (5′-ATTACCGCGGCTGCTGG-3′) (43). DGGE was performed with a D Gene System (Bio-Rad Laboratories, Hercules, Calif.). PCR samples (12.0 μl) were applied directly onto 6% (wt/vol) polyacrylamide gels in 1× TAE (40 mM Tris, 20 mM acetate, 1 mM EDTA), with denaturant gradient from 20 to 60% (where 100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was performed at a constant voltage of 200 V and a temperature of 60°C for 6 h. After electrophoresis, the gels were incubated for 15 min in ethidium bromide (0.5 mg/liter), rinsed for 10 min in distilled water, and photographed with a UV Foto Analyst system (Fotodyne, Inc., Hartland, Wis.). DGGE bands were gel purified and reamplified. The PCR products of the second amplification were loaded onto a DGGE gel to check the purity of the bands, and the sequence was determined for both strands.

Nucleotide sequence accession numbers. The sequences from this study are available through GenBank under accession numbers AF119123 to AF119147.

RESULTS

Depth-related abundance of *Archaea* in AC sediments. The relative contributions of bacterial and archaeal rRNA to the total prokaryotic community were estimated by slot blot hybridization. The total RNA was extracted from AC 1-cm sediment sections at depth intervals of 0, 4, 6, 8, and 16 cm. AC sediment was dominated by *Bacteria* at all depth intervals, comprising at least 92% of the prokaryotic rRNA. The relative contribution of *Archaea* was 2.5 and 2.4% at 0 and 4 cm, respectively, 5.7 and 7.9% at 6 and 8 cm, respectively, and decreased to 3.5% at 16 cm (Fig. 1).

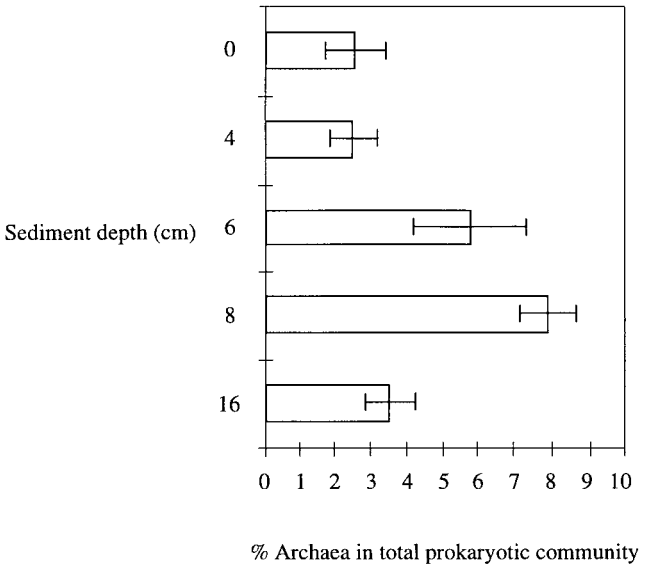

FIG. 1. Depth-related abundance of archaeal 16S rRNA as a fraction of total prokaryotic rRNA from an AC sediment core. Bars are means of two determinations, with error bars representing the range.

DNA extraction, PCR amplification of 16S rDNA, and library construction. Genomic DNA was extracted from a total of 10 depth intervals obtained from sediments collected at four sampling stations (Table 1). The concentration of DNA extracted from AP sediment was about half that extracted from CR sediment (data not shown). Furthermore, as the depth in the sediment increased, we observed a consistent decrease in the yield of the genomic DNA. Overall, the observed decrease in DNA yield suggested a decrease in biomass at deeper intervals in the sediment. Two archaeon-specific and two universal primers were used in different combinations to amplify the archaeal 16S rDNAs (see Materials and Methods) and to maximize the number of different sequences obtained. A 16S rDNA clone library was constructed for each depth interval (Table 1), and a total of 163 insert-containing clones were identified by direct PCR screening.

Vertical profiling of archaeal 16S rDNA by RFLP analysis. To estimate the depth-related diversity of *Archaea* in deep-sea sediments, a total of 128 rDNA clones were selected from different depth intervals and subjected to RFLP analysis. RFLP analysis revealed a striking depth-related pattern in archaeal rDNA diversity. The apparent diversity increased as the depth in the sediment increased. Details of the frequency of the RFLP pattern for 61 clones isolated from six depth intervals from AP and CR-1 sediments are shown in Fig. 2. Of the 25 unique RFLP patterns detected in these samples, patterns 2 and 3 were most abundant (33.1 and 18.6%, respectively), and they were found predominantly in the 0- to 2-cm depth interval. RFLP patterns 2 and 3 were also detected in the 11- to 13-cm depth intervals of both AP (1.6 and 3.2%, respectively) and CR-1 (10 and 1.6%, respectively) sediments. The distribution of RFLP patterns 1 and 4 was also limited to the 0- to 2-cm interval, but at significantly lower frequencies (1.6 and 3.2%, respectively). The remaining 21 unique RFLP patterns were only recovered from within or below the 11- to 13-cm interval, at frequencies ranging from 1.6 to 4.9% (Fig. 2). In particular, patterns 10 to 16 were detected exclusively in the AP sediment at the 15- to 17-cm interval, and patterns 19 to 25 were detected only in the CR-1 25- to 27-cm interval. Results from the RFLP analysis of the CR-2 sediments collected during DSV *Alvin* dive 3080 (Table 1) were consistent with the data shown in Fig. 2, whereas we detected significantly less diversity in the AC sediments (0 to 2 and 7 to 9 cm; data not shown).

Population structure and phylogenetic analysis of benthic *Archaea*. The sequence of representatives for each library (total, 41 clones) was determined. No chimeric molecules were detected in the 0- to 2-cm intervals, whereas potential chimeric artifacts (ca. 12%) were recognized in some of the deeper intervals and were excluded from further analysis. In order to obtain an accurate description of the phylogenetic relationships of the deep-sea benthic *Archaea*, we included in our analysis representatives of most of the environmental archaeal sequences available from the database, as well as sequences of archaeal isolates. Phylogenetic analyses that used the maximum likelihood and distance matrix methods revealed the presence of six clusters of archaeal sequences in deep-sea sediments (Fig. 3). Of the sequenced clones (Fig. 4), 63% grouped with the strongly supported monophyletic marine group I, which includes all the nonthermophilic marine planktonic *Cren-*

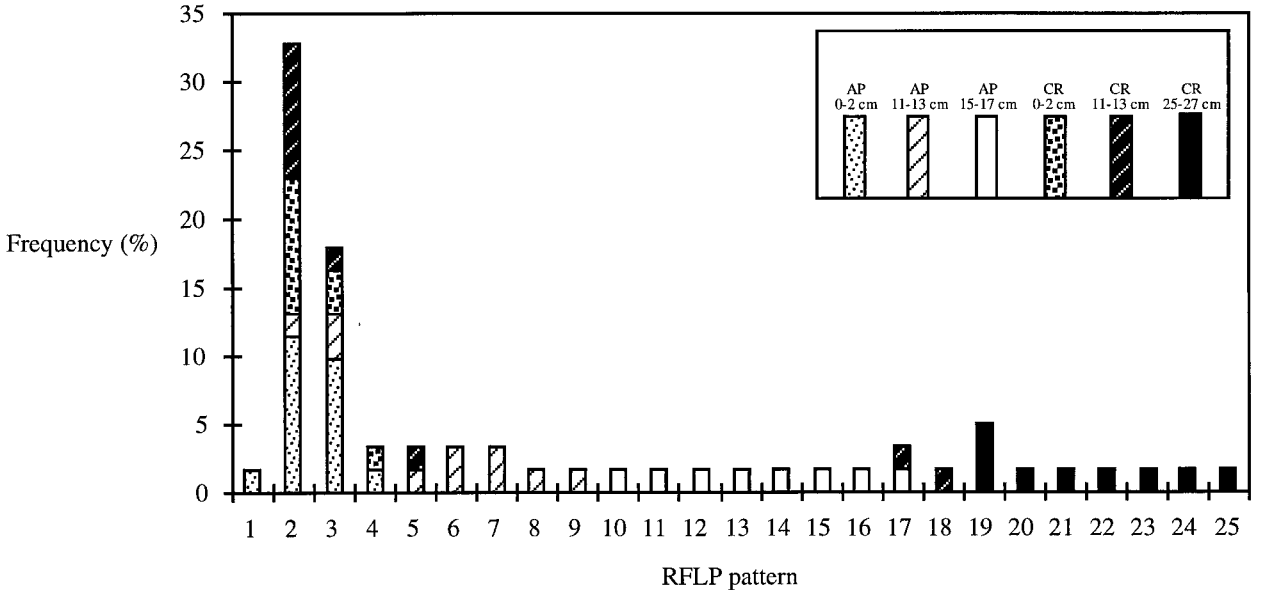

FIG. 2. RFLP pattern distribution for 61 16S rDNA clones isolated from different vertical depth intervals from the AP and CR-1 sediments (4,500- and 2,616-m depths, respectively).

archaeota described to date (9, 10, 19) (Fig. 3). Most (57%) of the marine group I-related benthic clones were isolated from the 0- to 9-cm intervals, whereas only 6% of the clones were isolated from the 11- to 13-cm intervals, and none came from the deeper intervals (Fig. 4). The remaining five clusters of sequences were not affiliated with any of the previously described archaeal planktonic lineages, namely, marine groups I, II, and III (9, 10, 19, 20), and they were grouped as independent clusters (Fig. 3). The average 16S rRNA sequence divergence between representatives of each cluster was 20 to 30%, a result similar to the typical sequence divergence values found between bacterial divisions (28), and each cluster exhibited unique group-specific signature sequences (Table 2). Thus, they have been designated as marine benthic *Archaea* groups A to E.

Phylogenetic analysis placed the marine benthic groups A, B, and C within the *Crenarchaeota*, whereas groups D and E were placed within the *Euryarchaeota* (Fig. 3). Group A (ca. 9% of the sequenced clones) is a cluster of sequences whose closest relatives (represented by FFSB1) were isolated from forest soil (30) and are specifically affiliated with a putative thermophilic clone (pSL12 in Fig. 3) isolated from a hot spring in Yellowstone National Park (5) (Fig. 3). Group A sequences were isolated from sediment depth intervals ranging from 0 to 17 cm (Fig. 4). Group B shares a common ancestry with two other marine benthic lineages previously isolated (56) and is represented by a cluster of closely related sequences (13% of the sequenced clones) that were isolated from both the AP and the CR, at intervals ranging from 11 to 27 cm (Fig. 3 and 4). The two lineages closely related to group B include clones previously isolated from continental shelf anoxic sediment (BBA 2, 4, and 6) (56) and freshwater sediment (pGrfB286) (26). The two group C clones (6%) were isolated from the deepest interval (25 to 27 cm) of the CR sediment and are closely related to a putative thermophilic clone (pSL17 in Fig. 3) isolated from a hot spring in Yellowstone National Park (5). Group C forms a paraphyletic group with a larger cluster of sequences isolated from freshwater anoxic sediments (26, 50), from deep subsurface paleosol (8), and from high-temperature environments (5) (Fig. 3). The branching topology of marine benthic groups A, B, and C was supported by high bootstrap values (see Fig. 6). The affiliation of marine benthic groups A, B, and C and group I-like lineage with the *Crenarchaeota* was confirmed by an intradomain nucleotide signature analysis (59) (Table 2). However, the presence of A-U at positions 504 and 541 is a feature common to all the deep-sea members of benthic groups A, B, and C, one not found in the marine group I lineage (Table 2).

Marine benthic group D consists of a cluster of closely related sequences affiliated with the *Euryarchaeota*, all of which were isolated from subsurface marine sediments (Fig. 3 and 4). This group includes a clone isolated from the 27-cm interval of the CR sediment and a group of clones previously isolated from continental shelf anoxic sediment (BBA) (56) and salt marsh subsurface sediment (MT) (42). Marine benthic group D and marine planktonic groups II and III all share a common ancestry with the aerobic moderate thermophile, *Thermoplasma acidophilum* (Fig. 3). Group E appears to be a monophyletic group of sequences isolated from CR and AP sediments (11- to 17-cm intervals) (Fig. 3) and represents 9% of the clones sequenced (Fig. 4). None of the group E clones appeared to be specifically related to any known archaeal isolate, although their phylogenetic position between members of the *Methanobacteriales* and the *Methanosarcinales* suggests a possible methanogenic phenotype. The intradomain signature analysis of groups D and E confirmed their affiliation with the *Euryarchaeota* and revealed the presence of G-U at positions 513 and 538 as a feature common to all members of marine benthic group D (Table 2).

DGGE profiling. To further investigate the depth-related distribution and spatial variability of the benthic *Archaea*, we analyzed the DGGE profiles of PCR-amplified fragments of the archaeal 16S rDNA V3 region from each depth interval of both the CR-1 and CR-2 cores (Fig. 5). Sixteen dominant bands, each representing a putative organism, were sequenced, and their phylogenetic affiliation was inferred by analyzing the nucleotide signatures contained in the region between positions 500 and 518 (*E. coli* numbering) (Table 3). According to

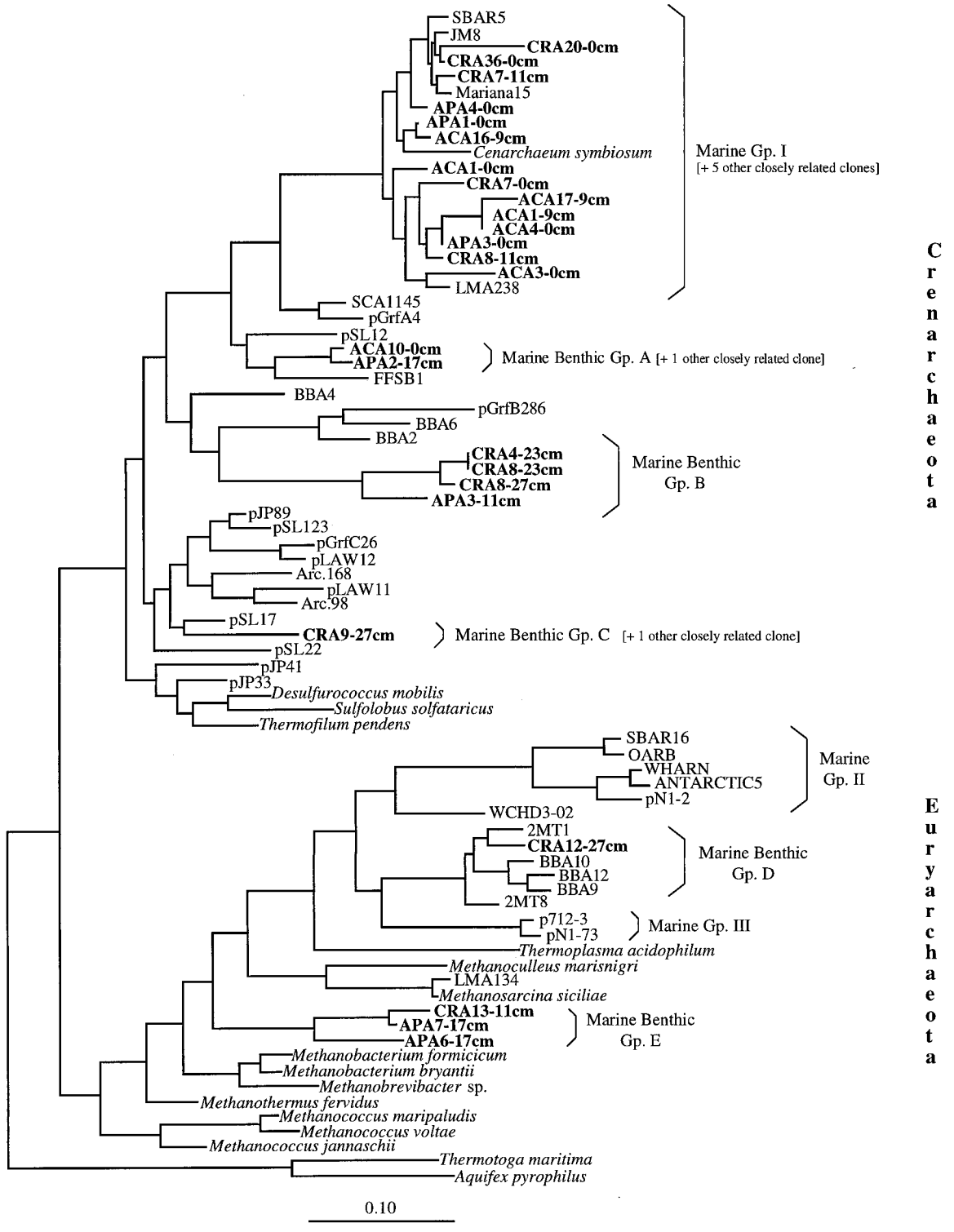

FIG. 3. Phylogenetic analysis of deep-sea benthic *Archaea*. A maximum likelihood tree was constructed for the archaeal sequences by using fastDNAml (17). The scale represents the expected number of changes per sequence position. Abbreviations for the benthic *Archaea* (in boldface) are formed by a combination of the sampling station abbreviation, followed by the letter A (*Archaea*), the phylotype number, and the vertical depth interval of the sediment core from which the specific phylotype was obtained. Abbreviations for as-yet-uncultivated phylotypes from various environments: BBA (AF004343 to AF004348), from continental shelf sediments (56); JM8 (L24201), from the gut of a deep-sea sediment feeder (38); Mariana15 (D87350), from Mariana Trench sediments (31); SBAR5 (M88075), SBAR16 (M88077), OARB (U11040), WHARN (M88078), ANTARCTIC5 (U11044), pN1-2 (U86455), pN1-73 (U86462), and p712-3 (U81540), from marine picoplankton (9, 20); FFSB1 (X96688) and SCA1145 (U62811), from soil (6, 30); pGrfA4 (U59968), pGrfC26 (U59986), pGrfB286 (U59984), LMA134 (U87515), LMA238 (U87517), pLAW11 (U77569), and pLAW12 (U77568), from freshwater sediments (26, 35, 50); Arc.98 (AF005760) and Arc.168 (AF005764), from a deep-subsurface paleosol (8); pJP33 (L25300), pJP41 (L25301), pJP89 (L25305), pSL17 (U63339), pSL22 (U63340), and pSL123 (U63445), from a Yellowstone National Park hot spring (5); 2MT1 (AF015981) and 2MT8 (AF015992), from salt marsh sediments (42); and WCHD3 (AF050616), from a contaminated aquifer (14).

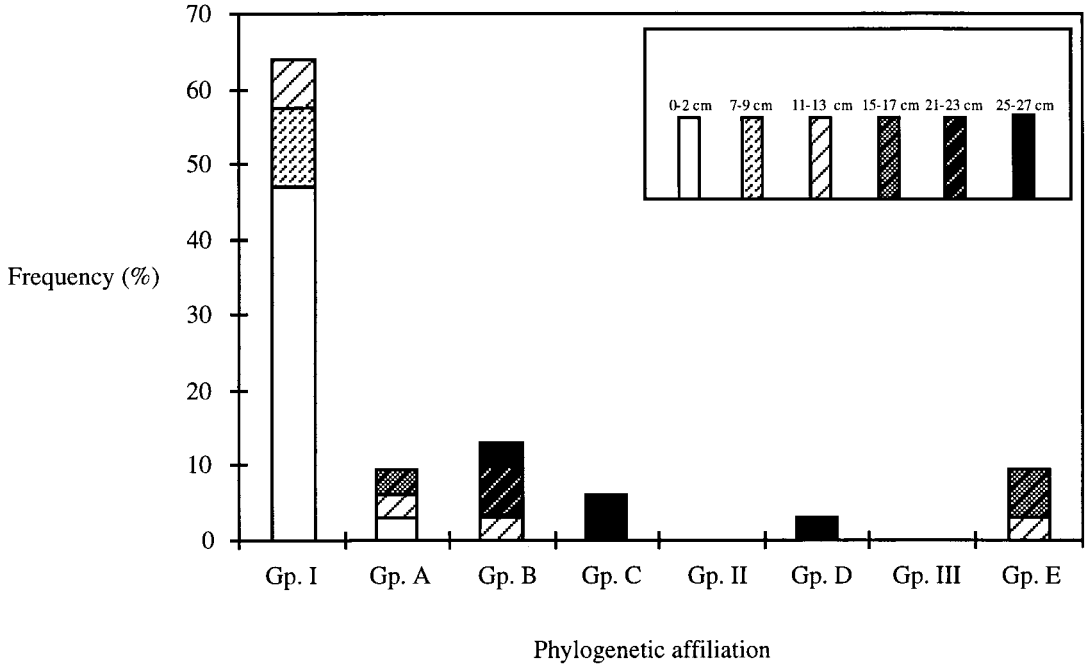

FIG. 4. Group distribution for 41 16S rDNA sequences obtained from six different vertical depth intervals from AC, CR, and AP sediments.

our analysis, the 16 sequences could be grouped into seven categories, each category containing sequences that shared identical signature features (Table 3). Bands a, m, and j were the only sequences to show crenarchaeal signature features, and they shared signature nucleotides with the marine benthic group B. Bands a and m were detected in the upper sediment of both CR-1 and CR-2 (0 to 2 cm), whereas band j was only detected in CR-2 (21 to 23 cm).

Bands i and p were of uncertain affiliation, whereas all of the bands that fell in the remaining categories showed euryarchaeal features. In particular, bands in category 3 shared their nucleotide signatures with group D, with the only exception of position 501, and bands in categories 4 and 5 shared their nucleotide signatures with group E, with the exception of position 501 for band e. The putative euryarchaeal band k was detected both in CR-1 (11 to 13 cm) and in CR-2 (21 to 23 cm) but disappeared in the CR-1 deeper horizon (25 to 27 cm) (Fig. 5). Bands e and n, whose signature sequences were also euryarchaeal, were detected in both the deeper horizons of CR-1 and CR-2 cores (25 to 27 cm and 21 to 23 cm, respectively), although the intensity of band e appeared to be significantly higher in CR-1 (25 to 27 cm) (Fig. 5).

TABLE 2. Intradomain nucleotide signature features for the benthic *Archaea* 16S rDNA sequences[a]

Position(s)[b]	Cren[c]	Eury[c]	Marine planktonic group I[d]	Marine benthic group:					
				Group I lineage	Group A[e]	Group B	Group C	Group D	Group E
27–556	C-G	G-C	Cren	Cren	Cren	Cren	Cren	Eury	Eury
28–555	C-G	G-Y	Cren	Cren	Cren	Cren	Cren	Eury	Eury
30–553	G-C	Y-R	Cren	Cren	Cren	Cren	**Eury**	Eury	Eury
501–544	C-G	R-Y	Eury	Eury (G-U)	Eury (G-C)	Eury (G-U)	Eury (G-C)	Eury	Eury
503–542	G-C	C-G	Cren	Cren	Cren	Cren	Cren	Eury	Eury
504–541	G-Y	Y-R	Eury	Eury (U-A)	**A-U**	**A-U**	**A-U**	Eury	Eury/**Cren (G-C)**[f]
513–538	U-A	C-G	Cren	Cren	Cren	Cren	Cren	**G-U**	Eury
518	U	C	Cren	Cren	Cren	**Eury**	Cren	Eury	Eury
658–747	G-C	Y-R	Cren	Cren	Cren	Cren	Cren	Eury	Eury/**Cren**[f]
692	C	U	Eury	Eury	Eury	Eury	Eury	Eury	Eury
965	G	Y	Cren	Cren[g]	**A**[g]	**Eury**	Cren	Eury	Eury
1,074–1,083	G-U	A-C	Cren	Cren[g]	ND	Cren	Cren	Eury	Eury
1,244–1,293	R-Y	Y-R	Cren	Cren[g]	ND	Cren	Cren	Eury (U-A)	Eury (C-G)
1,252	C	U	Eury	Eury[g]	ND	Eury	Eury	Eury	**A**/Eury[f]

[a] Differences between benthic groups A, B, and C and planktonic group I and between benthic groups D and E are indicated in boldface.
[b] Numbering (nucleotide position) is based on the *E. coli* 16S rRNA sequence.
[c] Signature features of 16S rRNA gene sequences for the two archaeal kingdoms, *Crenarchaeota* (Cren) and *Euryarchaeota* (Eury) (59). R and Y represent purines and pyrimidines, respectively.
[d] Group I marine planktonic *Archaea* signature features (9).
[e] Undetermined nucleotides are indicated by ND.
[f] Nucleotide signature features detected for clone APA6-17cm.
[g] Nucleotide signature features not detected for the ACA clones.

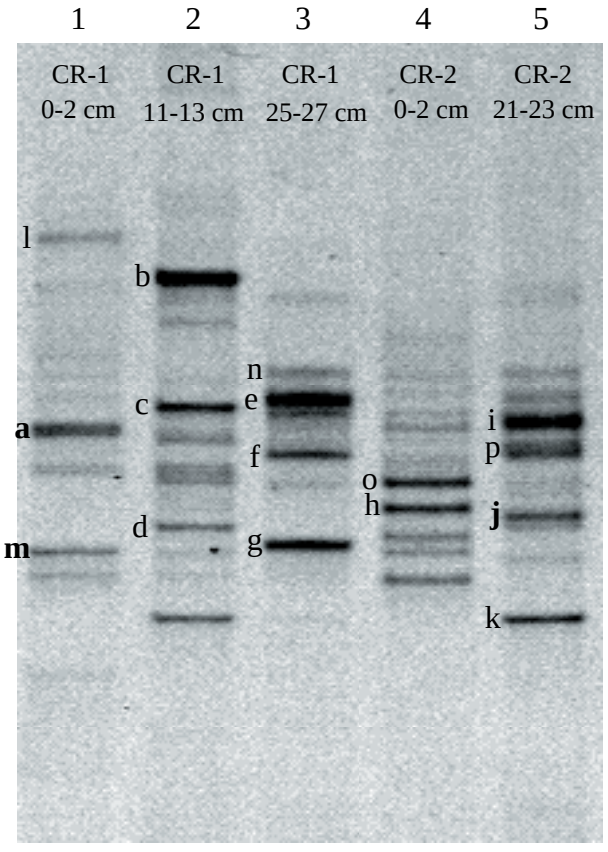

FIG. 5. Comparative DGGE profile analysis of archaeal 16S rDNA V3 fragments from two sampling stations and five vertical depth intervals. Lanes: 1, CR-1, 0 to 2 cm; 2, CR-1, 11 to 13 cm; 3, CR-1, 25 to 27 cm; 4, CR-2, 0 to 2 cm; 5, CR-2, 21 to 23 cm. Letters indicate sequenced 16S rDNA fragments; putative *Crenarchaeota* are indicated in boldface type, and *Euryarchaeota* are indicated in regular type (see text and Table 3).

DISCUSSION

Quantification of the benthic archaeal community. Although *Archaea* have been detected at up to 30% of total rRNA in marine picoplankton (10), in most cases the total contribution of this group is between 1 and 12% (9, 36, 49). Using quantitative probe hybridization, we estimated that the archaeal contributions in AC sediment ranged from 2.4 to 7.9% of the total prokaryotic 16S rRNA, a finding consistent with previous measurements in marine environments. The depth-related profiles of archaeal 16S rRNA abundances in sediments appear to vary depending on the study site. *Archaea* have been found to be more abundant in the oxic region of Lake Michigan sediments (35), whereas a general increase in archeal abundance in the deeper, anoxic regions have been documented in permanently cold marine sediments from the Arctic Ocean (49). Our data provide a preliminary estimate of the abundance of the archaeal 16S rRNA in deep-sea sediments. The precise quantification of the relative contributions and spatial distributions of each group of the benthic *Archaea* will require the design of group-specific probes based on unique consensus sequences.

Our results from hybridization experiments of RNA extracted from CR and AP sediments were not consistent (data not shown) and suggested that the RNA concentrations in these samples were below the detection threshold for the method. The limited amount of nucleic acids available in low biomass environments, such as oligotrophic deep-sea sediments, may be a limitation in quantitative analyses of microorganisms in situ (8), the detection threshold for nucleic acid hybridizations being approximately 10^5 to 10^6 target molecules (53).

Diversity of the benthic *Archaea*: ecological implications. Our study showed that the benthic *Archaea* could be phylogenetically assigned to six different groups, each of them identifiable by group-specific signature sequences. The average intergroup 16S rRNA sequence identities, measured between representatives of each defined group, were low, ranging from 70.2 to 76.8%. Overall, these data show that the benthic *Archaea* are phylogenetically diverse and suggest that each group is likely to represent ecologically distinct populations (45). For instance, most of the sequences recovered from the upper-sediment horizons were grouped with the marine group I planktonic *Archaea*, whereas groups B and D included sequences recovered from deeper, anoxic sediments, both from coastal and deep-sea sites.

In some instances we detected small clusters of very closely related sequences, such as the CRA4-23cm cluster within group B and the ACA17-9cm cluster within group I (Fig. 3), with sequence identities ranging from 97.1 to 99.1%. Although microheterogeneity may be attributed to multiple, nonidentical rRNA operons occurring within the same species, all members of the *Crenarchaeota* characterized to date contain only one rRNA operon (22). Furthermore, two closely related but distinct variants of the crenarchaeon *C. symbiosum* exhibited <0.7% sequence divergence in their rRNA genes (52). Overall, these data suggest that microheterogeneity among crenarchaeal 16S rRNA genes reflects authentic organismal genetic diversity. Microheterogeneity among closely related 16S rRNA sequences have been reported frequently in environmental surveys of microbial diversity (20, 23, 36, 39, 40, 52). Combining physiological and phylogenetic data, several studies linked molecular microdiversity to niche adaptation in marine cyanobacteria and suggested that small-scale diversity in 16S rRNA genes (in the order about 2% sequence divergence) probably represents adaptive radiation of species (18, 21, 40).

TABLE 3. Intradomain signature sequence features in the region from positions 500 to 518 (*E. coli* numbering) for archaeal 16S rDNA fragments retrieved from the DGGE profiles[a]

Position	Category						
	1, bands a, j, and m	2, bands b, c, n, and l	3, bands d, f, h, k, and o	4, band e	5, band g	6, band i	7, band p
501	G (Eury)	A (Eury)	G (Eury)	G (Eury)	A (Eury)	A (Eury)	G (Eury)
503	G (Cren)	C (Eury)	C (Eury)	C (Eury)	C (Eury)	G (Cren)	G (Cren)
504	A	U (Eury)	U (Eury)	U (Eury)	U (Eury)	G (Cren)	A
513	U (Cren)	C (Eury)	G	C (Eury)	C (Eury)	G	G

[a] Eury, *Euryarchaeota*; Cren, *Crenarchaeota*.

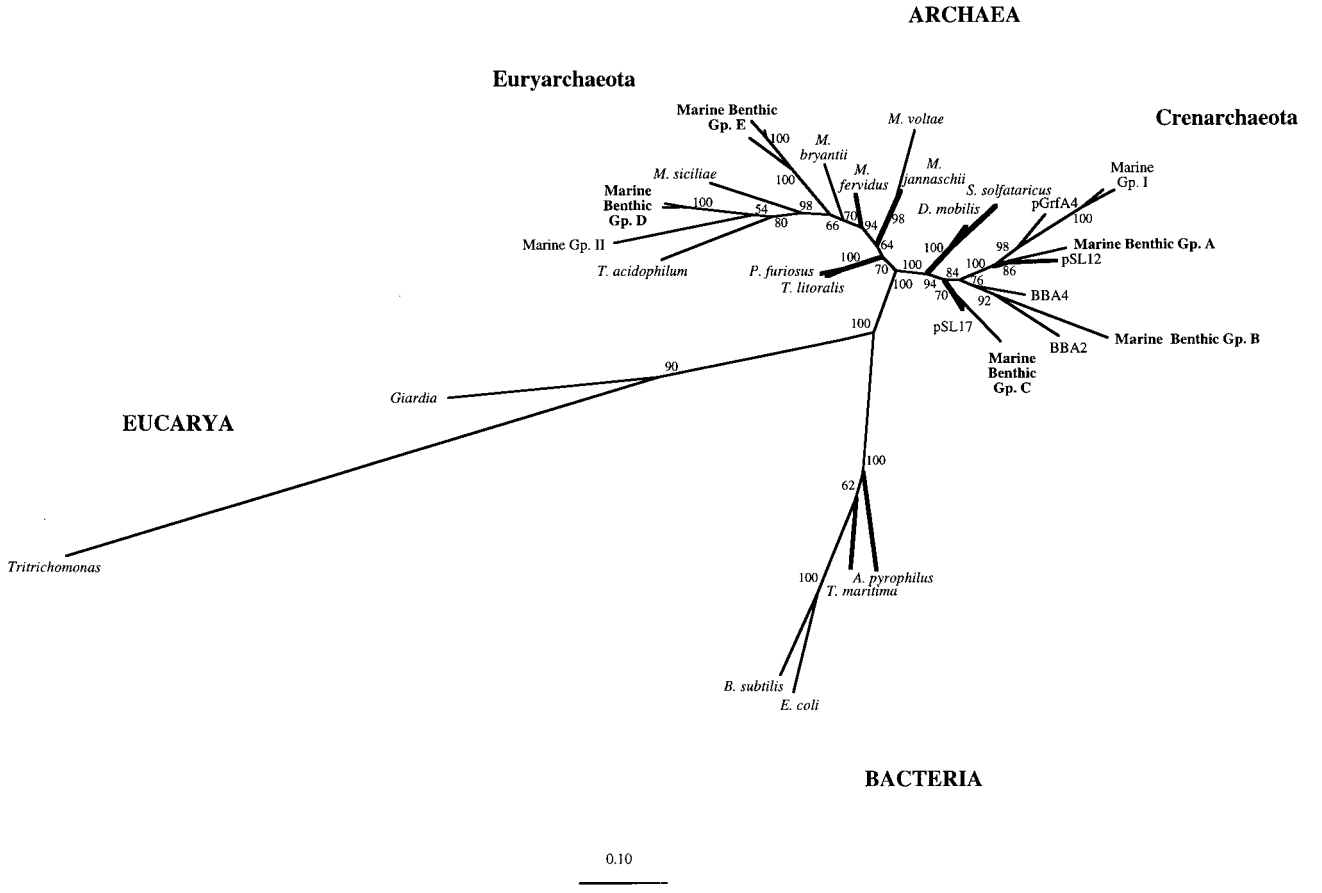

FIG. 6. Maximum-likelihood unrooted phylogenetic tree showing the position of the deep-sea benthic *Archaea* relative to members of the three main domains of life. Branches in boldface type indicate extremely thermophilic organisms. The scale represents the expected number of changes per sequence position. The numbers give the bootstrap values obtained for a bootstrap sampling of 100.

DGGE profiling reveals a complex euryarchaeal community. Comparative DGGE analysis of the benthic archaeal 16S rDNAs in different samples revealed a relatively higher complexity in the community structure of the deeper sediment horizons than in that of the surface sediments (Fig. 5). Direct comparison of the DGGE profiles from CR-1 and CR-2 at 0 to 2 cm (located about 50 miles apart on the CR) showed a significant variability in the composition of the rDNA fragments between the two sampling stations (Fig. 5).

Although DGGE only provided limited phylogenetic information, signature nucleotides in the V3 region of the 16S rDNA allowed us to infer the phylogenetic affiliation of most of the sequenced DGGE fragments (Table 3). Sequence analysis of the DGGE bands revealed that 11 of the 16 bands exhibited euryarchaeal signature sequences.

The presence of *Euryarchaeota* in marine sediments was not unexpected. Methanogenic *Archaea* are prevalent in sulfate-limited sediments, where sulfate-reducing bacteria are not competitors. However, even in sulfate-rich marine sediments methanogenesis may occur because some methanogens can use noncompetitive substrates that are inaccessible to sulfate reducers (44). Although none of the euryarchaeal sequences detected in this study were specifically related to any known methanogen, most of them were recovered from the deep, anoxic regions of the sediments, which suggests a specific association of these microorganisms with anaerobic microhabitats (Fig. 4 and 5). In three instances *Euryarchaeota* were also detected in surface sediments (bands h, l, and o), where anaerobic microniches may be associated with protozoan hosts, with the fecal pellets of deep-sea animals (38, 55), or with the sediment itself.

The identification of only three crenarchaeal bands in the DGGE profiles is not consistent with the wide crenarchaeal diversity detected with the clone library approach, and it may be explained by the intrinsic characteristics of universal primer 518R used in this study (43). The last nucleotide at the 3′ end of primer 518R (G) is complementary to position 518 (*E. coli* numbering) in the 16S rRNA molecule. This position is a U in most of the nonthermophilic *Crenarchaeota* sequenced to date, whereas it is a C in all marine planktonic and benthic *Euryarchaeota* and in the crenarchaeal benthic group B (Table 2) (9). Thus, the use of primer 518R in the nested-PCR approach may have failed to detect most of the crenarchaeal diversity, whereas it provided an in-depth characterization of the complex euryarchaeal populations in deep-sea sediments that would have otherwise escaped full detection. Our data confirmed the extreme sensitivity of the method to the PCR primers of choice and the possibility to target specific subpopulations within larger microbial communities.

High-temperature ancestry of nonthermophilic *Archaea*: evolutionary implications. It has been postulated that the ancestral archaeon was an extremely thermophilic anaerobe that probably derived its energy from the oxidation of molecular hydrogen and from the reduction of sulfur (60). This hypoth-

esis is corroborated by the apparent slow-evolving pace of thermophilic *Archaea* and by their common occurrence near the base of the rooted 16S rRNA phylogenetic tree (5). However, nonthermophilic *Archaea*, particularly marine groups I and II, appear to evolve faster than their thermophilic relatives (9).

Consistently with other reports (5, 8, 26), our phylogenetic analyses revealed that several distinct nonthermophilic crenarchaeal sequences were nested within presumptive thermophilic lineages previously recovered from a hot spring in Yellowstone National Park (5). In particular, our data revealed that marine benthic group A was closely related to pSL12 (5) and that group C was closely related to pSL17 (Fig. 3 and 6). In both cases these affiliations were sustained by high bootstrap values (Fig. 6). Furthermore, several other putative thermophilic lineages (pJP89, pSL123, and pSL22) (5) were found to be in close affiliation with nonthermophilic crenarchaeal clones recovered from freshwater sediments (pGrf26, pLAW11, and pLAW12) (26, 50) and deep subsurface paleosol (Arc.98 and Arc.168) (Fig. 3) (8). Among the *Euryarchaeota*, mesophilic members of the *Methanoccales*, such as *M. voltae* and *M. maripaludis*, are placed within the same lineage of their deeper-rooted thermophilic relative, *M. jannaschii* (Fig. 3 and 6).

The nesting of nonthermophilic *Archaea* within thermophilic lineages implies a high-temperature ancestry for the low-temperature organisms (26). Moreover, the observation that phylogenetically distinct nonthermophilic lineages are specifically affiliated with different thermophilic organisms suggests that the adaptation to low-temperature environments has arisen and evolved in several independent instances within the *Archaea*, although this conclusion requires further verification. The presumed evolution of independent low-temperature adaptive features from distinct thermophilic ancestral lineages is consistent with the wide phylogenetic diversity of the nonthermophilic *Archaea* that we reported here and with their putative broad ecological and metabolic potential.

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