Genetic Diversity of Archaea in Deep-Sea Hydrothermal Vent Environments

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Manuscript received January 26, 1999
Accepted for publication April 12, 1999

ABSTRACT

Molecular phylogenetic analysis of naturally occurring archaeal communities in deep-sea hydrothermal vent environments was carried out by PCR-mediated small subunit rRNA gene (SSU rDNA) sequencing. As determined through partial sequencing of rDNA clones amplified with archaea-specific primers, the archaeal populations in deep-sea hydrothermal vent environments showed a great genetic diversity, and most members of these populations appeared to be uncultivated and unidentified organisms. In the phylogenetic analysis, a number of rDNA sequences obtained from deep-sea hydrothermal vents were placed in deep lineages of the crenarchaeotic phylum prior to the divergence of cultivated thermophilic members of the crenarchaeota or between thermophilic members of the euryarchaeota and members of the methanogen-halophile clade. Whole cell *in situ* hybridization analysis suggested that some microorganisms of novel phylotypes predicted by molecular phylogenetic analysis were likely present in deep-sea hydrothermal vent environments. These findings expand our view of the genetic diversity of archaea in deep-sea hydrothermal vent environments and of the phylogenetic organization of archaea.

RECENT molecular phylogenetic analyses based on small subunit rRNA gene (SSU rDNA) sequencing have indicated that the microbial diversity of naturally occurring microbial communities is much greater than previously assumed based on standard cultivation and isolation methods (Stahl *et al.* 1984; Giovannoni *et al.* 1990; Delong 1992; Fuhrman *et al.* 1992; Ward *et al.* 1992; Barns *et al.* 1994, 1996; Delong *et al.* 1994; Reysenbach *et al.* 1994; Preston *et al.* 1996; Bintrim *et al.* 1997; Massana *et al.* 1997; Munson *et al.* 1997; Schleper *et al.* 1997a; Dojka *et al.* 1998; Hugenholtz *et al.* 1998). Although many studies focusing on the identification and diversity of prokaryotes of the domain Bacteria have been conducted, increasing attention is now being paid to the genetic diversity and ecological significance of prokaryotes of the domain Archaea (Delong 1992; Fuhrman *et al.* 1992; Barns *et al.* 1994, 1996; Delong *et al.* 1994; Preston *et al.* 1996; Bintrim *et al.* 1997; Kudo *et al.* 1997; Massana *et al.* 1997; Mcinerney *et al.* 1997; Munson *et al.* 1997; Schleper *et al.* 1997a; Vetriani *et al.* 1998; Takai and Sako 1999). Molecular phylogenetic surveys of archaeal diversity have focused on a variety of environments, for instance, ocean water (Fuhrman *et al.* 1992; Mcinerney *et al.* 1997), coastal water (Delong 1992; Massana *et al.* 1997), a polar sea (Delong *et al.* 1994), sediments from a continental shelf (Vetriani *et al.* 1998), a salt marsh (Munson *et al.* 1997), a freshwater lake (Schleper *et al.* 1997a), agricultural soils (Bintrim *et al.* 1997), paddy field soil (Kudo *et al.* 1997), Yellowstone hot springs (Barns *et al.* 1994, 1996), shallow marine hydrothermal vent water, and a Japanese acidic hot spring (Takai and Sako 1999). The results of these studies suggested that a number of previously unknown and uncharacterized members of the archaea exist in such environments.

To date, a number of extreme thermophiles or hyperthermophiles have been isolated from terrestrial solfataric fields and hot springs, and shallow or deep marine hydrothermal vent environments (Stetter *et al.* 1990; Adams 1993; Adams *et al.* 1995; Sako *et al.* 1996a,b). The diversity of thermophilic microorganisms in terrestrial hot springs and in a shallow marine hydrothermal vent has been investigated by both the cultivation-isolation technique and the molecular phylogenetic approach (Stetter *et al.* 1990; Adams 1993; Adams *et al.* 1995; Barns *et al.* 1994, 1996; Sako *et al.* 1996a,b; Takai and Sako 1999). Since the discovery of deep-sea hydrothermal vents in 1977, such environments have been regarded as one of the major habitats of thermophiles and have been also a major source of novel deep-sea thermophilic archaea and bacteria (Gonzalez *et al.* 1995, 1998; Prieur *et al.* 1995; Blöchl *et al.* 1997). However, the genetic diversity of archaea in deep-sea hydrothermal vent environments is poorly understood, and molecular phylogenetic surveys of the archaeal populations in such environments have not yet been undertaken.

In this study, we sought to determine the genetic diversity of archaea in deep-sea hydrothermal vent environments such as effluent vent water and chimney structures. The hydrothermal vent systems in this study were located at Suiyo Sea Mount and Myojin Knoll in the Ogasawara area and at Iheya Basin in the Okinawa area,

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Japan, at depths in the range of 1000–1350 m. These hydrothermal vent systems are geographically and geochemically different from other hot water environments studied to date and from one another (Table 1; Ishibashi and Urabe 1995). The archaeal populations in these deep-sea hydrothermal vent environments were compared with those of other high-temperature ecosystems, and the genetic diversity and phylogenetic organization of the archaea were examined.

MATERIALS AND METHODS

Sample collection and DNA extraction: Samples from various deep-sea hydrothermal vent environments were obtained from the hydrothermal fields at Suiyo Sea Mount and Myojin Knoll in the Ogasawara area and from the hydrothermal fields at Iheya Basin in the Okinawa area, Japan, by means of the manned submersible Shinkai 2000 in several dives (Dives 977, 1007, 1016, and 1111). Effluent black smoker vent water (11 liters; ~300°; designated as SSMVW) and the tip of a black smoker chimney (~700 g; designated as SSMC) were collected from a hydrothermal vent site at Suiyo Sea Mount. The tip of a black smoker chimney (~500 g; the temperature of the vent water was ~180°; designated as MC2) and the tip of a clear smoker chimney (~300 g; the temperature of the vent water was ~100°; designated as MC1) were obtained from a hydrothermal vent site at Myojin Knoll. Effluent black smoker vent water (12 liters; ~300°; designated as IVW) and sediment from the upper 1–10-cm layer at the water simmering point (~300 g; the temperature of the sediment was 100°; designated as IS) were obtained from the hydrothermal vent site at Iheya Basin. The sample sources, locations, and properties are summarized in Table 1. Water samples were immediately cooled on ice and stored at 4°, and the tips of chimneys and sediments were immediately frozen at −85° until processed.

Nucleic acids were extracted by physical disruption (the elaborate grinding of filter with mortar and pestle) and chemical lysis of the cells according to the method of Takai and Sako (1999). Approximately 10 liters of effluent black smoker vent water from each of the hydrothermal vent sites at Suiyo Sea Mount or Iheya Basin was sequentially filtered through 10-μm-pore-size Nitex filters and 6.0-μm-pore-size filter paper (Advantec, Tokyo, Japan). Microbial particles were collected from these filtered water samples on 0.22-μm-pore-size, 47-mm-diameter cellulose acetate filters (Advantec). The filters were then washed with NET buffer (50 mm Tris-HCl, pH 8.0/150 mm NaCl/100 mm EDTA) twice and stored at −20°. In the case of the tips of chimneys and sediments, ~300 g (wet) of the sample material was resuspended in 500 ml of MJ synthetic sea water (Sako *et al.* 1996b), which had been filtered through a 0.22-μm-pore-size filter and autoclaved, and the sample material was crushed into fine pieces with a mortar and pestle. The resuspended sample was sonicated for 10 sec twice to remove microbial cells from the crushed chimney and sediment particles and then screened through a 10-μm-pore-size Nitex filter and a 6.0-μm-pore-size filter paper. Microbial fractions were collected on a 0.22-μm-pore-size, 47-mm-diameter cellulose acetate filter. This filter was washed as described above and stored at −20°. To check for experimental contaminants (Tanner *et al.* 1998), a filter without microbial particles was washed with NET buffer twice and used for the following experiment as a negative control containing no environmental DNA. High molecular weight DNA was extracted and purified from all filters including the filter em-

TABLE 1

List of deep-sea hydrothermal vent samples used in this study

Sample (abbreviation)	Source	Location	Temperature/depth	Characteristics[a]
Black smoker vent water (SSMVW)	Suiyo seamount, Izu-Ogasawara arc	28°34.090′N, 140°38.630′E	~300°/1369 m	High temperature fluid containing relatively high concentrations of Ca, Sr, Ba, Al
Black smoker chimney (SSMC)	Suiyo seamount, Izu-Ogasawara arc	28°34.090′N, 140°38.630′E	~300° inside/1369 m	Composed of chalcopyrite, sphalerite, anhydrite, barite. High Au content
Clear smoker chimney (MC1)	Myojin Knoll, Izu-Ogasawara arc	32°06.208′N, 139°52.004′E	~100° inside/1338 m	~100° clear fluid discharged from
Black smoker chimney (MC2)	Myojin Knoll, Izu-Ogasawara arc	32°06.208′N, 139°52.004′E	~180° inside/1330 m	~180° black-colored fluid discharged from. Composed of barite, pyrite, and other minor sulfides
Black smoker vent water (IVW)	Iheya Basin, Middle Okinawa trough	27°47.220′N, 126°53.900′E	~300°/972 m	High temperature fluid containing quite high concentrations of CO_2, CH_4, and other carbon hydrates, and H_2S
Water simmering sediments (IS)	Iheya Basin, Middle Okinawa trough	27°32.800′N, 126°58.300′E	~100°/1398 m	Composed of carbonate-rich hydrothermal precipitates with disseminated sulfides

[a] Characteristics were for the large part referred from Ishibashi and Urabe (1995).

ployed as a negative control as described previously (Takai and Sako 1999).

Microscopic observation: The vent water and chimney samples filtered through 6.0-μm-pore-size filter paper were fixed for 12 hr in 3.7% formaldehyde, and the fixed microbial particles were collected on 0.22-μm-pore-size, 13-mm-diameter polycarbonate filters (Advantec). Each filter was rinsed twice in MJ synthetic sea water (Sako *et al.* 1996b), which had been filtered through a 0.22-μm-pore-size filter and autoclaved, and then stained by treatment with MJ sea water containing 4′, 6-diamidino-2-phenylindole (DAPI; 10 μg/ml) or ethidium bromide (10 μg/ml) at 4° for 20 min. The filter was briefly rinsed in MJ sea water and examined under epifluorescence using a Leica DMRB microscope with a Leica MPS 30 camera system.

PCR amplification of rDNA: Small subunit ribosomal RNA genes (rDNAs) were amplified by PCR using LA Taq polymerase with GC buffer (TaKaRa, Kyoto, Japan), as recommended by the manufacturer. Reaction mixtures were prepared in which the concentration of each oligonucleotide primer was 0.1 μm and that of the DNA template was 1 ng/μl. Thermal cycling was performed using the GeneAmp PCR system 9600 (Perkin-Elmer, Foster City, CA) and the conditions were as follows: denaturation at 96° for 20 sec, annealing at 50° for 45 sec, and extension at 72° for 120 sec for a total of 30 cycles. The oligonucleotide primer sequences and combinations used were as follows: Arch21F (Fuhrman *et al.* 1992) and 1492R (Lane 1985) for archaeal rDNA, and Uni515F and Uni1408R (Takai and Sako 1999) for all microbial rDNAs.

Cloning and sequencing of amplified rDNA: Amplified rDNAs from five separate reactions were pooled, fractionated by electrophoresis on a 1.2% (w/v) agarose gel, then extracted from the gel slices sequentially with phenol, phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol, and precipitated with ethanol. After centrifugation, DNA pellets were resuspended in sterile distilled water. The purified rDNAs were cloned in the vector pCR2.1 using the original TA cloning kit (Invitrogen, Carlsbad, CA), and then the archaeal and universal primer PCR libraries were built from DNA obtained from each deep-sea hydrothermal vent sample. Clones containing inserts of the appropriate size were identified by electrophoresis of alkaline lysis plasmid preparations (Maniatis *et al.* 1982). Denatured, double-stranded plasmid templates were sequenced by the dideoxynucleotide chain-termination method with Taq DNA polymerase FS (Perkin-Elmer) following the manufacturer's recommendations. Universal rRNA-specific primers (Lane 1985) and M13 forward and reverse primers (Maniatis *et al.* 1982) were used in sequencing reactions.

Sequence and phylogenetic analyses: The partial rDNA sequences were analyzed using SIMILARITY_RANK and ALIGN_SEQUENCE from the Ribosomal Database Project (RDP; Larsen *et al.* 1993) and the gapped-BLAST search algorithm (Altschul *et al.* 1997; Benson *et al.* 1998) to estimate the degree of similarity to other rDNA sequences. Sequences of 400 bp in length determined by means of Uni515F primer in one strand were used in the similarity analysis. The databases used for RDP and gapped-BLAST analysis were the prokaryotic SSU rRNA database and the nonredundant nucleotide sequence database from GenBank, EMBL, and DDBJ, respectively. In this study, we tentatively defined that >98% similarity in Uni515F-dependent sequences represented the same rDNA clone type among rDNA sequences obtained from deep-sea hydrothermal vent environments. In addition, error frequency, which presumably occurred through PCR amplification, cloning, and partial sequencing, was roughly estimated. The 16S rDNA of *Pyrococcus horikoshii* JCM9974 (Gonzalez *et al.* 1998) was amplified, cloned, and partially sequenced in the same manner as described in this study. A total of 26 of 33 clones revealed the correct identical rDNA sequence of this organism. A total of 6 of 33 clones had an error such as a single nucleotide substitution, insertion, or deletion, and 1 of 33 clones had both a single nucleotide substitution and a single nucleotide insertion. The overall error frequency of partial sequencing analysis was calculated to be 0.063 error per 100 nucleotides.

Sequences were manually aligned to SSU rDNA data from the RDP on the basis of primary and secondary structure considerations and were also submitted to analysis using the CHECK_CHIMERA program to detect the presence of chimeric artifacts. Phylogenetic analyses were restricted to nucleotide positions that were unambiguously alignable in all sequences. Least-squares distance matrix analysis (Olsen 1988), based on evolutionary distances, was performed using the correction of Kimura (1980). Neighbor-joining analysis was accomplished using the ODEN software package (version 1.1; National Institute of Genetics, Mishima, Japan). Maximum-likelihood analyses were performed using the PHYLIP package (version 3.5; obtained from J. Felsenstein, University of Washington, Seattle). Bootstrap analysis was used to provide confidence estimates for phylogenetic tree topologies.

Whole-cell hybridization analysis: Ribosomal RNA-targeted oligonucleotide probes were designed for detection of archaeal members that were potentially predominant in the deep-sea hydrothermal vent environments. They were the MGI probe (5′-AAAYCACTCGGAYTAACCTT-3′) and AAG probe (5′-CCTAGCACTCGGGCTCGCGG-3′), which corresponded to positions 391–411 and 401–421 in *Escherichia coli* 16S rDNA, respectively, and were designed for detection of marine group I (MGI) crenarchaeotic members and a few ancient archaeal group (AAG) members based on the alignment in the phylogenetic analysis. Both probe sequences were analyzed using the PROBE_CHECK from the RDP (Larsen *et al.* 1993) and the gapped-BLAST search algorithm (Altschul *et al.* 1997; Benson *et al.* 1998) to examine whether any other rDNA sequences have similarity to these probe sequences. In addition, dot-hybridization analysis was carried out with representative archaeal rDNA clones obtained from the deep-sea hydrothermal vent environments to check the specificity of the probes toward the MGI crenarchaeotic and AAG members, respectively. The purified plasmid DNAs (100 ng) containing ~1.5 kb of rDNA sequences were blotted onto positively charged nylon membranes (Boehringer Mannheim, Indianapolis). The membranes were hybridized with the MGI-D probe or the AAG-D probe (5 ng/μl), which was labeled at the 5′-end with digoxigenin (DIG) and purified by HPLC (Amersham Pharmacia Biotech). After hybridization, the membranes were washed, and the DIG-labeled oligonucleotide probes hybridized with the targeted rDNA sequences were detected by using the DIG luminescent detection kit for nucleic acids (Boehringer Mannheim). All hybridization and washing procedures were carried out in the same conditions as described below.

For whole-cell hybridization experiments, the vent water and chimney samples filtered through 6.0-μm-pore-size filter paper were fixed for 12 hr in 3.7% formaldehyde, and the fixed microbial particles were collected on 0.22-μm-pore-size, 13-mm-diameter polycarbonate filters (Advantec). Each filter was rinsed sequentially in MJ synthetic sea water that had been filtered through a 0.22-μm-pore-size filter and autoclaved in 1:1 (v/v) MJ sea water/deionized distilled water (DDW) and DDW. The filter was dehydrated in an ethanol series (30, 60, 80, 90, and 95%, v/v) and air-dried (Amann *et al.* 1990). The hybridization was carried out in hybridization solution containing 20 mm Tris-HCl (pH 7.2), 0.9 m NaCl, 0.1% (w/v) sodium dodecyl sulfate, and 50% (v/v) formamide for 12 hr at 48° in the case of the MGI-F probe (5 ng/μl) or at

55° in the case of the AAG-F probe (5 ng/μl), which were each labeled at the 5′-end with fluorescein and purified by HPLC (Amersham Pharmacia Biotech). After hybridization, the filter was washed in hybridization solution lacking the probes, at the same temperature as that employed for hybridization, for 20 min twice and stained with a 10 μg/ml solution of DAPI in DDW at 4° for 20 min. The filter was briefly rinsed in DDW and was examined under epifluorescence using a Leica DMRB microscope with a Leica MPS 30 camera system. Two types of negative controls were employed in each experiment, using identical cell preparations and hybridization conditions. One type of negative control consisted of samples in which the unlabeled MGI-F or AAG-F probe was added in 50-fold excess (250 ng/μl) of the fluorescein-labeled MGI-F or AAG-F probe added at the standard concentration (5 ng/μl). The other type of negative control consisted of samples employed to examine hybridization with cultivated thermophilic microorganisms, including *Aeropyrum pernix* JCM9820 (Sako *et al.* 1996a), *Thermococcus peptonophilus* JCM9653 (Gonzalez *et al.* 1995), *P. horikoshii* JCM9974 (Gonzalez *et al.* 1998), *Methanococcus jannashii* DSM2661 (Jones *et al.* 1983), *Rhodothermus obamensis* JCM9785 (Sako *et al.* 1996b), and *Thermaerobacter marianensis* (Takai *et al.* 1999). Cells were grown as described in the references given above, and cells in the stationary phase of growth were used for these hybridization experiments.

Nucleotide sequence accession numbers: The sequences reported here have been deposited in the DDBJ database under the accession nos. AB019714–AB019759.

RESULTS

Characterization of microbial rDNA recovered from deep-sea hydrothermal vent environments: DNA was isolated directly from samples collected from various deep-sea hydrothermal vent environments except for the effluent water from the black smoker vent (SSMVW) at the hydrothermal vent site at Suiyo Sea Mount. In that sample, low DNA recovery was expected due to the very small number of microorganisms (<10 cells/ml) observed in the sample by epifluorescence microscopy after staining with DAPI or ethidium bromide (Table 2). Microbial ribosomal RNA genes were selectively amplified by PCR and cloned. PCR amplification was also carried out using extracts prepared from negative control samples to check for possible contaminants introduced during the DNA extraction and purification procedures, as described by Tanner *et al.* (1998). However, no rDNA was amplified from the negative control samples using either the archaea-specific primers or the universal primers under the conditions of this study.

The first step in our strategy was to examine the archaeal populations in naturally occurring microbial communities in various deep-sea hydrothermal vent environments on the basis of partial nucleotide sequence information obtained through analysis of a number of insert-containing plasmids. First, a 400-nt sequence (corresponding to positions 536–935 of *E. coli* rDNA numbering) determined in one strand from each plasmid was obtained from universal and archaeal primed PCR libraries of each sample. In this study, 132, 116, 110, 102, and 124 clones were partially sequenced from the universal primer PCR libraries established from the deep-sea hydrothermal vent samples of SSMC, MC1, MC2, IVW, and IS, respectively (Table 2). Furthermore, 104, 164, 139, 145, and 188 clones were partially sequenced from the archaeal primer PCR libraries of SSMCA (SSMC-Archaeal library), MC1A, MC2A, IVWA, and ISA (Table 3). Gapped-BLAST analysis of these sequences provided a profile of the PCR-dependent rDNA composition of the various environmental samples (Tables 2 and 3). As described in materials and methods, we tentatively defined that >98% similarity in Uni515F-dependent sequences represented an identical rDNA clone type among rDNA sequences obtained from deep-sea hydrothermal vent environments. The error frequency during the partial sequencing analysis was estimated to be <0.1%.

From the black smoker vent chimney of Suiyo Sea Mount (SSMC), 128 of the 132 clones (97%) with inserts amplified by means of universal primers were found to be bacterial SSU rDNA sequences (Table 2). Archaeal rDNA sequences were detected in a library of sequences derived from SSMC prepared by PCR using the universal primer. All 4 of these clones were found to have the same rDNA sequence, and this sequence was the predominant rDNA sequence (pSSMCA1) found in the archaeal popu-

TABLE 2

Ribosomal DNA composition of samples from various deep-sea hydrothermal vent environments

Sample (no. of clones sequenced)	Bacterial (%)	Archaeal (%)	Eucaryal (%)	Microbial cells[a]
SSMVW	ND	ND	ND	ND
SSMC (132)	97.0	3.0	0	10^4/g wet
MC1 (116)	82.8	17.2	0	10^7/g wet
MC2 (110)	95.5	4.5	0	10^7/g wet
IVW (102)	73.5	26.5	0	10^4/ml
IS (124)	96.8	3.4	0	10^7/g wet

Ribosomal DNA composition was determined by partial sequencing analysis of rDNA clones of universal primer PCR libraries. ND, not detected.

[a] Direct counts of microbial cells stained with DAPI and ethidium bromide.

lation of this sample. The archaeal rDNA clones derived from SSMC consisted of only two clone types, represented by pSSMCA1 (99 of 104 clones) and pSSMCA108 (5 of 104 clones), both of which showed relatively low similarity (<80%) to any other rDNA sequences from cultivated or uncultivated archaea (Table 3).

The proportions of archaeal rDNA sequences were similar in the universal primer PCR libraries prepared from the black smoker chimney sample collected from Myojin Knoll (MC2) and the clear water simmering sediment sample from Iheya Basin (IS; Table 2). The archaeal primer PCR library prepared from MC2 consisted of a variety of rDNA sequences, and the same clone type as that recovered from SSMC was found also in this library (Table 3). The archaeal rDNA composition of IS differed significantly from that of the black smoker chimney samples of SSMC and MC2. In the case of the archaeal primer PCR library prepared from IS, a high proportion of Euryarchaeota was found (Table 3). These euryarchaeotic rDNA clones were unidentified sequences and consisted of a variety of clone types (Table 3).

A relatively high proportion of archaea was found in the universal primer PCR libraries prepared from the clear smoker chimney sample obtained from Myojin Knoll (MC1) and the effluent black smoker vent water sample from Iheya Basin (IVW). In the universal primer PCR library prepared from MC1 and IVW, 20 of 116 clones (17.2%) and 30 of 102 clones (26.5%) were archaeal rDNA sequences, respectively (Table 2). All of these archaeal rDNA clones were closely related to marine planktonic clones, designated as MGI (Delong 1992; Fuhrman *et al.* 1992; Delong *et al.* 1994; Massana *et al.* 1997; Mcinerney *et al.* 1997); however, none of these sequences showed >98% similarity to the sequences of MGI clones described previously. In addition, the archaeal primer PCR library prepared from MC1 contained only two clone types, both of which were categorized within the MGI (39 clones for pMC1A103 type or 71 clones for pMC1A11 type of 164 clones), and another major unidentified clone type (pMC1A4) categorized within the Euryarchaeota (54 of 164 clones), while the archaeal primer PCR library prepared from IVW mainly consisted of a variety of rDNA clone types categorized within the MGI (Table 3).

Phylogenetic analyses of rDNA sequences representing unidentified microorganisms: To infer phylogenetic affiliations of archaeal rDNA clones obtained from various deep-sea hydrothermal vent environments, nearly full-length sequences (~1400 nt) of representative rDNA clone types were determined and used for appropriate phylogenetic analysis. Phylogenetic analysis by the neighbor-joining and maximum-likelihood methods resulted in trees with similar topologies (Figure 1). In an overview of the tree, the archaeal rDNA sequences obtained not only from deep-sea hydrothermal vent environments but also other hot water environments were found to constitute two large phylogenetic assemblages; one consisted of the very deep lineages of rDNA sequences within the Crenarchaeota and the other was placed in an intermediate position between the thermophilic Euryarchaeota and the methanogens-halophiles (Figure 1). Most of the rDNA clones obtained from deep-sea hydrothermal vent environments were placed in novel phylogenetic branches distinct from those of cultivated members of the archaea and other environmental clones (Figure 1).

Of the many novel rDNA sequences found, several rDNA sequences represented by pMC2A256, pMC2A249, and pMC2A14/pSSMCA1 (AAG) were placed in the deepest branches within the crenarchaeotic phylum. In this phylogenetic tree, the crenarchaeotic phylum accommodated korarchaeotic rDNA sequences and clone pOWA133, which were previously denoted as possible outgroups prior to the divergence of Crenarchaeota and Euryarchaeota, respectively (Barns *et al.* 1994; Takai and Sako 1999). The preference in placement of these deep lineages of rDNA sequences into the crenarchaeotic phylum was strongly influenced by the rDNA sequence of *Methanopyrus kandleri*. When the *M. kandleri* rDNA sequence was omitted from the phylogenetic analysis, the phylogenetic trees obtained based on the neighbor-joining and maximum-likelihood methods showed five possible separate archaeal phyletic groups at the kingdom level; the deepest outgroup was represented by the AAG rDNA clones, the second outgroup by the pOWA133 clone, and the third outgroup by the korarchaeotic clones, prior to the divergence of Crenarchaeota and Euryarchaeota (data not shown). These very deep branches of rDNA sequences obtained from various hot water environments had relatively low bootstrap significance and were sensitive to the sequences used; however, base composition disparities had little influence on the topology, which was supported by transversion distance-matrix analysis (Woese *et al.* 1991). These results indicated that the AAG rDNA clones recovered from deep-sea hydrothermal vent environments undoubtedly represent one of the deepest lineages of any archaeal rDNA sequences derived from cultivated members or unidentified environmental clones, although whether the phylogenetic classification of these rDNA clones into the kingdom Crenarchaeota is appropriate remains uncertain.

In addition, a great diversity of rDNA sequences were allied in positions close to the Thermoplasma-Picrophilus clade and in intermediate positions between thermophilic Euryarchaeota branches and methanogen-halophile branches (DHV euryarchaeotic groups I and II; Figure 1). These sequences were mainly obtained from the black smoker chimney at Myojin Knoll (MC2) and the clear water simmering sediments at IS, and most of them showed no significant similarity to any other rDNA sequences known to date. When the phylogenetic tree was reconstructed by neighbor-joining methods to in-

TABLE 3

Distribution of representative archaeal rDNA clone types in various deep-sea hydrothermal environments

Phylogenetic group	Clone type (position on blot)	SSMC	MC1	MC2	IVW	IS
Crenarchaeota		39/99[a]	90/110	46/63	103/141	5/6
Ancient archaeal group[b]		39/99	—	14/22	—	—
DHVA1[b]						
	pMC2A256 (1e)	—	—	1/2	—	—
	pMC2A249 (1d)	—	—	1/1	—	—
	pMC2A14 (1c)/pSSMCA1 (1a/1b)	39/99	—	12/19	—	—
DHV crenarchaeotic group[b]		—	—	12/14	—	—
DHVC1[b]						
	pMC2A15 (1f)	—	—	1/1	—	—
	pMC2A209 (1g)	—	—	4/5	—	—
	pMC2A36 (1h)	—	—	4/4	—	—
	pMC2A308 (1i)	—	—	3/4	—	—
Marine group I (MGI)		—	90/110	20/27	103/141	—
	pMC1A103 (2a)	—	31/39	—	—	—
	pMC1A11 (2b)	—	59/71	—	—	—
	pMC2A1 (2c)	—	—	20/27	—	—
	pIVWA1 (2d)	—	—	—	21/23	—
	pIVWA2 (2e)	—	—	—	28/39	—
	pIVWA3 (2f)	—	—	—	11/16	—
	pIVWA5 (2g)	—	—	—	29/47	—
	pIVWA11 (2h)	—	—	—	3/4	—
	pIVWA101 (2i)	—	—	—	8/9	—
	pIVWA108 (2j)	—	—	—	3/3	—
THS crenarchaeotic group[b]		—	—	—	—	5/6
THSC1[b]						
	pISA9 (1j)	—	—	—	—	4/5
	pISA7 (3a)	—	—	—	—	1/1
Euryarcheaota		3/5	36/54	66/76	3/4	144/182
Methanococcales		—	—	1/1	—	—
	pMC2A384 (3b)	—	—	1/1	—	—
Archaeglobales		—	—	3/3	—	—
	pMC2A228 (3c)	—	—	3/3	—	—
DHV euryarchaeotic group I[b]		3/5	—	39/46	—	52/56
DHVE1[b]						
	pMC2A24 (3d)	—	—	17/18	—	—
	pMC2A203 (3e)	—	—	3/3	—	—
	pMC2A33 (3f)	—	—	2/2	—	—
DHVE2[b]						
	pMC2A10/pSSMCA108 (3g)	3/5	—	17/23	—	—
	pISA12 (3h)	—	—	—	—	23/24
	pISA42 (3i)	—	—	—	—	29/32
DHV euryarchaeotic group II[b]		—	36/54	23/26	3/4	59/78
DHVE3[b]						
	pMC2A25 (3j)	—	—	2/3	—	—
	pMC2A21 (4a)	—	—	4/4	—	—
	pMC2A232 (4b)	—	—	8/8	—	—
	pMC2A211 (4c)	—	—	6/8	—	—
DHVE4[b]						
	pMC2A17 (4d)	—	—	1/1	—	—
	pISA35 (4e)	—	—	—	—	1/1
DHVE5[b]						
	pISA18 (4f)	—	—	—	—	6/7
	pISA38 (4g)	—	—	—	—	4/4
	pISA1 (4h)	—	—	—	—	4/4

(*continued*)

TABLE 3

(Continued)

Phylogenetic group	Clone type (position on blot)	SSMC	MC1	MC2	IVW	IS
DHVE6[b]						
	pISA3 (4i)	—	—	—	—	3/5
	pMC2A35 (4j)	—	—	2/2	—	—
	pMC1A4 (5a)	—	36/54	—	—	—
	pISA48 (5b)	—	—	—	—	31/41
	pISA13/pIVWA104 (5c)	—	—	—	3/4	10/16
DHV euryarchaeotic group III[b]		—	—	—	—	33/48
DHVE7[b]						
	pISA16 (5d)	—	—	—	—	26/37
	pISA14 (5e)	—	—	—	—	7/11
Total		**42/104**	**126/164**	**112/139**	**106/145**	**149/188**

Archaeal rDNA composition was determined by partial sequencing analysis of rDNA clones of archaeal primer PCR libraries.

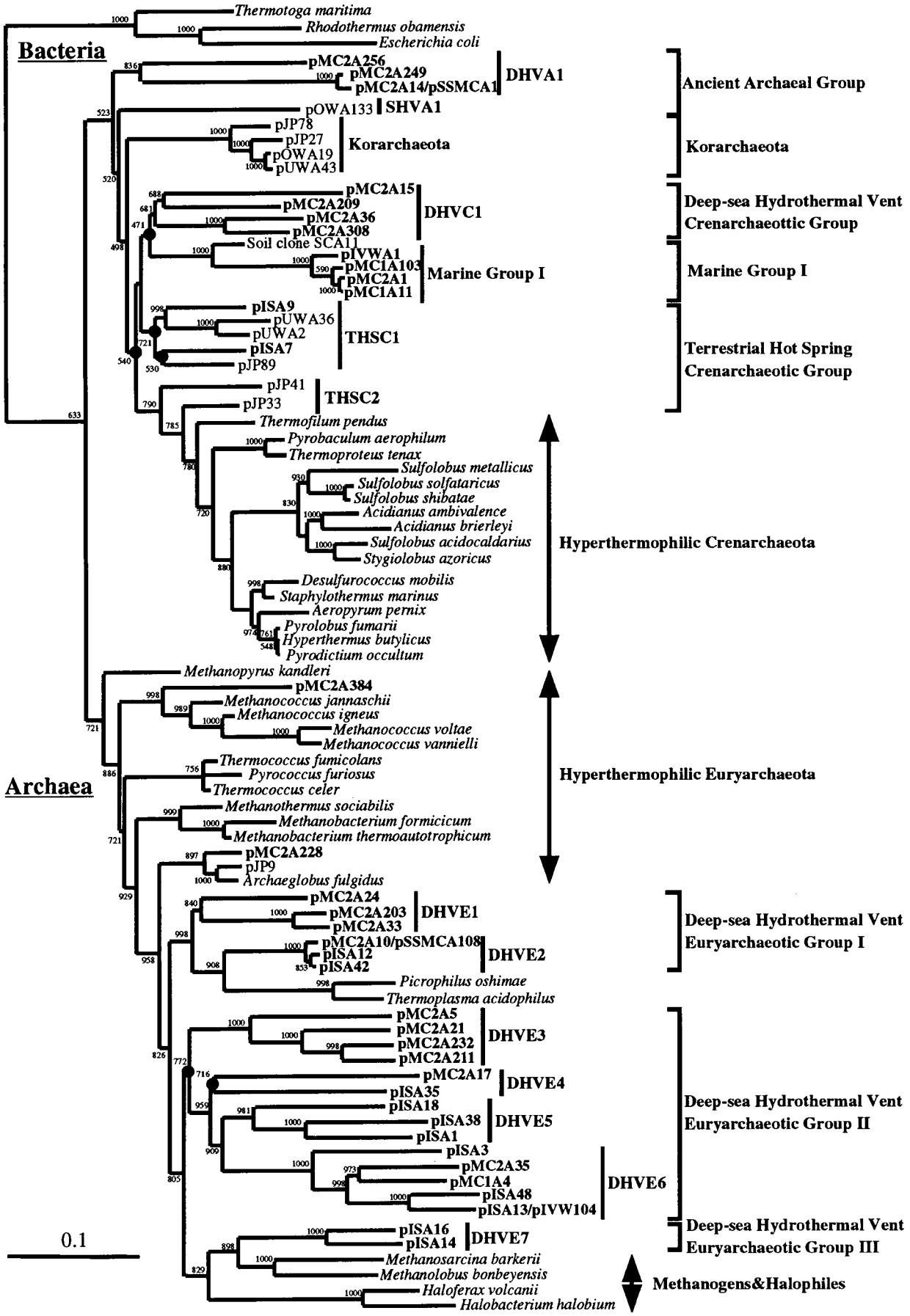
[a] This indicates the number of sequence variations per total number of clones designated into each phylogenetic group or clone type on the basis of partial sequencing analysis.

[b] Novel uncultivated phylogenetic groups shown in Figure 1 and abbreviations are as follows: DHVA, deep-sea hydrothermal vent ancient archaea; DHVC, deep-sea hydrothermal vent crenarchaeota; THSC, terrestrial hot spring crenarchaeota; DHVE, deep-sea hydrothermal vent euryarchaeota.

clude other environmental, unidentified euryarchaeotic clones based on the partial sequences (corresponding to positions 438–887 in *E. coli* 16S rDNA), some phylotypes of sequences (DHVE1) within the DHV euryarchaeotic group I were closely related with some unidentified euryarchaeotic clones obtained from microbial communities in coastal salt marsh and continental shelf sediments (salt marsh and marine benthic euryarchaeota; Munson *et al.* 1997; Vetriani *et al.* 1998; Figure 2). However, no significant phylogenetic relatedness was observed between a variety of euryarchaeotic rDNA sequences from the deep-sea hydrothermal vent environments and the marine group II obtained from planktonic microbial communities in ocean water, coastal water, and polar sea water (MGII; Delong 1992; Delong *et al.* 1994). It seems likely, therefore, that uncultivated and unidentified members of the euryarchaeota displaying great genetic diversity are present in the microbial communities of various coastal and deep sea sedimentary environments, as in the case of members of MGI in planktonic microbial communities (Delong 1992; Fuhrman *et al.* 1992; Delong *et al.* 1994; Massana *et al.* 1997; Mcinerney *et al.* 1997).

Figure 3 shows a phylogenetic tree prepared focusing on the MGI rDNA clones. These MGI rDNA clones are the most frequently occurring rDNA clones in marine planktonic microbial communities (Delong 1992; Fuhrman *et al.* 1992; Delong *et al.* 1994; Massana *et al.* 1997; Mcinerney *et al.* 1997) and have often been recovered from marine sediment samples and even sediment adjacent to a hydrothermal vent (Takai and Sako 1999). A variety of archaeal rDNA sequences closely related to the sequences of the MGI clones were also obtained from the black smoker chimney (pMC2A) and the clear smoker chimney (pMC1A) at Myojin Knoll, and the black smoker vent water (pIVWA) at Iheya Basin (Figure 3). When the phylogenetic tree was reconstructed including these new MGI clones, two evolutionary arrays were found in the MGI cluster. The finding of further genetic diversity among the members of the MGI in deep-sea hydrothermal vent environments serves as an important clue to understanding the ubiquity and origin of these uncultivated members of the archaea in marine environments.

Whole-cell hybridization analysis: Ribosomal RNA-targeted oligonucleotide probes were employed in visualization of formalin-fixed microbial cells from the deep-sea hydrothermal vent environments to facilitate identification of members of MGI or the AAG represented by pMC2A249, pMC2A14, and pSSMCA1. The designed probes were confirmed to have no specificity to any other rDNA sequences by analyses using the PROBE_CHECK from the RDP (Larsen *et al.* 1993) and the gapped-BLAST search algorithm (Altschul *et al.* 1997; Benson *et al.* 1998), and were also confirmed to hybridize specifically with the targeted rDNA sequences of MGI and AAG members by dot-hybridization analysis (Figure 4). Using these probes, whole-cell hybridization analysis was carried out with the samples of SSMC, MC1, MC2, and IVW (Figure 5). Hybridization with the MGI-specific fluorescent oligonucleotide probe was observed in the samples of MC1 and IVW, and hybridization with the AAG-specific probe was observed in the case of SSMC and MC2. The archaeal cells in the case of members of the AAG were irregular cocci, on average 1.0 μm in diameter (Figure 5, C and D, G and H), and


Bacteria
Archaea
Thermotoga maritima
Rhodothermus obamensis
Escherichia coli
pMC2A256
pMC2A249
pMC2A14/pSSMCA1
DHVA1
pOWA133
SHVA1
pJP78
pJP27
pOWA19
pUWA43
Korarchaeota
pMC2A15
pMC2A209
pMC2A36
pMC2A308
DHVC1
Soil clone SCA11
pIVWA1
pMC1A103
pMC2A1
pMC1A11
Marine Group I
pISA9
pUWA36
pUWA2
pISA7
pJP89
THSC1
pJP41
pJP33
THSC2
Thermofilum pendus
Pyrobaculum aerophilum
Thermoproteus tenax
Sulfolobus metallicus
Sulfolobus solfataricus
Sulfolobus shibatae
Acidianus ambivalence
Acidianus brierleyi
Sulfolobus acidocaldarius
Stygiolobus azoricus
Desulfurococcus mobilis
Staphylothermus marinus
Aeropyrum pernix
Pyrolobus fumarii
Hyperthermus butylicus
Pyrodictium occultum
Methanopyrus kandleri
pMC2A384
Methanococcus jannaschii
Methanococcus igneus
Methanococcus voltae
Methanococcus vannielli
Thermococcus fumicolans
Pyrococcus furiosus
Thermococcus celer
Methanothermus sociabilis
Methanobacterium formicicum
Methanobacterium thermoautotrophicum
pMC2A228
pJP9
Archaeglobus fulgidus
pMC2A24
pMC2A203
pMC2A33
DHVE1
pMC2A10/pSSMCA108
pISA12
pISA42
DHVE2
Picrophilus oshimae
Thermoplasma acidophilus
pMC2A5
pMC2A21
pMC2A232
pMC2A211
DHVE3
pMC2A17
DHVE4
pISA35
pISA18
pISA38
DHVE5
pISA1
pISA3
pMC2A35
pMC1A4
pISA48
pISA13/pIVW104
DHVE6
pISA16
pISA14
DHVE7
Methanosarcina barkerii
Methanolobus bonbeyensis
Haloferax volcanii
Halobacterium halobium
0.1
Ancient Archaeal Group
Korarchaeota
Deep-sea Hydrothermal Vent Crenarchaeottic Group
Marine Group I
Terrestrial Hot Spring Crenarchaeotic Group
Hyperthermophilic Crenarchaeota
Hyperthermophilic Euryarchaeota
Deep-sea Hydrothermal Vent Euryarchaeotic Group I
Deep-sea Hydrothermal Vent Euryarchaeotic Group II
Deep-sea Hydrothermal Vent Euryarchaeotic Group III
Methanogens&Halophiles

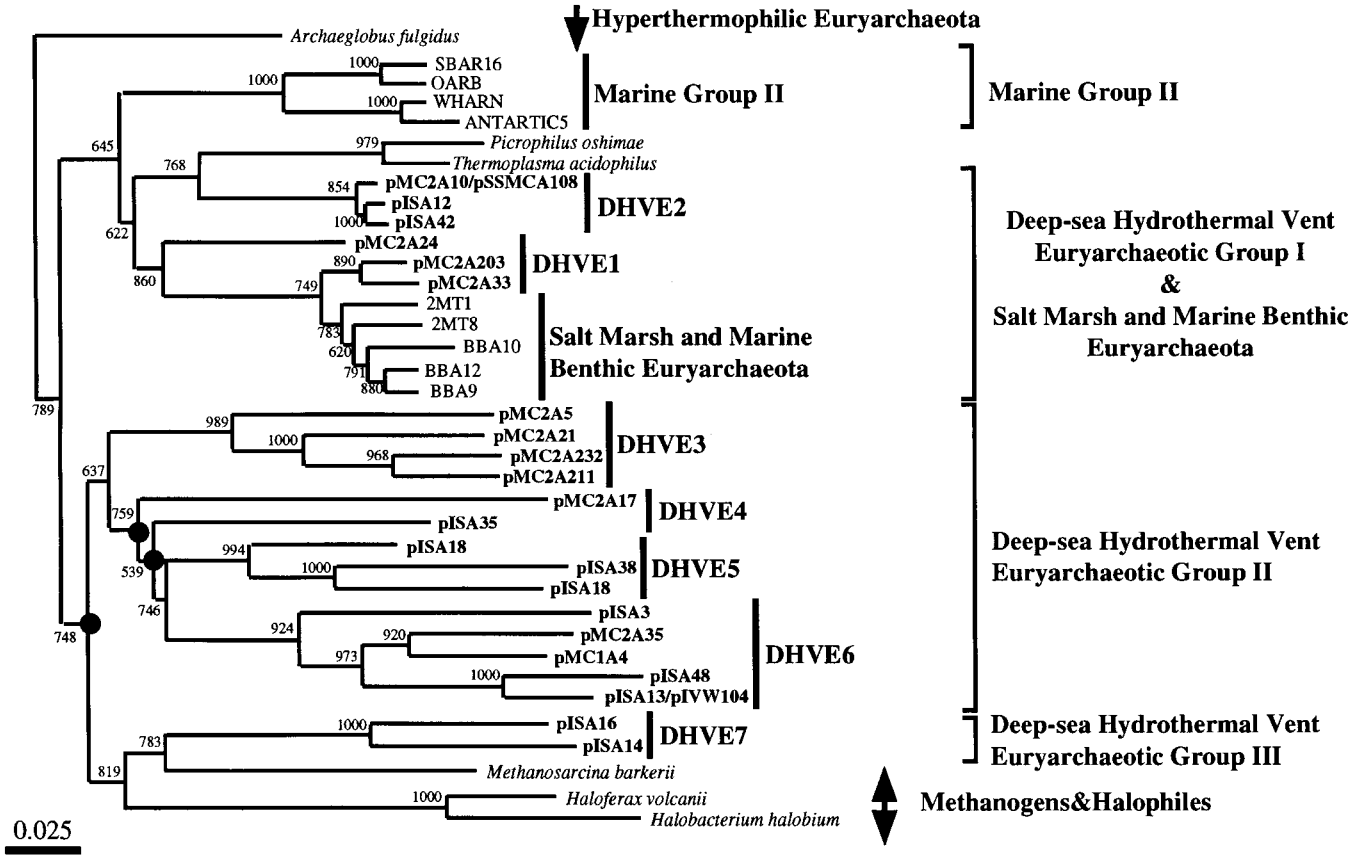


Figure 2.—Phylogenetic tree of rDNA clones focused on a variety of environmental, unidentified euryarchaeotic rDNA sequences. The tree was inferred by neighbor-joining analysis of 436 homologous positions of the rDNA sequence in the case of each organism or clone. Abbreviations indicate rDNA clones corresponding to uncultivated organisms derived from the following environments: SBAR16, OARB, and WHARN, from coastal water in North America (Delong 1992); ANTARCTIC5, from antarctic marine picoplankton (Delong *et al.* 1994); 2MT, from salt marsh sediment (Munson *et al.* 1997); and BBA, from continental shelf sediments (Vetriani *et al.* 1998). Bold letters indicate rDNA clones obtained from deep-sea hydrothermal vent environments. The scale bar represents 0.025 nucleotide substitutions per sequence position. The percentage of 1000 bootstrap resamplings is indicated. The filled circles indicate the branch points not supported by maximum-likelihood analysis.

those in the case of the MGI were irregular cocci, ~1.0 μm in diameter and rod shaped, on average 2.0 μm long and 0.6 μm wide (Figure 5, A and B, E and F). Preston *et al.* (1996) reported that marine sponge-associated MGI cells were rod shaped, 0.8 μm long, and 0.5 μm wide, and the fluorescence signals of probes labeled with Texas Red were detected at both cellular poles. In this study, no fluorescent signals were detected at the poles of the short rod-shaped cells. No fluorescence was observed when hybridization was performed using the MGI-specific and AAG-specific probes in the case of cultivated bacterial and archaeal thermophiles or in hybridization experiments in which MGI-specific or AAG-specific probes lacking the fluor were included simultaneously with fluor-labeled probes, but at a 50-fold higher concentration.

Of the total prokaryotic cells counted after DAPI staining, 0.4 and 12.5% were identified as members of the MGI in the case of MC1 (7 of 1753) and IVW (59 of 474), respectively, and 2.2 and 0.8% were identified as members of the AAG in the case of MMSC (29 of 1319) and MC2 (11 of 1375), respectively. The percentages of certain rDNA clone types among the total rDNA sequences, based on PCR-mediated partial sequencing

Figure 1.—Phylogenetic tree of rDNA clones derived from various deep-sea hydrothermal vent environments. The tree was inferred by neighbor-joining analysis of 1211 homologous positions of the rDNA sequence in the case of each organism or clone. Abbreviations indicate rDNA clones corresponding to uncultivated organisms derived from the following environments: pOWA and pUWA, from shallow marine hydrothermal vent water and terrestrial acidic hot spring water, respectively (Takai and Sako 1999); pJP, from sediment in a Yellowstone National Park hot spring (Barns *et al.* 1994); and soil clone SCA, from agricultural soil (Bintrim *et al.* 1997). Bold letters indicate rDNA clones obtained from deep-sea hydrothermal vent environments. The scale bar represents 0.1 nucleotide substitutions per sequence position. The percentage of 1000 bootstrap resamplings is indicated. The filled circles indicate the branch points not supported by maximum-likelihood analysis.

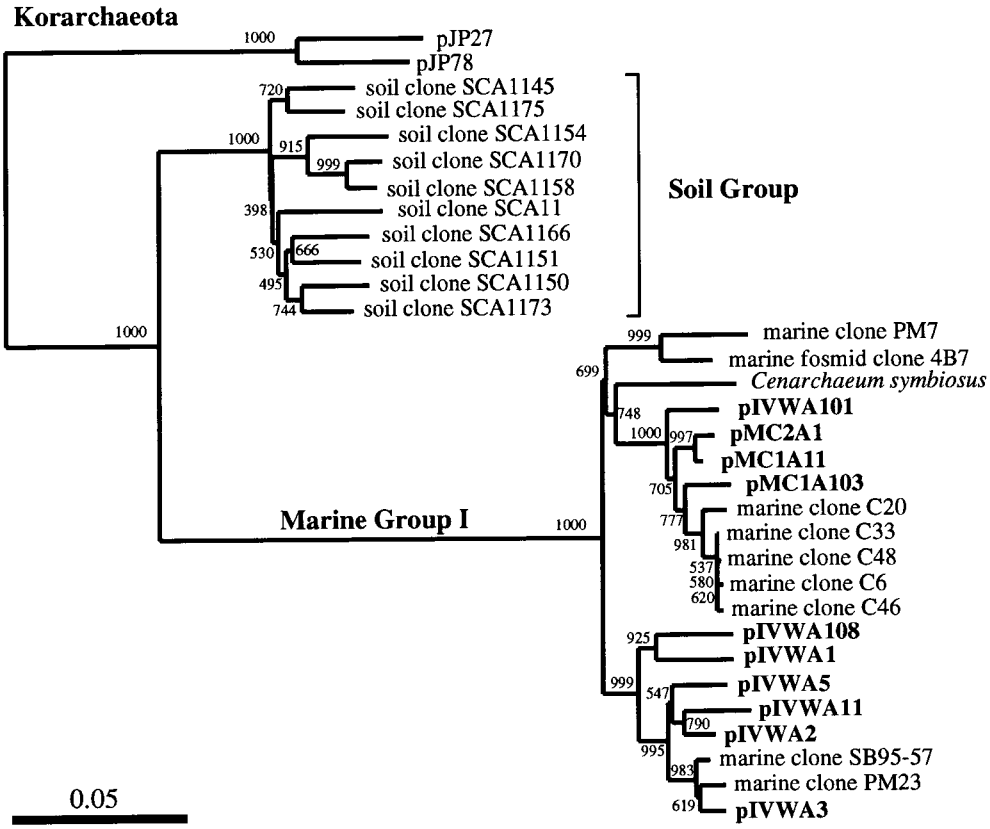


Figure 3.—Phylogenetic tree of rDNA clones within marine group I (MGI) derived from various deep-sea hydrothermal vent environments. The tree was inferred by neighbor-joining analysis of 1211 homologous positions of the rDNA sequence in the case of each organism or clone. Abbreviations indicate rDNA clones corresponding to uncultivated organisms derived from the following environments: pJP, from sediment in a Yellowstone National Park hot spring (Barns *et al.* 1994); soil clone SCA, from agricultural soil (Bintrim *et al.* 1997); marine clones C and PM, from water of the northeast Atlantic ocean at 500 m depth (Mcinerney *et al.* 1997); marine fosmid clone 4B7, from Pacific ocean water (Stein *et al.* 1996); and marine clone SB95-57, from coastal water of the Santa Barbara channel (Massana *et al.* 1997). Bold letters indicate rDNA clones obtained from deep-sea hydrothermal vent environments. The scale bar represents 0.05 nucleotide substitutions per sequence position. The percentage of 1000 bootstrap resamplings is indicated. The topology of this tree was supported by maximum-likelihood analysis.

analysis, were 11.5% MGI cells, 25.8% MGI cells, 2.9% AAG cells, and 0.7% AAG cells in the whole microbial communities of MC1, IVW, SSMC, and MC2, respectively. Although there were differences found between the rDNA population and the fluorescence-labeled cell population, both experiments strongly indicated that uncultivated and unidentified archaeal members are present as a certain proportion of the microbial communities in deep-sea hydrothermal vent environments, and the rDNA from these organisms was successfully recovered in certain proportions from the bulk of DNA directly extracted from these environments.

DISCUSSION

A great genetic diversity of archaeal rDNA clones was evident upon analysis of DNA recovered from various deep-sea hydrothermal vent environments. Most of the archaeal rDNA sequences obtained from black smoker vent water and chimneys, a clear smoker chimney, and clear water simmering sediments represented the sequences of as-yet-uncultivated phylotypes distinct from any other cultivated archaea and environmental clones, and some were closely related to previously discovered environmental archaeal clones. Whole-cell hybridization analysis to facilitate identification of representative uncultivated environmental clones revealed the existence of organisms of such uncultivated phylotypes in the naturally occurring microbial communities of the deep-sea hydrothermal vent environments. This is an outstanding example showing that a great variety of archaea remain undiscovered and uncharacterized in various natural microbial habitats on the earth.

Samples have been collected from various environments with different geologic, physical, and chemical properties (Table 1; Ishibashi and Urabe 1995). Environmental characteristics have an impact on the microbial communities in a given environment and might be important factors in determining the magnitude, content, and diversity of the microbial communities. For instance, in the case of the samples of MC2 and IS, the samples examined contained almost the same number of microbial cells, and the universal primer PCR libraries showed similar rDNA composition (Table 2). These samples also displayed a greater diversity of archaeal rDNA clones than the other deep-sea hydrothermal

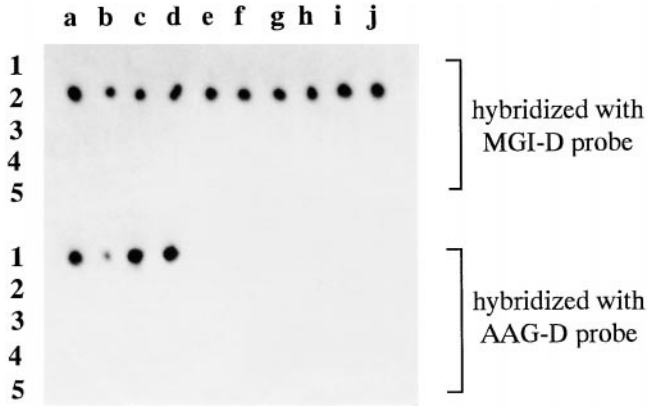


Figure 4.—Dot-hybridization analysis of the probe specificities. Hybridization of the marine group I-specific probe (MGI-D; top) and the ancient archaeal group (AAG-D; bottom) labeled with digoxigenin (DIG) was carried out with 44 plasmid DNAs containing target and nontarget rDNA sequences. A total of 100 ng of each plasmid DNA (except for 1b) was blotted on the membrane at the position shown in Table 3. The pSSMCA1 clone was blotted at 1a (100 ng) and 1b (1 ng).

vent samples (Table 3). However, the presence of various crenarchaeotic rDNA sequences was evident only in the case of MC2. The different archaeal populations may reflect differences in environmental conditions such as the temperature, the geologic location, and the chemical composition between the two hydrothermal vent systems (Ishibashi and Urabe 1995). Likewise, a predominance of rDNA clones of the AAG was observed in the sample of SSMC while rDNA clones of MGI were the major constituent in the archaeal population of the sample IVW (Table 3). MGI clones have been recovered from cold- or moderate-temperature marine environments such as ocean water (Fuhrman *et al.* 1992; Mcinerney *et al.* 1997), coastal water (Delong 1992; Massana *et al.* 1997), polar sea (Delong *et al.* 1994), and surface sediments adjacent to hydrothermal vents (Takai and Sako 1999), and are regarded as being of planktonic origin. In this case, therefore, it seems more likely that the members of the MGI, which comprised a large population, were present as contaminants from the sea water around the black smoker vent water rather than being inhabitants of the superheated vent water or the sedimentary layers through which the vent water had passed. On the other hand, the AAG clones were present only in the superheated black smoker chimneys such as SSMC and MC2 (Table 3). No rDNA sequence similar to AAG clones has been discovered in any other environments, but pOWA133 clones from a shallow marine hydrothermal vent (Takai and Sako 1999) and some korarchaeotic clones from terrestrial hot springs (Barns *et al.* 1994; Takai and Sako 1999) showed the closest phylogenetic affiliations. Although these very deep branches of rDNA sequences had relatively low bootstrap significance, and although the matter of whether the phylogenetic classification of these rDNA clones

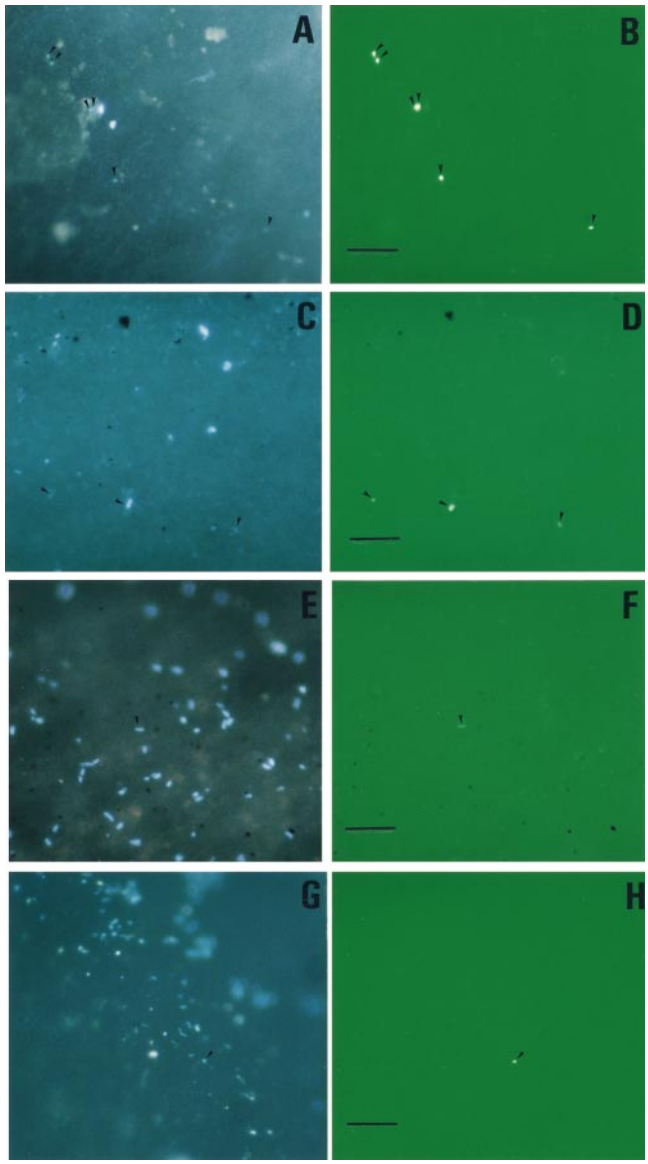


Figure 5.—Photomicrographs of microbial cells stained with DAPI (A, C, E, and G) and archaeal cells in the same field visualized through hybridization with the MGI-F (B and F) or the AAG-F (D and H). Cells visualized through hybridization with the MGI-F probe were observed in samples of IVW (A and B) and MC1 (E and F), and cells visualized through hybridization with the AAG-F probe were observed in the samples of SSMC (C and D) and MC2 (G and H). Arrows indicate cells visualized through hybridization with probes in the same field. Bar, 10 μm.

into the kingdom Crenarchaeota is appropriate remains uncertain, they apparently represent deep lineages in the phylogenetic tree constructed on the basis of archaeal rDNA sequences of cultivated members and unidentified environmental clones. Furthermore, these clones were all obtained from hot water environments. These results suggest that the rDNA sequences in the very deep branches are derived from uncultivated and unidentified (hyper-)thermophilic archaea. This prediction is consistent with the hypothesis of the thermo-

philic origin of Bacteria and Archaea (Achenbach-Richter *et al.* 1987). Hence, the presence of the AAG clones, pOWA133-like clones, and korarchaeotic clones can serve as an index of occurrence of the hyperthermophilic archaeal populations, and each may represent a specific phylotype adapted to deep-sea, shallow marine, and terrestrial hot water environments, respectively.

A variety of novel rDNA clones categorized within the euryarchaeotic kingdom were recovered from the deep-sea hydrothermal vent environments. Phylogenetic analysis indicated that most of these clones were allied in positions close to the Thermoplasma-Picrophilus clade and in intermediate positions between the thermophilic euryarchaeotic branches and methanogen-halophile branches (DHV euryarchaeotic groups I and II; Figure 1). Although these sequences were mainly obtained from geologically and geochemically different hydrothermal vent samples of MC2 and IS, most of them shared several similar phylogenetic affiliations (DHVE2, DHVE3, DHVE4, and DHVE6) showing no significant similarity to any other rDNA sequences known to date (Figure 1). When the phylogenetic tree based on shorter rDNA sequences was reconstructed to include other environmental, unidentified euryarchaeotic clones, some phylotypes of sequences (DHVE1) within the DHV euryarchaeotic group I were closely related with some unidentified euryarchaeotic clones obtained from microbial communities in coastal salt marsh and continental shelf sediments (salt marsh and marine benthic euryarchaeota; Munson *et al.* 1997; Vetriani *et al.* 1998; Figure 2). However, no significant phylogenetic relatedness was observed between a variety of euryarchaeotic rDNA sequences from the deep-sea hydrothermal vent environments and the unidentified environmental clones from planktonic microbial communities in ocean water, coastal water, and polar sea water (MGII; Delong 1992; Delong *et al.* 1994; Figure 2). Considering that both MGII and salt marsh and marine benthic euryarchaeota were obtained from moderate-temperature and cold environments, the DHV euryarchaeotic groups I and II clones may be derived from uncultivated and unidentified mesophilic archaea. However, it is clear that members of the euryarchaeota showing great genetic diversity are widely distributed in marine environments, from top to bottom and from cold to hot habitats, and that such uncultivated and unidentified archaea are one of the most ubiquitous and predominant archaeal groups in global marine environments.

Our molecular phylogenetic survey of deep-sea hydrothermal vent environments has revealed a number of as-yet-uncultivated and undescribed groups of archaea of potential importance and has expanded our view of the genetic diversity of archaea. The discovery of additional archaeal lineages through further molecular phylogenetic analyses will be helpful to predict the precise evolutionary relationships among these groups of novel phylotypes. Although determination of the phylogenetic affiliation of these groups was possible, it might be difficult to predict their phenotypic characteristics solely on the basis of phylogenetic placement. The combination of elaborated enrichment (Burggraf *et al.* 1997; Huber *et al.* 1998), group-specific, rRNA-targeted hybridization (Burggraf *et al.* 1994), and microscopic manipulation with an optical tweezer (Huber *et al.* 1995, 1998) should facilitate cultural isolation of microorganisms of such novel phylotypes from natural environments. For instance, Burggraf *et al.* (1997) reported the successful mixed cultivation of microorganisms of the korarchaeotic group that had been predicted by molecular phylogenetic analysis from a Yellowstone National Park hot spring and showed that the microorganisms were consistently cultured with other hyperthermophilic archaea under hyperthermal anaerobic conditions. Huber *et al.* (1998), by means of a contrived and delicate enrichment technique, also succeeded in cultivation and isolation of a pink-filament-forming hyperthermophilic bacterium, of which the rDNA sequence had been obtained from Octopus Spring in Yellowstone National Park (Reysenbach *et al.* 1994). For obligately symbiotic or syntropic microorganisms, it is a good approach to identify their rDNA gene-containing clones in genomic libraries of mixed populations (Olsen *et al.* 1986; Schmidt *et al.* 1991; Stein *et al.* 1996; Schleper *et al.* 1997), and it may be possible to determine simultaneously whole nucleotide sequences of several replicons through application of the whole-genome-shotgun method from mixed populations. These genetic approaches should also help in obtaining further insight into the nature of the uncultivated and undescribed phylotypes of microorganisms.

We thank Dr. Wayne R. Bellamy for editing English usage in the manuscript. This work was supported in part by a domestic research fellowship provided by the Japan Science and Technology Corporation.

LITERATURE CITED

Achenbach-Richter, L., R. Gupta, K. O. Stetter and C. R. Woese, 1987 Were the original eubacteria thermophiles? Syst. Appl. Microbiol. **9:** 34–39.

Adams, M. W. W., 1993 Enzymes and proteins from organisms that grow near and above 100°. Annu. Rev. Microbiol. **47:** 627–658.

Adams, M. W. W., F. B. Perler and R. M. Kelly, 1995 Extremozyme: expanding the limits of biocatalysis. Bio / technology **13:** 662–668.

Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang *et al.*, 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:** 3389–3402.

Amann, R. I., L. Krumholtz and D. A. Stahl, 1990 Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. **172:** 762–770.

Barns, S. M., R. E. Fundyga, M. W. Jeffries and N. R. Pace, 1994 Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc. Natl. Acad. Sci. USA **91:** 1609–1613.

Barns, S. M., C. F. Delwiche, J. D. Palmer and N. R. Pace, 1996 Perspectives on archaeal diversity, thermophily and monophyly

from environmental rRNA sequences. Proc. Natl. Acad. Sci. USA **93:** 9188–9193.

Benson, D. A., M. S. Boguski, D. J. Lipman, J. Ostell and B. F. F. Ouellette, 1998 Genbank. Nucleic Acids Res. **26:** 1–7.

Bintrim, S. B., T. J. Donohue, J. Handelsman, G. P. Roberts and R. M. Goodman, 1997 Molecular phylogeny of Archaea from soil. Proc. Natl. Acad. Sci. USA **94:** 277–282.

Blöchl, E., R. Rachel, S. Burggraf, D. Hafenbradl, H. W. Jannasch *et al.*, 1997 *Pyrolobus fumarii*, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113°. Extremophiles **1:** 14–21.

Burggraf, S., T. Mayer, R. Amann, S. Schadhauser, C. R. Woese *et al.*, 1994 Identifying members of the domain *Archaea* with rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. **60:** 3112–3119.

Burggraf, S., P. Heyder and N. Eis, 1997 A pivotal archaea group. Nature **385:** 780.

Delong, E. F., 1992 Archaea in coastal marine environments. Proc. Natl. Acad. Sci. USA **89:** 5685–5689.

Delong, E. F., K. Y. Wu, B. B. Prezelin and R. V. M. Jovine, 1994 High abundance of Archaea in Antarctic marine picoplancton. Nature **371:** 695–697.

Dojka, M. A., P. Hugenholtz, S. K. Haack and N. R. Pace, 1998 Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. **64:** 3869–3877.

Fuhrman, J. A., K. McCallum and A. A. Davis, 1992 Novel major archaebacterial group from marine plankton. Nature **356:** 148–149.

Giovannoni, S. J., T. B. Britschgi, C. L. Moyer and F. G. Field, 1990 Genetic diversity of Sargasso Sea bacterioplankton. Nature **345:** 60–65.

Gonzalez, J. M., C. Kato and K. Horikoshi, 1995 *Thermococcus peptonophilus* sp. nov., a fast-growing, extremely thermophilic archaebacterium isolated from deep-sea hydrothermal vents. Arch. Microbiol. **164:** 159–164.

Gonzalez, J. M., Y. Masuchi, F. T. Robb, J. W. Ammerman, D. L. Maeder *et al.*, 1998 *Pyrococcus horikoshii* sp. nov., a hyperthermophilic archaeon isolated from a hydrothermal vent at the Okinawa Trough. Extremophiles **2:** 123–130.

Huber, R., S. Burggraf, T. Mayer, S. M. Barns, P. Rossnagel *et al.*, 1995 Isolation of a hyperthermophilic archaeum predicted by *in situ* RNA analysis. Nature **376:** 57–58.

Huber, R., W. Eder, S. Heldwein, G. Wanner, H. Huber *et al.*, 1998 *Thermocrinis ruber* gen. nov., sp. nov., a pink-filament-forming hyperthermophilic bacterium isolated from Yellowstone National Park. Appl. Environ. Microbiol. **64:** 3576–3583.

Hugenholtz, P., C. Pitulle, K. L. Hershberger and N. R. Pace, 1998 Novel division level bacterial diversity in a Yellowstone hot spring. J. Bacteriol. **180:** 366–376.

Ishibashi, J., and T. Urabe, 1995 Hydrothermal activity related to Arc-Backarc Magmatism in the Western Pacific, pp. 451–495 in *Backarc Basins: Tectonics and Magmatism*, edited by B. Taylor. Plenum Press, New York.

Jones, W. J., J. A. Leigh, F. Mayer, C. R. Woese and R. S. Wolfe, 1983 *Methanococcus jannaschii* sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. Arch. Microbiol. **136:** 254.

Kimura, M., 1980 A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequence. J. Mol. Evol. **16:** 111–120.

Kudo, Y., S. Shibata, T. Miyaki, T. Aono and H. Oyaizu, 1997 Peculiar archaea found in Japanese paddy soils. Biosci. Biotech. Biochem. **61:** 917–920.

Lane, D. J., 1985 16S/23S sequencing, pp. 115–176 in *Nucleic Acid Techniques in Bacterial Systematics*, edited by E. Stackbrandt and M. Goodfellow. John Wiley & Sons, New York.

Larsen, N., G. J. Olsen, B. L. Maidak, M. J. McCaughey, R. Overbeek *et al.*, 1993 The ribosomal database project. Nucleic Acids Res. **21:** 3021–3023.

Maniatis, T., E. F. Fritsch and J. Sambrook, 1982 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Massana, R., A. E. Murray, C. M. Preston and E. F. Delong, 1997 Vertical distribution and phylogenetic characterization of marine planktonic Archaea in the Santa Barbara channel. Appl. Environ. Microbiol. **63:** 50–56.

Mcinerney, J. O., M. Mullarkey, M. E. Wernecke and R. Powell, 1997 Phylogenetic analysis of group I marine archaeal rRNA sequences emphasizes the hidden diversity within the primary group Archaea. Proc. R. Soc. Lond. Ser. B **264:** 1663–1669.

Munson, M. A., D. B. Nedwell and T. M. Embley, 1997 Phylogenetic diversity of Archaea in sediment samples from a coastal salt marsh. Appl. Environ. Microbiol. **63:** 4729–4733.

Olsen, G. J., 1988 Phylogenetic analysis using ribosomal RNA. Methods Enzymol. **164:** 793–812.

Olsen, G. J., D. J. Lane, S. J. Giovannoni, N. R. Pace and D. A. Stahl, 1986 Microbial ecology and evolution: a ribosomal RNA approach. Annu. Rev. Microbiol. **40:** 337–365.

Preston, C. M., K. Y. Wu, T. F. Molinski and E. F. Delong, 1996 A psychrophilic crenarchaeon inhabits a marine sponge: *Cenarchaeum symbiosum* gen. nov., sp. nov. Proc. Natl. Acad. Sci. USA **93:** 6241–6246.

Prieur, D., G. Erauso and C. Jeanthon, 1995 Hyperthermophilic life at deep-sea hydrothermal vents. Planet. Space Sci. **43:** 115–121.

Reysenbach, A.-L., G. S. Wichham and N. R. Pace, 1994 Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. Appl. Environ. Microbiol. **60:** 2113–2119.

Sako, Y., N. Nomura, A. Uchida, Y. Ishida, H. Morii *et al.*, 1996a *Aeropyrum pernix* gen. nov., sp. nov., a novel aerobic hyperthermophilic archaeon growing at temperatures up to 100°C. Int. J. Syst. Bacteriol. **46:** 1070–1077.

Sako, Y., K. Takai, A. Uchida, Y. Ishida and Y. Katayama, 1996b *Rhodothermus obamensis* sp. nov., a modern lineage of extremely thermophilic marine bacterium. Int. J. Syst. Bacteriol. **46:** 1099–1104.

Schleper, C., W. Holben and H.-P. Klenk, 1997a Recovery of crenarchaeotal ribosomal DNA sequences from freshwater-lake sediments. Appl. Environ. Microbiol. **63:** 321–323.

Schleper, C., R. V. Swanson, E. J. Mathur and E. F. Delong, 1997b Characterization of a DNA polymerase from the uncultivated psychrophilic archaeon Cenarchaeum symbiosum. J. Bacteriol. **179:** 7803–7811.

Schmidt, T. M., E. F. Delong and N. R. Pace, 1991 Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J. Bacteriol. **173:** 4371–4378.

Stahl, D. A., D. J. Lane, G. J. Olsen and N. R. Pace, 1984 Analysis of hydrothermal vent associated symbionts by ribosomal RNA sequences. Science **244:** 409–411.

Stein, J. L., T. L. Marsh, K. Y. Wu, H. Shizuya and E. F. Delong, 1996 Characterization of uncultivated prokaryotes: isolation and analysis of a 40-kilobase-pair genome fragment from a planktonic marine archaeon. J. Bacteriol. **178:** 591–599.

Stetter, K. O., G. Fiala, G. Huber, R. Huber and A. Segerer, 1990 Hyperthermophilic microorganisms. FEMS Microbiol. Rev. **75:** 117–124.

Takai, K., and Y. Sako, 1999 A molecular view of archaeal diversity in marine and terrestrial hot water environments. FEMS Microbiol. Ecol. **28:** 177–188.

Takai, K., A. Inoue and K. Horikoshi, 1999 *Thermaerobacter marianensis* gen. nov., sp. nov., an aerobic extremely thermophilic marine bacterium from the 11,000 m deep Mariana Trench. Int. J. Syst. Bacteriol. **49:** 619–628.

Tanner, M. A., B. M. Grobel, M. A. Dojka and N. R. Pace, 1998 Specific ribosomal DNA sequences from diverse environmental settings correlate with experimental contaminants. Appl. Environ. Microbiol. **64:** 3110–3113.

Vetriani, C., A.-L. Reysenbach and J. Dore, 1998 Recovery and phylogenetic analysis of archaeal rRNA sequences from continental shelf sediments. FEMS Microbiol. Lett. **161:** 83–88.

Ward, D. M., M. M. Bateson, R. Weller and A. L. Ruff-Roberts, 1992 Ribosomal RNA analysis in microorganisms as they occur in nature. Adv. Microbiol. Ecol. **12:** 219–286.

Woese, C. R., L. Achenbach, P. Rouviere and L. Mandelco, 1991 Archaeal phylogeny: reexamination of the phylogenetic position of Archaeglobus fulgidus in light of certain composition induced artifacts. Syst. Appl. Microbiol. **14:** 364–371.

Communicating editor: B. Whitman