Extremophiles (2001) 5:385–392
Digital Object Identifier (DOI) 10.1007/s007920100211

ORIGINAL PAPER

Fumio Inagaki · Ken Takai · Tetsushi Komatsu
Toshiya Kanamatsu · Kantaro Fujioka · Koki Horikoshi

Archaeology of Archaea: geomicrobiological record of Pleistocene thermal events concealed in a deep-sea subseafloor environment

Received: January 8, 2001 / Accepted: May 8, 2001 / Published online: September 20, 2001

Abstract A record of the history of the Earth is hidden in the Earth's crust, like the annual rings of an old tree. From very limited records retrieved from deep underground, one can infer the geographical, geological, and biological events that occurred throughout Earth's history. Here we report the discovery of vertically shifted community structures of Archaea in a typical oceanic subseafloor core sample (1410 cm long) recovered from the West Philippine Basin at a depth of 5719 m. Beneath a surface community of ubiquitous deep-sea archaea (marine crenarchaeotic group I; MGI), an unusual archaeal community consisting of extremophilic archaea, such as extreme halophiles and hyperthermophiles, was present. These organisms could not be cultivated, and may be microbial relicts more than 2 million years old. Our discovery of archaeal rDNA in this core sample, probably associated with the past terrestrial volcanic and submarine hydrothermal activities surrounding the West Philippine Basin, serves as potential geomicrobiological evidence reflecting novel records of geologic thermal events in the Pleistocene period concealed in the deep-sea subseafloor.

Key words Subseafloor environment · Pleistocene · Archaea · 16S rDNA · T-RFLP · DGGE

Communicated by W.D. Grant

F. Inagaki (✉) · K. Takai · T. Komatsu · K. Fujioka · K. Horikoshi
Subground Animalcule Retrieval (SUGAR) Project, Frontier Research System for Extremophiles, Japan Marine Science and Technology Center (JAMSTEC), Yokosuka 237-0061, Japan
Tel. +81-468-679687; Fax +81-468-666364
e-mail: inagaki@jamstec.go.jp

T. Kanamatsu
Deep-sea Research Department, Japan Marine Science and Technology Center (JAMSTEC), Yokosuka, Japan

Introduction

Recent progress in exploring the deep-sea subseafloor biosphere, in research projects such as the Ocean Drilling Project (ODP), has revealed that microbial populations are consistently present in drilling core samples recovered from the deep oceanic subsurface (Stetter et al. 1993; Parkes et al. 1994; Bale et al. 1997; Wellsbury et al. 1997). Results of calculations suggest that the terrestrial and oceanic subsurface biosphere is the largest reservoir of biomass on the Earth (Whitman et al. 1998). Since the detection of microorganisms in most early published investigations was mainly dependent on microscopic observations and conventional cultivation techniques (Stetter et al. 1993; Parkes et al. 1994; Bale et al. 1997; Wellsbury et al. 1997), the microbial components and the species diversity of deep-sea subseafloor ecosystems have been poorly demonstrated.

Culture-independent, molecular phylogenetic analysis is a powerful approach recently applied to the investigation of naturally occurring microbial communities (Giovannoni et al. 1990; Fuhrman et al. 1992; Hinrichs et al. 1999; Takai and Horikoshi 1999a,b). A combination of newly developed molecular analytic techniques has allowed investigators to detect, quantify, and identify the microbial components in samples with scarce and low biomass. Using these molecular approaches, it has been revealed that the phylogenetic diversity of Archaea, the predominant microorganisms in extreme environments, is greater than previously assumed and that their phylogenetic features are strongly associated with the environmental constraints of their habitats such as temperature, pH, oxidation–reduction potential, and salt concentration (Fuhrman et al. 1992; DeLong et al. 1994; Barns et al. 1996; Buckley et al. 1998; Hinrichs et al. 1999; Takai and Sako 1999; Takai and Horikoshi 1999a,b; McGenity et al. 2000; Reysenbach et al. 2000). On the basis of these observations, we first proposed the hypothesis of Archaea as geomicrobiological signatures reflecting certain environmental conditions at present or in the past. If the structure of an archaeal community can be revealed through analysis of the particular lithology of a core sample, this might provide valuable insight into the environmental

conditions at the time of deposition and the impact of geologic and biological events associated with sedimentation. Analysis of vertical shifts in archaeal community structures could also be helpful to estimate the lithological and microbiological changes and disturbances occurring thereafter. Using a combination of molecular analyses including terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu et al. 1997), denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993; Vetriani et al. 1999), and sequencing of rDNA in archaeal rDNA clone libraries (Giovannoni et al. 1990; Fuhrman et al. 1992), we report here the archaeal ribotype structures in an oceanic subseafloor core sample.

Materials and methods

Sampling of the subseafloor core sediments

A deep-sea subseafloor piston core sample was recovered at the PC-4 site, 18°10.7921′ N, 129°19.9915′ E, at a depth of 5719 m in the center of the West Philippine Basin by the Japan and Marine Science Technology Center R/V *Kairei* STEPS-III'99 cruise express, October 1999. The length of the core was 1410 cm. Nineteen sections as subsamples from different depths were collected entirely from inside the core. These samples were immediately stored at –80°C on board.

Magnetostratigraphic analysis

Continuous magnetic measurements on the PC-4 core sediments were performed in order to construct the polarity magnetostratigraphy to serve as a geological time scale. Subsamples, 2 × 2 cm in cross section, were removed from the PC-4 core, and then magnetic polarities were analyzed sequentially at 2-cm intervals using a horizontal 2G cryogenic magnetometer (2G Enterprises, Mountain View, CA, USA). Clear polarity changes were obtained from PC-4 core sediments under demagnetization values between 20 mT and 60 mT, and then these polarities were compared with the polarity time scale as described by Cande and Kent (1995).

Water content and total organic carbon concentration

The water content of each sediment core sample was measured by the weight difference after incubation at 90°C for 2 days. To obtain the total organic carbon (TOC) concentration, 5 mg of each sediment sample was incubated in HCl-saturated vapor phase overnight to remove the inorganic carbon by decalcification, and then TOC was estimated by an elemental analyzer, model 2400CHN (Perkin-Elmer, Norwalk, CT, USA).

Total cell counts

Total microbial mass was estimated by direct count using epifluorescence microscopy of 4′,6-diamidino-2-phenylindole (DAPI)-stained cells (Porter and Feig 1980). The sample was suspended in MJ synthetic seawater (Sako et al. 1996) containing 3.7% formaldehyde for 10 min. DAPI was added to the mixture to a final concentration of 10 mg/ml and briefly mixed by vortexing. The suspension was incubated for 20 min on ice, and the number of cells per gram of sediment was calculated from an average of 50 fields by epifluorescence microscope observation.

DNA extraction and polymerase chain reaction amplification of 16S rDNA

Bulk DNA was extracted from 2-g portions of the crushed rock using the Ultra Clean Large Scale Soil DNA kit (Mo Bio, Solana Beach, CA, USA) following the manufacturer's instructions. Bacterial 16S rDNA was amplified by polymerase chain reaction (PCR) using LA Taq polymerase with GC buffer I (TaKaRa, Kyoto, Japan) as recommended by the manufacturer. Archaeal 16S rDNA was amplified by nested PCR using the Arch21F (DeLong et al. 1994) and 1492R (Lane 1985) primers for the first PCR, and Arch21F and Arch958R for the second PCR of the rDNA in clone libraries. Thermal cycling was performed using the GeneAmp PCR system 9600 (Perkin-Elmer) under the following conditions: denaturation at 96°C for 20 s, annealing at 54°C for 45 s, and extension at 72°C for 120 s for a first reaction of 20 cycles and a second of 35 cycles.

T-RFLP analysis

The 16S rDNA for T-RFLP analysis was amplified using the Arch21F and Arch958R (DeLong et al. 1994) FAM primers for the second PCR as described above. After the PCR amplification, the products were gel-purified using an Ultra Clean Gelspin DNA Purification kit (Mo Bio) according to the manufacturer's instructions. Purified rDNAs were then digested with *Hha*I at 37°C for 8 h. Terminal lengths of digested rDNAs recovered from each section of the deep-sea rock were determined by electrophoresis with a model 377 automated sequencer [Applied Biosystems Instruments (ABI), Foster City, CA, USA]. After electrophoresis, the lengths of the FAM-labeled fragments were determined by comparison with internal standards (Rox 1000; ABI) by using GeneScan software (ABI).

Sequence and phylogenetic rDNA analyses

Amplified rDNAs from the core samples were gel-purified as described above. 16S rDNA libraries were constructed for each section of the PC-4 core sediments. The purified rDNA fragments were cloned into the vector pCR2.1 using the original TA cloning kit (Invitrogen, San Diego, CA, USA). The Arch21F primer was used in sequencing reactions to determine the rDNA clone type. Partial rDNA sequences (300 bp corresponding to positions 21 to 321 of *Escherichia coli* rRNA) were determined using a model 377 automated sequencer (ABI) and then analyzed with the FASTA3 and gapped-BLAST search algorithms to estimate

the degree of similarity to other rDNA sequences. We tentatively defined for representative clones >97% similarity as a species-level relationship in 21F primer-dependent sequences obtained from the PC-4 core sediments (Stackebrandt and Goebel 1994). The representative rDNA clone sequences corresponding to *E. coli* rDNA positions 21 to 958 were determined for both strands. Phylogenetic analyses were restricted to nucleotide positions that were unambiguously alignable in all sequences. Least-squares distance matrix analysis, 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). Bootstrap analysis was carried out for 100 trial replications to provide confidence estimates for the phylogenetic tree topologies.

DGGE analysis

DGGE was performed with a D-Code Gene apparatus (Bio-Rad, Hercules, CA, USA). The 16S rDNA for DGGE analysis was amplified using Arch344F with a GC clamp (Muyzer et al. 1993; Vetriani et al. 1999) and Arch 785R (Lane 1985) for the second PCR as described above. Samples containing approximately equal amounts of PCR amplicons were loaded onto an 8% (wt/vol) polyacrylamide gel in 1 × TAE [40 mM Tris, 20 mM acetate, 1 mM ethylenediaminetetraacetate (EDTA)], with a denaturing gradient ranging from 30% to 60% denaturant (100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was performed at 60°C, initially at 50 V for 10 min and then at 150 V for 180 min. After electrophoresis, the gels were incubated for 15 min in ethidium bromide (0.5 mg/l), rinsed for 10 min in distilled water, and visualized on a UV transillumination table and photographed.

Nucleotide sequence accession number

The representative 16S rDNA sequences of pPCA clones have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB049029–AB049052.

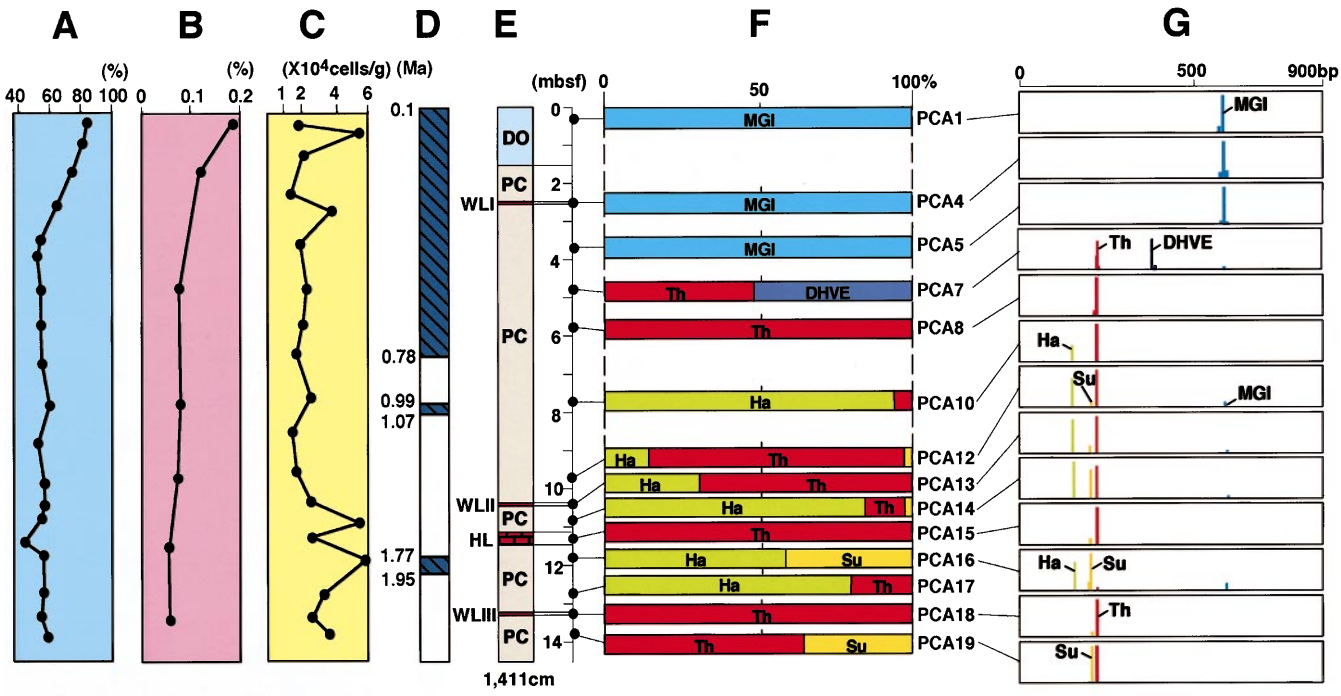

Fig. 1A–G. Vertical characterization of the PC-4 core. **A** Water content of the sediments. **B** Concentration of total organic carbon (TOC). **C** Direct cell counts by epifluorescence microscopic observation after 4′,6-diamidino-2-phenylindole (DAPI) staining. Total cell densities were calculated from an average of 50 microscopic fields. **D** Magnetostatic shift and putative stratigraphic ages of the sediments. **E** Lithology and depth from the surface of the seafloor. Abbreviations for lithology are as follows: *DO*, diatom ooze; *PC*, pelagic clay; *WL*, white layer; *HL*, hard layer. **F** Archaeal ribotype structure. Single-strand sequences 300 bp in length determined by means of the Arch21F primer (48 clones from each section) were used in a similarity analysis by the FASTA3 and gapped-BLAST systems. Abbreviations for the phylotypes of clones from PC-4 core sediments are as follows: *MGI*, marine crenarchaeotic group I; *Th*, genus *Thermococcus* members; *DHVE*, deep-sea hydrothermal vent euryarchaeotic groups; *Ha*, genus *Haloarcula* members; *Su*, genera *Sulfolobus* and *Sulfurisphaera* members. **G** Ribotype-fingerprint patterns of the archaeal rDNA community based on terminal restriction fragment length polymorphism (T-RFLP) analysis. Archaeal polymerase chain reaction (PCR) product was amplified using Arch21F and Arch958R-FAM primers and digested with *Hha*I at 37°C for 8 h. The positions of each peak correspond to the terminal length of *Hha*I-digested rDNA from the Arch958R-FAM primer end. Abbreviations indicate putative phylotypes based on the *Hha*I site of rDNA sequences of representative clones. The colors of the peaks and the abbreviations correspond to those of the clone types shown in (**F**)

Results and discussion

Lithology and magnetostratigraphy

The core most intensively investigated was a 1410-cm-long core obtained below the surface of the seafloor at the PC-4 site at a depth of 5719 m. The PC-4 core was found to be composed of diatom ooze and pelagic clay with three intervening white layers (WL-I, -II, and -III) and a hard, consolidated layer (HL) (Fig. 1E). Since pumice was detected in the WL-I, WL-III, and HL layers, the sediments in these layers were likely derived largely from particulate matter released through geothermal activity at the margin of the Philippine Sea. Magnetostratigraphic examination of the PC-4 core showed that the deepest part of the PC-4 core, at a depth of 14 m, was deposited 2–2.5 million years ago and the sedimentation rate was relatively constant (Fig. 1D).

Microbial population

Epifluorescence microscopic observation of samples stained with 4′,6-diamidino-2-phenylindole (DAPI) indicated that the microbial population density was almost constant (0.9–5.2×10^4 cells/g wet weight) throughout the core. There was no apparent correlation between the microbial population density and the total organic carbon (Figs. 1B,C).

Archaeal community structures

Vertical shifts in archaeal community structures throughout the core were demonstrated by T-RFLP analysis (Fig. 1G), DGGE analysis (Fig. 2), and sequencing of rDNA in clone libraries (Figs. 1F, 3). Archaeal ribotype structures obtained by both T-RFLP and DGGE analyses were drastically changed at a point in the pelagic clay layer corresponding to a depth of 480 cm below the seafloor surface (Fig. 1G). According to Krumholtz et al. (1997) and Takai et al. (2000a), shifts in microbial population densities and community structures are likely to occur in association with transitions in the lithology and geochemistry in subsurface environments. In this case, there appeared to be little association between the alterations in the archaeal community structures and the lithologic transitions in the PC-4 core (Fig. 1). In the analysis of the surface of sections PCA1 to PCA6, a single ribotype signature was most prominently detected by T-RFLP and DGGE analysis, and all of the archaeal rDNA clones sequenced (in total 216 clones) were closely related to the marine crenarchaeotic group I (MGI) (Figs. 1F, 2, 3A), those most frequently recovered from global marine environments (Fuhrman et al. 1992; DeLong et al. 1994; Mcinerney et al. 1997). In terms of lithology, the surface zone consisted of highly permeable, porous diatom ooze overlying a layer of pelagic clay. Both of these sediment layers in the surface zone were found to have a relative high water content (Fig. 1A). It seems likely, therefore, that the interstitial water in the surface zone was for the most part attributable to permeation of water from the deep-sea bottom into the sediment. This may account for the high abundance of MGI ribotypes, which are typical archaeal components of deep-sea sediments, in the surface zone of the core.

In the deeper sediments, from a point between the sections PCA5 and PCA7, the archaeal ribotype structures were different from those in the surface zone and were relatively stable with only minor alterations detected throughout the deeper zone (Fig. 1G). The major archaeal components in the deeper core samples were thermophilic archaea (members of the genera *Thermococcus* and *Sulfolobus*) and extreme halophiles (members of the genus *Haloarcula*) according to the results of the sequencing of the rDNA clone libraries (Fig. 1F). A phylotype related to the deep-sea hydrothermal vent euryarchaeotic group (DHVE) was detected only in section PCA7 (Figs. 1F,G, 3) (Takai and Horikoshi 1999a). The in situ temperature of the core was measured on board and found to be consistently 4°–5°C, and the salinity of the interstitial water in the deeper zone of the core was the same as that of the interstitial water in the surface zone and that of the seawater. It seems unlikely that the presence of thermophilic and halophilic archaeal rDNA in the deeper zone was representative of current microbial activity. We attempted to culture the thermophilic and halophilic archaea from the core sample under various conditions commonly used for *Thermococcus*, *Sulfolobus,* and *Haloarcula* isolates (Robb and Place 1995), but our attempts were not successful. This suggested that the archaeal rDNAs had been recovered from either nonviable or viable but unculturable populations, i.e., the extremophilic archaea might be microbial relicts. The occurrence of thermophilic and halophilic archaeal rDNA likely reflects

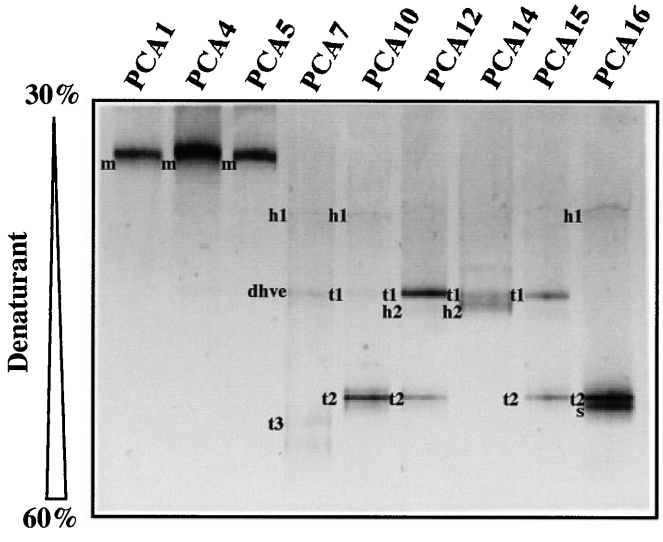

Fig. 2. Comparative denaturing gradient gel electrophoresis (DGGE) profiles of PCR-mediated archaeal 16S rDNA fragments from vertical PC-4 core sections. Letters indicate the positions of rDNA fragments corresponding to the following representative clones; *m*, pPCA4.9 (Marine Group I); *h1*, pPCA17.1 (*Haloarcula*); *dhve*, pPCA7.6 (DHVE); *t1*, pPCA13.11 (*Thermococcus*); *h2*, pPCA14.16 (*Haloarcula*); *t2*, pPCA15.21 (*Thermococcus*); *s*, pPCA16.11 (*Sulfolobus–Sulfurisphaera*); and *t3*, pPCA7.22 (*Thermococcus*)

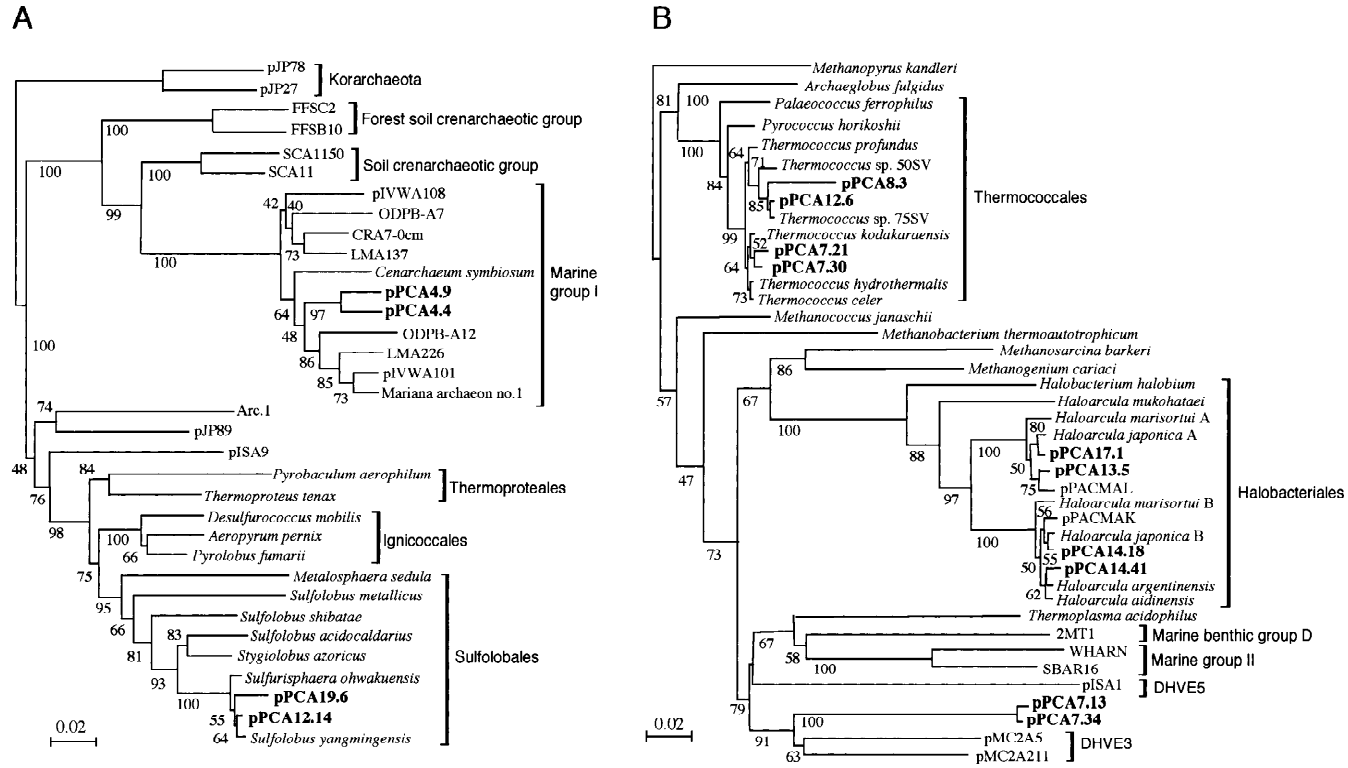

Fig. 3A,B. Phylogenetic relationship between the representative 16S rDNA clones from PC-4 core sediments and 16S rDNA sequences of related pure cultures and environmental clones among kingdoms Crenarchaeota (**A**) and Euryarchaeota (**B**). The trees were inferred by neighbor-joining analysis based on 716 bp (**A**) and 681 bp (**B**) homologous positions of the rDNA sequences. The values of 100 bootstrap trial replications are given for the nodes in the trees. The *scale bar* represents 0.02 nucleotide substitutions per sequence position. Representative rDNA clones obtained from PC-4 core sediments are indicated by *bold letters*. Abbreviations indicate rDNA clones corresponding to as-yet-uncultivated phylotypes derived from the following environments: (**A**) *pJP* from sediments in a Yellowstone National Park hot spring (Barns et al. 1994); *FFSC* and *FFSB* from boreal forest soils (Jurgens et al. 1997; Jurgens and Saano 1999); *SCA* from agricultural soil (Bintrim et al. 1997); *pIVWA* and *pISA* from deep-sea hydrothermal vent environments (Takai and Horikoshi 1999a); *CRA* and *LMA1* from deep-sea sediments (Vetriani et al. 1999); *ODPB-A* from subseafloor sediments and water (Bidle et al. 1999); *Mariana archaeon no. 1* from sediments in the Mariana Trench (Kato et al. 1997); *Arc. 1* from a deep subsurface paleosol (Chandler et al. 1998); (**B**) *pPACMA* from a hydrothermal vent in the Manus Basin (Takai et al. 2001); *2MT1* from salt marsh sediments (Munson et al. 1997; Vetriani et al. 1999); *WHARN* and *SBAR* from coastal water (DeLong 1992); *pISA* and *pMC2A* from deep-sea hydrothermal vent environments (Takai and Horikoshi 1999a)

the microbial activity and species distribution during the period of deposition over the past two million years.

Among the archaeal community structures in the deeper zone of the core, members of the genus *Thermococcus* were predominantly and consistently present (Fig. 1F). *Thermococcus* species are ubiquitous microorganisms in marine hydrothermal vent systems, and a number of them have been isolated from hydrothermal environments throughout the world (Takai et al. 2000b). Sequence similarity analysis and phylogenetic analysis indicated that representative thermococcal rDNA clones from DNA obtained from the sections in the deeper zone were clustered into two phylotypes within the genus *Thermococcus*. The PC-4 thermococcal group I contained the clone types pPCA7.8, pPCA7.21, and pPCA7.30, and was most closely related to *T. hydrothermalis* (Godfroy et al. 1997) and *T. kodakaraensis* (formerly *Pyrococcus kodakaraensis*, Morikawa et al. 1994) isolated from hydrothermal fields in the eastern and western Pacific Ocean (Fig. 3B). The PC-4 thermococcal group II contained the clone types pPCA8.3, pPCA12.6, pPCA13.4, and pPCA15.21, which were phylogenetically associated with *Thermococcus* sp. strains 75SV and 50SV from the Manus Basin in the western Pacific Ocean (Takai et al. 2001) (Fig. 3B). The phylogenetic relatedness found between the PC-4 thermococcal rDNA clones and the known isolates possibly serves as biogeographical evidence that these microbial relicts were originally derived from marine hydrothermal vent systems such as those in the Izu-Bonin Arc, the Mariana Trough, the Manus Basin, and the Okinawa Trough encompassing the Philippine Plate (Fig. 4). Furthermore, from the sections obtained from the WL-III and HL layers containing a considerable amount of pumice, only a thermococcal rDNA signature was obtained in all molecular analyses (Figs. 1F,G). Since the presence of pumice in these layers is considered to be important geological evidence of vigorous hydrothermal activity, the increased abundance of *Thermococcus* rDNA might be strongly associated with past hydrothermal activity at certain intervals. Similarly, the stable occurrence of thermococcal rDNA throughout the deeper zone may serve as a geomicrobio-

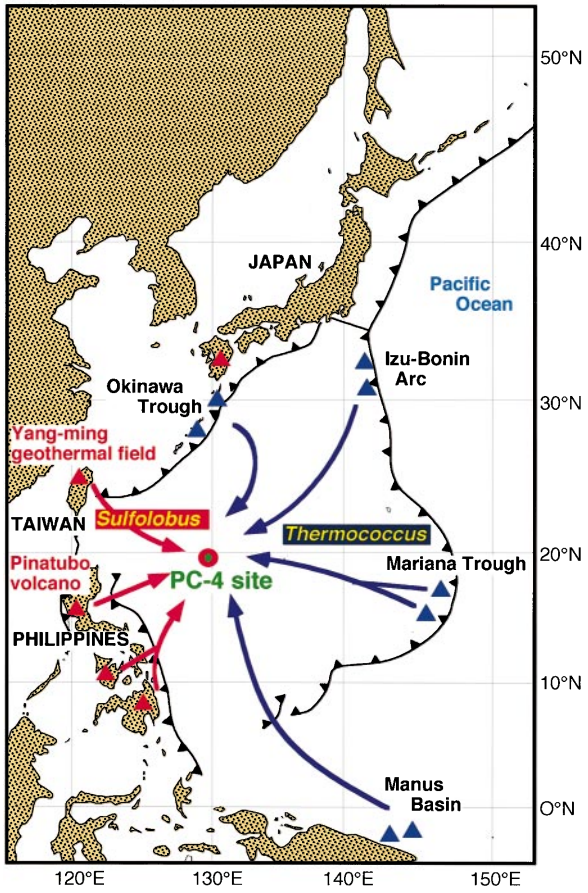

Fig. 4. A schematic diagram illustrating the sampling site and the possible sources of Archaea found in the subseafloor sediments

logical clue pointing to persistent hydrothermal activity along the margin of the Philippine Plate over the past two million years (Fig. 4).

The rDNA signature of the extremely halophilic genus *Haloarcula* was also detected in several sections of the deeper zone. The members of the genus *Haloarcula* are cosmopolitan microorganisms, often found in surface hypersaline environments such as salt lakes, salt mines, and solar salterns (Kamekura 1998; McGenity et al. 2000), and even in subsurface salt deposits and salt mines (Vreeland et al. 1998). The finding of members of *Haloarcula* in subseafloor environments might suggest the formation/existence of hypersaline microhabitats in typical pelagic sediments. However, our recent investigation has revealed a remarkably high proportion of *Haloarcula* rDNA in a chimney structure at a deep-sea hydrothermal site in the Manus Basin, and this discovery suggests the occurrence of halophilic archaeal populations associated with hydrothermal vent activity (Takai et al. 2001). In addition, the results of phylogenetic analysis indicated a strong genetic similarity between the rDNA sequences obtained from the PC-4 subseafloor sediments and the black smoker chimney in the Manus Basin (Fig. 3B). Strains of *Haloarcula* contain two defined rDNA operons in their genomes, and this heterogeneity of rDNA is affiliated with two different phylogenetic branches (Fig. 3B) (Mylvaganam and Dennis 1992). Given the possibility that members of *Haloarcula* inhabit deep-sea hydrothermal vent environments, and considering the phylogenetic relatedness between the subseafloor clones and the deep-sea hydrothermal vent clones, the rDNA signature of extremely halophilic archaea in the subseafloor sediments may be further geomicrobiological evidence for submarine hydrothermal activity in the margin of the Philippine Plate during the Pleistocene period.

The discovery of rDNA clones closely related to *Sulfolobus* and *Sulfurisphaera* was the most striking finding concerning the features of the archaeal community structures in the core sample. The rDNA signatures of these thermoacidophilic archaea were predominantly recovered from the sections (PCA16 and PCA19) immediately below the HL and WL-III layers (Figs. 1F,G). Both of these layers contained a high abundance of thermococcal rDNA, and it was inferred that these were sediments resulting from submarine hydrothermal activity. All previously known members of *Sulfolobus* and *Sulfurisphaera* have been found only in terrestrial acidic hot springs and solfataric fields (Kurosawa et al. 1998; Jan et al. 1999). This is the first report of *Sulfolobus* and *Sulfurisphaera* rDNA being detected in marine environments. Since the thermoacidophilic archaea are presumably associated with terrestrial volcanic activities, the occurrence of *Sulfolobus* and *Sulfurisphaera* rDNA in sections PCA16 and PCA19 might not be directly associated with the submarine hydrothermal events represented by the HL and WL-III layers. Sections PCA16 and PCA19 consisted of pelagic clay and had no apparent geological features indicative of active volcanism. In addition, geologic records provide no evidence that large-scale volcanic eruptions occurred at the margin of the Philippine Plate (e.g., the Pinatubo volcano in the Philippines) around 2.5 to 1.8 million years ago. However, the existence of *Sulfolobus* and *Sulfurisphaera* rDNA strongly suggests the occurrence of unidentified terrestrial volcanic eruptions or other volcanic activity during that period. In addition, the results of phylogenetic analysis indicated that the rDNA sequences of representative clones obtained from the subseafloor sediments were closely related to the rDNA sequences of *Sulfolobus yangmingensis* (Jan et al. 1999) and *Sulfurisphaera ohwakuensis* (Kurosawa et al. 1998) isolated from the Yangming volcanic field in Taiwan and the Hakone volcanic field in Japan, respectively (Fig. 3A). This phylogenetic similarity reinforces the inference that the thermoacidophilic archaea are derived from volcanic fields in the island arc encircling the Philippine Plate (Fig. 4). Although the ribotype structures do not directly indicate the population sizes or abundances, the rDNA signature of thermoacidophilic archaea may provide important insight into previously unknown geologic thermal events of the Pleistocene period.

In conclusion, the members of the extremophilic archaeal community discovered in the subseafloor sediments may be nonviable or nonculturable populations from the Pleistocene period, more than 2 million years ago. These microorganisms serve as geomicrobiological evidence of past geologic thermal events concealed in the subseafloor sediments that have not yet been revealed by conventional geological approaches. In this study, we adopted a new strategy for elucidating Earth's history through geological,

geochemical, and microbiological surveys focusing on relicts of Archaea confined in the subseafloor stratigraphy. In the future, we intend to apply this strategy to a much longer ocean drilling core retrieved by the new Ocean Drilling Project. The archaeology of Archaea will allow us to discover unexpected answers concealed in terrestrial and oceanic subsurface environments.

Acknowledgements We are very grateful to the R/V *Kairei* STEPS-III'99 operation team for helping us to collect the deep-sea subseafloor samples. We also thank Dr. W. R. Bellamy for editing English usage in the manuscript.

References

Bale SJ, Goodman K, Rochelle PA, Marchesi JR, Fry JC, Weightman AJ, Parkes RJ (1997) *Desulfovibrio profundus* sp. nov., a novel barophilic sulfate-reducing bacterium from deep sediment layers in the Japan Sea. Int J Syst Bacteriol 47:515–521

Barns SM, Fundyga RE, Jeffries MW, Pace NR (1994) Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc Natl Acad Sci U S A 91:1069–1613

Barns SM, Delwiche CF, Palmer JD, Pace NR (1996) Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. Proc Natl Acad Sci U S A 93:9188–9193

Bidle KA, Kastner M, Bartlett DH (1999) A phylogenetic analysis of microbial communities associated with methane hydrate containing marine fluids and sediments in Cascadia margin (ODP site 892B). FEMS Microbiol Lett 177:101–108

Bintrim SB, Donohue TJ, Handelsman J, Robert GP, Goodman RM (1997) Molecular phylogeny of Archaea from soil. Proc Natl Acad Sci U S A 94:277–282

Buckley DH, Graber JR, Schmidt TM (1998) Phylogenetic analysis of nonthermophilic members of the Kingdom Crenarchaeota and their diversity and abundance in soils. Appl Environ Microbiol 64:4333–4339

Cande SC, Kent DV (1995) Revised calibration of the geomagnetic polarity time scale for Cretaceous and Cenozoic. J Geophys Res 100:6093–6095

Chandler DP, Brockman FJ, Bailey TJ, Fredrickson JK (1998) Phylogenetic diversity of Archaea and Bacteria in a deep subsurface paleosol. Microb Ecol 36:37–50

DeLong EF (1992) Archaea in coastal marine environments. Proc Natl Acad Sci U S A 89:5685–5689

DeLong EF, Wu KY, Prezelin BB, Jovine RVM (1994) High abundance of Archaea in Antarctic marine picoplankton. Nature 371:695–697

Fuhrman JA, McCallum K, Davis AA (1992) Novel major archaebacterial group from marine plankton. Nature 356:148–149

Giovannoni SJ, Britschgi TB, Moyer CL, Field FG (1990) Genetic diversity of Sargasso Sea bacterioplankton. Nature 345:60–65

Godfroy A, Lesongeur F, Raguenes G, Querellou J, Antoine E, Meunier JR, Guezennec J, Barbier G (1997) *Thermococcus hydrothermalis* sp. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. Int J Syst Bacteriol 47:622–626

Hinrichs KU, Hayes JM, Sylva SP, Brewer PG, Delong EF (1999) Methane-consuming archaebacteria in marine sediments. Nature 398:802–805

Jan RL, Wu J, Chaw SM, Tsal CW, Tsen SD (1999) A novel species of thermoacidophilic archaeon, *Sulfolobus yangmingensis* sp. nov. Int J Syst Bacteriol 49:1809–1816

Jurgens GN, Saano A (1999) Diversity of soil Archaea in boreal forest before, and after clear-cutting and prescribed burning. FEMS Microbiol Ecol 29:205–213

Jurgens GN, Lindstrom K, Saano A (1997) Novel group within the kingdom Crenarchaeota from boreal forest soil. Appl Environ Microbiol 63:803–805

Kamekura M (1998) Diversity of extremely halophilic bacteria. Extremophiles 2:289–296

Kato C, Li L, Tamaoka J, Horikoshi K (1997) Molecular analysis of the sediment of the 11,000 m depth Mariana Trench. Extremophiles 1:117–120

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

Krumholz LR, McKinley JP, Ulrich GA, Suflita JM (1997) Confined subsurface microbial communities in Cretaceous rock. Nature 386:64–66

Kurosawa N, Itoh YH, Iwai T, Sugai A, Uda I, Kimura N, Horiuchi T, Itoh T (1998) *Sulfurisphaera ohwakuensis* gen. nov., sp. nov., a novel extremely thermophilic acidophile of Sulfolobales. Int J Syst Bacteriol 48:451–456

Lane DJ (1985) In: Stackebrandt E, Goodfellow M (ed) Nucleic acid techniques in bacterial systematics. Wiley, New York, pp. 115–176

Liu W-T, Marsh TL, Cheng H, Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol 63:4516–4522

McGenity TJ, Gemmell RT, Grant WD, Stan-Lotter H (2000) Origins of halophilic microorganisms in ancient salt deposits. Environ Microbiol 2:243–250

Mcinerney JO, Mullarkey M, Wernecke ME, Powell R (1997) Phylogenetic analysis of group I marine archaeal rRNA sequences emphasizes the hidden diversity within the primary group Archaea. Proc R Soc Lond 264:1663–1669

Morikawa M, Izawa Y, Rashid N, Hoaki T, Imanaka T (1994) Purification and characterization of a thermostable thiol protease from a newly isolated hyperthermophilic *Pyrococcus* sp. Appl Environ Microbiol 60:4559–4566

Munson MA, Nedwell DB, Embley TM (1997) Phylogenetic diversity of Archaea in sediment samples from a coastal salt marsh. Appl Environ Microbiol 63:4729–4733

Muyzer G, DeWaal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Mylvaganam S, Dennis PP (1992) Sequence heterogeneity between the two genus encoding 16S rRNA from the halophilic archaebacterium *Haloarcula marismortui*. Genetics 130:399–410

Parkes RJ, Cragg BA, Bale SJ, Getliff JM, Goodman K, Rochelle PA, Fry JC, Weightman AJ, Harvey SM (1994) Deep bacterial biosphere in Pacific Ocean sediments. Nature 371:410–413

Porter KG, Feig YS (1980) The use of DAPI for identifying and counting microflora. Limnol Oceanogr 25:943–948

Reysenbach A-L, Longnecker K, Kirshtein J (2000) Novel bacterial and archaeal lineages from an in situ growth chamber deployed at Mid-Atlantic Ridge hydrothermal vent. Appl Environ Microbiol 66:3798–3806

Robb FT, Place AR (1995) Media for thermophiles. In: Robb FT, Place AR, Sowers KR, Schreier HJ, DasSarma S, Fleischmann EM (eds) Archaea: a laboratory manual. Cold Spring Harbor Laboratory Press, New York, pp. 197–188

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

Stackebrandt E, Goebel BM (1994) Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44:846–849

Stetter KO, Huber R, Blöchl E, Kurr M, Eden RD, Fielder M, Cash H, Vance I (1993) Hyperthermophilic archaea are thriving in deep North Sea and Alaskan oil reservoirs. Nature 365:743–745

Takai K, Horikoshi K (1999a) Genetic diversity of Archaea in deep-sea hydrothermal vent environments. Genetics 152:1285–1297

Takai K, Horikoshi K (1999b) Molecular phylogenetic analysis of archaeal intron-containing genes coding for rRNA obtained from a deep-subsurface geothermal water pool. Appl Environ Microbiol 65:5586–5589

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

Takai K, Mormile MR, McKinley JP, Brockman FJ, Fredrickson JK (2000a) Shifts in subsurface microbial community structure associated with lithological and geochemical variations in Cretaceous rock. Abstracts of the American Society of Microbiology 100th general meeting, May 21–25, 2000, Los Angeles, p 459

Takai K, Sugai A, Itoh T, Horikoshi K (2000b) *Palaeococcus ferrophilus* gen. nov., sp. nov., a barophilic hyperthermophilic archaeon from a deep-sea hydrothermal vent chimney. Int J Syst Evol Microbiol 50:489–500

Takai K, Komatsu T, Inagaki F, Horikoshi K (2001) Distribution of Archaea in a black smoker chimney structure. Appl Environ Microbiol 67:3618–3629

Vetriani C, Jannasch HW, MacGregor BJ, Stahl DA, Reysenbach A-L (1999) Population structure and phylogenetic characterization of marine benthic archaea in deep-sea sediments. Appl Environ Microbiol 65:4375–4384

Vreeland RH, Piselli Jr. AF, McDonnough S, Meyers SS (1998) Distribution and diversity of halophilic bacteria in a subsurface salt formation. Extremophiles 2:321–332

Wellsbury P, Goodman K, Barth T, Cragg BA, Barnes SP, Parkes RJ (1997) Deep marine biosphere fueled by increasing organic matter availability during burial and heating. Nature 388:573–576

Whitman WB, Coleman DC, Wiebe WJ (1998) Prokaryotes: the unseen majority. Proc Natl Acad Sci U S A 95:6578–6583