Geobiology (2004), **2**, 151–161

Archaeal phylotypes in a metal-rich and low-activity deep subsurface sediment of the Peru Basin, ODP Leg 201, Site 1231

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ABSTRACT

Site 1231 of the Ocean Drilling Project (ODP) was characterized by low concentrations of organic carbon, as well as low cell numbers and biological activity rates. A 16S rRNA survey was performed in order to analyse the microbial community composition of these central oceanic sediments. Archaeal 16S rRNA genes from subsurface sediments at Site 1231 (1.8, 9.0, and 43 mbsf) were affiliated with uncultured lineages from subsurface or hydrothermal vent habitats. Members of the Marine Group I (MGI) found in the 1.8 mbsf sediment formed distinct clusters, some dominated by phylotypes from Site 1231 and other subsurface environments. The archaeal community survey at Site 1231 indicated that several archaeal lineages were widespread in subsurface environments, marine sediments as well as hydrothermal habitats.

Received 14 February 2004; accepted 29 June 2004

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INTRODUCTION

Studies on samples collected during Deep Sea Drilling Project (DSDP) and Ocean Drilling Program (ODP) cruises have consistently demonstrated microbial activity in deep marine sediments millions of years after their initial deposition on the seafloor and several hundreds of meters below the sediment surface (Parkes *et al.*, 1994, 2000; D'Hondt *et al.*, 2002). Though cell densities in deep marine sediments are typically orders of magnitudes lower than that of surface, the amount of living carbon in the deep biosphere has been estimated to constitute between one-tenth and one-third of Earth's biomass (Parkes *et al.*, 2000; Whitman *et al.*, 1998). Deep subsurface organisms play a significant role in global carbon cycling. For example, the amount of carbon buried in marine sediments as biogenic gas hydrates seems to equal between four and eight times the amount of carbon in all living organisms on Earth (Kvenvolden, 1993). Apart from expanding the recognized biosphere on Earth itself, the discovery of life deep below the seafloor has led to speculations about the possibility of finding similarly buried life forms in water-containing subsurface Martian sediments (Mancinelli, 2000).

Concentration profiles of biologically relevant porewater constituents (Parkes *et al.*, 2000; D'Hondt *et al.*, 2002), direct rate measurements of microbial processes (Cragg *et al.*, 1992; Wellsbury *et al.*, 2000, 2002), and cultivations of subsurface bacteria and archaea (Parkes *et al.*, 1995; Bale *et al.*, 1997; Barnes *et al.*, 1998) have led to some insight into the metabolic activities and capabilities of deep marine subsurface microbial communities. However, these data give only limited information about the structure and diversity of the microbial community. As contamination controls and technologies for sampling of subsurface sediments have been developed, extraction and analysis of prokaryotic genomic DNA has become an increasingly promising approach for the study of microbial communities in deep-sea sediment. Thus, a number of molecular studies have now been performed on continental shelf and margin sediments, as well as gas hydrate rich and/or hydrothermally influenced subsurface sediments (Rochelle *et al.*, 1992, 1994; Juniper *et al.*, 2001; Marchesi *et al.*, 2001; Reed *et al.*, 2002; Teske *et al.*, 2002; Kormas *et al.*, 2003; Rogers *et al.*, 2003). However, little is yet known about organic-poor, recalcitrant cold sediments that are characteristic for a major part of the deep-sea bottom.

This communication reports on a phylogenetic study of the archaeal community in samples of deep subsurface sediment collected at ODP Site 1231, characterized by low cell numbers and organic carbon content (Shipboard Scientific Party, 2003). Archaeal 16S rRNA genes from three depths in the sediment were PCR amplified, cloned and sequenced, demonstrating the presence of distinct and novel archaeal clades in different sediment layers.

MATERIALS AND METHODS

Field site and sampling

Site 1231 is located in the Peru Basin at approximately 4800 m water depth, about 400 km from the coast of Peru. The total sediment thickness is about 120 m, of which the upper half consists of Oligocene to Holocene clay, and the lower half Eocene to early Oligocene nannofossil ooze (Dymond *et al.*, 1976). Biological activity rates were not directly measured at Site 1231, but porewater concentration profiles of oxygen (present in the upper 60 cm of the sediment), and sulphate (>28 mM at the sediment surface, gradually decreasing to 27 mM at the sediment base) suggested low rates of aerobic respiration and sulphate reduction, respectively (Shipboard Scientific Party, 2003). The presence of small amounts of methane as well as peaks of dissolved (reduced) iron and manganese indicated that methanogens and metal-reducing prokaryotes were active in the upper half of the sediment column (Fig. 1). Total acridine orange cell counts were between 10^6 and 10^7 cells ml^{-1} sediment throughout most of the sediment column (Shipboard Scientific Party, 2003). Sampling was performed using Advanced Hydraulic Piston Coring, with a perfluorocarbon chemical tracer added to the drilling water as a seawater contamination control (House *et al.*, 2003). Sediment samples were immediately frozen at −80 °C upon sampling.

Extraction of genomic DNA

Unless stated otherwise, chemicals were purchased from Fisher Scientific, USA. Cell lysis and DNA extraction were performed successfully using two different protocols, one including lysozyme and Proteinase K treatment of the sediment samples (protocol ENZ) and the other including mechanical homogenization and cell shearing by bead beating (protocol BB). A blind extraction without sample but otherwise identical was included as contamination control during all sediment extractions.

Protocol ENZ

Sediment samples of 3 g (2 ML) were suspended in two volumes of extraction buffer (200 mM NaCl, 200 mM TRIS, 2 mM sodium citrate, 10 mM $CaCl_2$, 50 mM EDTA, 0.5 mg mL^{-1} 5′-polyadenylic acid (PolyA), 5 mM sodium pyrophsophate (NaPh), pH 8). This mixture was subjected to four freeze/thaw cycles, alternating in liquid nitrogen and in a water bath at 65 °C, followed by a 2 h incubation on a shaking table at 37 °C with 20 mg mL^{-1} lysozyme, and a second 2 h incubation at 55 °C in a water bath with 5 mg mL^{-1} sodium dodecyl sulphate (SDS) and 1 mg mL^{-1} Proteinase K. SDS was then added to a final concentration of 5% (w/v) and the samples were subjected to another four freeze/thaw cycles. The samples were centrifuged at 6000 g for 10 min and the supernatant was extracted with pH neutral phenol/chloroform/isoamylalcohol (25 : 24 : 1). DNA was precipitated by adding 0.1 volume of 5 M NaCl and 2.5 volumes of ice-cold ethanol, followed by overnight precipitation at –20 °C. After centrifugation of the samples (25 min, 4 °C, 10 000 g), pellets were washed once in 100 µL ice-cold 70% ethanol, air dried, and resuspended in 50 µL double autoclaved Milli-Q water (PCR water).

Protocol BB

This protocol was an adaptation of the method developed by Hurt *et al.* 2001. Initially, three grams of sediment was suspended in 5 mL of denaturing solution (4 M guanidine isothiocyanate, 10 mM TRIS, 1 mM EDTA, 5 mL l^{-1} 2-mercaptoethanol, pH 7.0) by vortexing for 1 minute. 1.8 mL aliquots of the slurry was transferred to 2 mL bead-beating tubes containing 0.1 mm glass beads (BioSpec Products, Inc., Bartlesville, OK) and homogenized on a FastPrep™ FP120 homogenizer (Qbiogene, Inc., Carlsbad, CA) for 40 s at level 4.5. The tubes were then frozen and thawed four times in liquid nitrogen and a water bath at 65 °C, respectively. After pooling of the lysates, 20 mL of extraction buffer (100 mM sodium pyrophosphate, 100 mM TRIS, 100 mM EDTA, 1.5 M NaCl, 1% hexadecyltrimethylammonium bromide, 2% sodium

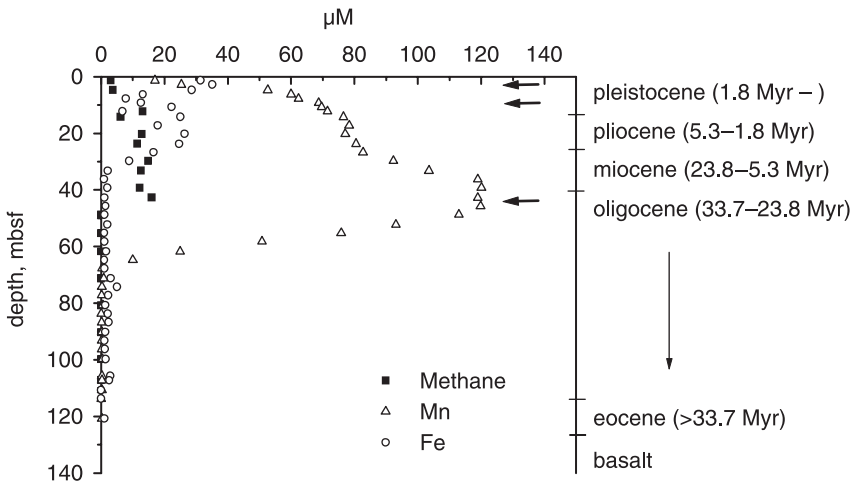

Fig. 1 Profiles of methane and dissolved iron and manganese in sediment at Site 1231, ODP Leg 201 (redrawn from Shipboard Scientific Party, 2003). The right axis indicates the age of the sediment. Arrows indicate the depth of the three samples analysed in this study.

dodecyl sulphate) was added and the mixture was incubated for 30 min at 65 °C. Following a 10 minute centrifugation at 6000 g, the supernatant was extracted with an equal volume of ice-cold pH neutral phenol/chloroform/isoamylalcohol (25 : 24 : 1). The water phase was transferred to a new tube and after addition of one volume of isopropanol, DNA was precipitated overnight at –20 °C. Centrifugation, washing, and resuspension of the DNA pellet were performed as described above.

Purification procedure

The amount of DNA extracted from pure sediment was generally below detection limit on agarose gels, and in order to detect coextraction of PCR-inhibitors and evaluate the efficiency of different purification protocols, we performed three parallel control extractions: (1) 5 mL of a pure culture of an anaerobic, sulphur disproportionating bacterium, *Desulfocapsa* sp. (supplied by Kai Finster, University of Aarhus, Denmark) (2) 5 g sediment mixed with 5 mL of bacterial culture, and (3) a negative control extraction without any sample. Similar amounts of DNA were extracted from pure culture and from culture/sediment mixtures (evaluated on 1% agarose gels, data not shown). Co-extracted PCR-inhibiting compounds from the sediment prevented amplification of genomic DNA from the culture/sediment mixtures. Thus, further purification of the extracts was necessary.

The following four purification protocols were tested on extracts from culture/sediment mixtures in order to improve the quality of the DNA extraction products. (1) Gel purification was performed by loading the DNA extract on sterile 1% low melting point (LMP) agarose gels, then running the gels for 2 h at 70 V in sterile 0.5 × TAE buffer (20 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 7.8), and excising the area of the gel containing genomic DNA. Genomic DNA was subsequently extracted from the gels using the QIAEX®II gel extraction kit (QIAGEN, Valencia, CA) according to the manufacturers instructions. (2) The commercial UltraClean Soil DNA Kit (Mobio Laboratories, Carlsbad, CA) has previously been employed successfully in extractions of genomic DNA from deep-sea sediments (e.g. Inagaki *et al.*, 2001; Kormas *et al.*, 2003). The kit involves the removal of humic substances by addition of an inhibitor removal solution of unknown composition (IRS) followed by precipitation of humic acids present in the sample by centrifugation. In an attempt to take advantage of the IRS and purify our extraction products of possible humic compounds, we added DNA extracts rather than sediment samples and used the kit according to the manufacturers instructions except that the bead-beating step was avoided. (3) An equal volume of sterile pH neutral 3% Na_2S solution was added to extraction products in order to precipitate coextracted metals as metal/sulphide complexes. The mixture was incubated overnight at 4 °C and centrifuged for 10 min at 10 000 g. (4) DNA Extraction products were purified using the Wizard Plus Miniprep DNA Purification System (Promega) according to the manufacturers instructions. Visualization of the purified genomic DNA on 1% agarose gels ran at 70 V for 2 h and stained with ethidium bromide indicated whether DNA had been damaged or lost during purification. The efficiency of PCR inhibitor removal was verified by preparing a dilution series of each purification product and adding 1 μL of each dilution to a PCR reaction.

PCR reactions

The extracted and purified DNA was amplified using the FailSafe™ PCR system (Epicentre, Madison, WI). Reactions of 25 μL were prepared with 0.25 μL FailSafe enzyme mix, 12.5 μL 2 × PREMIX G (supplied by the manufacturer), 25 pmol of each primer, 12 μL PCR water, and template DNA. Selective amplification of archaeal 16S rRNA genes was performed using the primer pair 8f (5′-TTCCGGTTGATCCTGCCGGA-3′) and 1492r (5′-GGCTACCTTGTTACGACTT-3′) in an initial reaction followed by a second round of nested amplification with either the forward primer 8f or 344f (5′-A(C/T)GGGG(C/T)GCA(G/C)CAGG(G/C)G-3′) in combination with the primer 915r (5′-GTGCTCCCCCGCCAATTCCT-3′). PCR reactions were carried out in a BIORAD i-cycler and the reaction sequence consisted of 2 min at 94 °C followed by 32 cycles of alternating temperatures as follows: 94 °C for 30 s, 60 s at 58 °C, and 3 min at 72 °C. The final elongation step at 72 °C was extended to 10 min.

Cloning and sequencing

PCR products were gel purified on 1% LMP agarose gels. About 50 ng of PCR product was loaded on the gel, ran for 30 min at 70 V, and stained with SYBR gold (Invitrogen Corportion, Carlsbad, CA). The PCR products were visualized on a Dark Reader transilluminator (Clare Chemicals, Dolores, CO), excised, and DNA was extracted from the gel using the QIAEX®II gel extraction kit according to the manufacturers instructions. The purified DNA samples were a-tailed to improve cloning efficiency by mixing the purified PCR product (40 μL) with 5 μL deoxynucleoside triphosphate (2 mM), 5 μL 10 × reaction buffer B, and 1 unit of Taq DNA polymerase (Promega, Madison, WI). The mixture was incubated at 72 °C for 10 min, extracted with phenol/chloroform/isoamylalcohol (25 : 24 : 1), and centrifuged at 6000 g for 5 min. The aqueous phase was then transferred to a fresh microcentrifuge tube and precipitated with 2.5 volumes of ethanol and 0.1 vol of 5 M NaCl. After centrifugation (10 min, 10 000 g) the pellet was washed once with 20 μL 70% ethanol, air-dried and resuspended in 4 μL PCR water. These PCR products were cloned using the TOPO XL PCR cloning kit (Invitrogen Corporation, Carlsbad, CA) and transformed by electroporation according to the manufacturers specifications. Sequence data were obtained on the DNA sequencing facility at the Medical Department,

University of North Carolina at Chapel Hill or the sequencing facility at Marine Biological Laboratory in Woods Hole, using sequencing primers M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′). Forward and reverse reads were assembled, aligned, and checked for chimeras by comparing phylogenetic trees constructed with the first and last half (each about 300 bp) of the sequences (Hugenholtz & Huber, 2003).

Phylogenetic analysis

Sequence data were BLAST analysed against the entire GenBank database (Altschul *et al.*, 1997). The sequences were then aligned with their closest relatives and representative cultured and uncultured archaea using the CLUSTAL W program followed by manual alignment in Seqpup (Gilbert, 1996). Phylogenetic trees were constructed from the alignment sequences, using Jukes-Cantor distance matrices for inferring the tree topology and neighbour joining and maximum parsimony for bootstrap analysis (1000 replica) of the branching pattern using the PHYLIP software package (Felsenstein, 1988, 1989).

RESULTS AND DISCUSSION

Protocol development

As mentioned above, coextracted inhibiting compounds prevented PCR amplification of genomic DNA from culture/sediment mixtures. This PCR inhibition was not observed in extracts of pure cell culture, indicating that the interfering substance(s) originated from the sediment. A common solution to such PCR inhibition is to dilute extraction products to minimize the amount of inhibiting compounds in the reaction mixture (e.g. Kormas *et al.*, 2003; Webster *et al.*, 2003). However, this approach depends on the DNA to inhibitor ratio since sufficient template for amplification has to remain in the reaction while the inhibitor was diluted. In sediments with low cell numbers this ratio is likely to be low, and the inhibitor may prevail after the template has been diluted to extinction. Furthermore, dilution of the already small amount of template in such samples may cause a loss of diversity and/or inconsistent PCR-results due to random amplification (Webster *et al.*, 2003). In this study, dilution of the DNA-enriched template extracted from sediment/culture mixtures up to 10 000 times did not improve PCR results. We used 3 g of sediment in each extraction which, based on shipboard cell counts, corresponds to roughly 3×10^6 cells. Assuming 100% cell lysis and extraction efficiency, the final 50 μL of extraction product contains 6×10^4 genomes μL^{-1}. A PCR reaction in which 1 μL of a 10 000 times dilution of the extract is added thus contains only about 6 DNA templates, and further dilution is not a feasible solution to the inhibitor problems in the samples.

As described in the methods section, various purification protocols were employed and evaluated, including (1) gel purification; (2) the Ultra Clean Soil DNA Extraction Kit, Mobio, in a modified protocol, (3) sulphide addition; (4) and the Wizard Miniprep kit. The purified extraction products of sediment/culture mixtures were evaluated on agarose gels, revealing that little DNA was lost during Wizard Miniprep and gel-purification, whereas only a small fraction of the DNA initially present in the sample was recovered after the modified Mobio purification or the sulphide precipitation protocol. Possibly, DNA in the sulphide treatment was either coprecipitated with formed sulphide-metal complexes and aggregates of partly oxidized sulphur species, e.g. elemental sulphur, during centrifugation, or degraded by DNAses in the sample during the overnight incubation at 4 °C.

The purified DNA was tested for function in PCR by using between 2 and 10^{-4} μL as template in 25 μL reactions. Wizard Miniprep and gel-purified extracts were now readily amplifiable with up to 2 μL template per 25 μL PCR reactions. Products that were treated with sulphide or the Mobio kit could not be amplified, which was not unexpected because most of the DNA was lost during the purification step. In conclusion, the optimal purification procedure evaluated on DNA extracts from bacteria-enriched Site 1231 sediments was either the Wizard Miniprep Purification Kit or gel purification on LMP agarose.

DNA extraction

Genomic DNA was extracted and purified from three grams of sediment from 1.8 mbsf, 9.0 mbsf, and 43.0 mbsf (Site 1231, Hole E, core 1 section 2, core 2 section 5, and core 6 section 2, respectively) using the two extraction protocols described above followed by purification on the Wizard Miniprep Kit. The position of each of these three samples in the sediment profile is indicated in Fig. 1. Protocol ENZ yielded genomic DNA suitable for PCR amplification from 1.8 mbsf as well as 43 mbsf but not from 9.0 mbsf, while protocol BB was successful in extracting DNA from 1.8 mbsf and 9.0 mbsf but not from 43 mbsf. Irrespective of sample and extraction protocol used, PCR products were obtained using down to but not less than 0.1 μL extraction product, indicating that DNA was diluted to extinction at smaller template concentrations. Thus, the two extraction protocols seemed to extract similar amounts of DNA from the samples. After purification, the volume of each extract was 100 μL. Based on direct cell counts that indicated *c.* 10^6 cells ml^{-1} at Site 1231 (Shipboard Scientific Party, 2003), three grams of sediment contain roughly 3×10^6 cells, and 0.1 μL DNA extract corresponds to 3000 prokaryotic cells. In a recent study, the archaeal component of the subsurface communities at ODP Sites 1227 (Peru Margin) and 1230 (Peru Trench) was found to be less than 10% of the total prokaryotic population in the upper 10–20 m of sediment, and was decreasing to less than 0.01% below this (Inagaki *et al.*

Table 1 A summary of the archaeal sequences retrieved from sediment samples from ODP Site 1231

Origin (mbsf)	Affiliation	Name*	Acc. no.†	Freq. ‡	Closest match§	Similarity¶
1.8	TMEG	1.8mENZ.1	AY661821	15/32	HTA-C7, AF498931	86%
		1.8mENZ.2	AY661822	6/32	HTA-C7, AF498931	86%
		1.8mENZ.3	AY661823	9/32	HTA-C7, AF498931	85%
		1.8mENZ.4	AY661824	2/32	HTA-C7, AF498931	87%
	SAGMEG1	1.8mBB.1	AY661826	9/54	SAGMA-E, AB050209	91%
		1.8mBB.2	AY661827	1/54	SAGMA-E, AB050209	92%
	DHVE6	1.8mBB.3	AY661828	1/54	pMC1A4, AB019754	85%
		1.8mBB.4	AY661829	1/54	pMC1A4, AB019754	87%
	MG1	1.8mBB.5	AY661830	2/54	Mar arc. 1, D87348	98%
		1.8mBB.6	AY661831	1/54	JTA266, AB015278	97%
		1.8mBB.7	AY661832	1/54	JTA266, AB015278	97%
		1.8mBB.8	AY661833	7/54	pPCA4.21, AB049032	95%
		1.8mBB.9	AY661834	5/54	pPCA4.21, AB049032	98%
		1.8mBB.10	AY661835	1/54	pPCA4.21, AB049032	97%
		1.8mBB.11	AY661836	1/54	19b-5, AJ294854	95%
		1.8mBB.12	AY661837	1/54	19b-5, AJ294854	96%
		1.8mBB.13	AY661838	2/54	19a-18, AJ294878	94%
		1.8mBB.14	AY661839	1/54	19a-18, AJ294878	95%
		1.8mBB.15	AY661840	1/54	19a-18, AJ294878	96%
		1.8mBB.16	AY661841	1/54	SAGMA-9, AB050239	97%
		1.8mBB.17	AY661842	1/54	SAGMA-9, AB050239	96%
		1.8mBB.18	AY661843	1/54	SAGMA-9, AB050239	98%
		1.8mBB.19	AY661844	1/54	SAGMA-9, AB050239	95%
		1.8mBB.20	AY661845	1/54	pHAuB-30, AB072725	98%
		1.8mBB.21	AY661846	4/54	19a-5, AJ294876	96%
		1.8mBB.22	AY661847	2/54	JTB153, AB015276	94%
		1.8mBB.23	AY661848	2/54	JTB153, AB015276	99%
		1.8mBB.24	AY661849	2/54	JTB153, AB015276	97%
		1.8mBB.25	AY661850	1/54	JTB153, AB015276	97%
		1.8mBB.26	AY661851	1/54	JTB153, AB015276	98%
		1.8mBB.27	AY661852	2/54	19b-52, AJ294873	96%
9.0	DSAG	9.0mBB.1	AY661825	46/46	pHAuA-5, AB072723	98%
43	43 m cluster I	43mENZ.1	AY661819	9/34	20a-6, AJ299151	89%
		43mENZ.2	AY661820	4/34	20a-6, AJ299151	88%
		43mENZ.3	AY661818	1/34	20a-6, AJ299151	89%
	43 m cluster II	43mENZ.4	AY661816	11/34	33-P27A98, AF355901	86%
		43mENZ.5	AY661815	8/34	33-P27A98, AF355901	86%
		43mENZ.6	AY661817	1/34	33-P27A98, AF355901	86%

* Phylotype names indicate the depth from which they were retrieved, the extraction procedure used, and an individual number, † for the GenBank Accession number, ‡ for the frequency with which the sequence was observed in the clone library, § for the closest match found by BLAST analysis, and ¶ for the similarity between the sequence and its closest match.

submitted). The number of archaeal cells represented by 0.1 μL extraction product may thus be several orders of magnitude lower than the estimated total number of prokaryotes.

Archaeal phylotypes from site 1231

The cloning and sequencing results from the three sediment depths are summarized in Table 1, listing the phylotype names, origin, and frequency in the clone libraries, as well as the closest GenBank match based on BLAST analysis (Altschul *et al.*, 1997).

1.8 mbsf

DNA was successfully extracted from the 1.8 mbsf sample using both the ENZ and the BB protocol. A total of 51 clones obtained from this sample with protocol ENZ yielded 32 high quality sequences, while 96 clones obtained with protocol BB yielded 55 sequences (Table 1). The phylotypes detected using the ENZ protocol formed a tight cluster (1.8 m Cluster) within the Euryarchaeota (Fig. 2A). The closest relatives were uncultured organisms from a variety of terrestrial, aquatic, and subsurface habitats, including clones HTA-C7 and HTA-H9 from metal-rich particles formed in the water column of a freshwater reservoir (Stein *et al.*, 2002), clone WCHD3-02 from the methanogenic zone in terrestrial aquifer sediments contaminated with jet fuel aromatic hydrocarbons and chlorinated C2-solvents (Dojka *et al.*, 1998), and clone KuA16 from oil-contaminated groundwater (Watanabe *et al.*, 2002). Other related sequences include clone 33–1 from waste water sludge (Williams *et al.* unpublished), clone SAGMA-T from

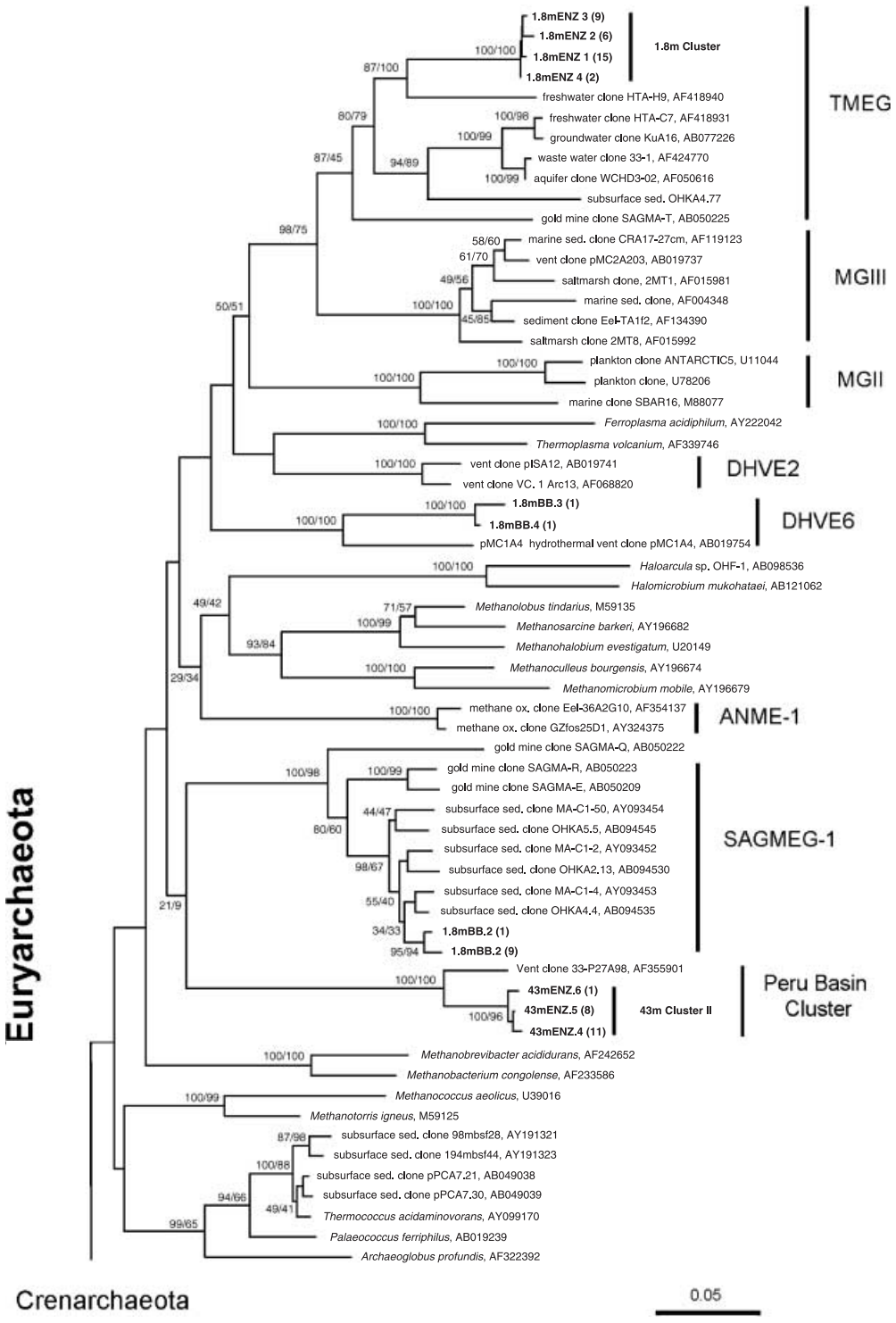

Fig. 2 Distance tree with subsurface sequences from ODP Site 1231, their closest GenBank relatives, and representatives of various archaeal lineages. The number of times each phylotype was encountered in the clone libraries is indicated in parentheses. (A) Euryarchaeota (B) Crenarchaeota. The trees were rooted with each other. Bootstrap numbers for neighbour joining (left) and parsimony (right) are based on 1000 replicates each. The scale bar indicates 5% 16 rRNA sequence difference (Jukes-Cantor). TMEG: Terrestrial Miscellaneous Euryarchaeotal Group (Takai *et al*., 2001b), MGIII: Marine Group III (Delong, 1998), MGII: Marine group II (Delong, 1998), DHVE2/6: Deep-sea Hydrothermal Vent Euryarchaeota 2/6 (Takai & Horikoshi, 1999), DSAG: Deep-Sea Archaeal Group (Inagaki *et al*., 2003a), UCIIb: Uncultured Crenarchaeotal Group IIb (Schrenk *et al*., 2003), TMCG: Terrestrial Miscellaneous Crenarchaeotic Group (Takai *et al*., 2001b), THSCG: Terrestrial Hot Spring Crenarchaeotic Group (Takai *et al*., 2001b), MGI: Marine Group I (Fuhrmann *et al*., 1992), SAGMEG-1: South African Gold Mine Euryarchaeotal Group 1 (Takai *et al*., 2001b).

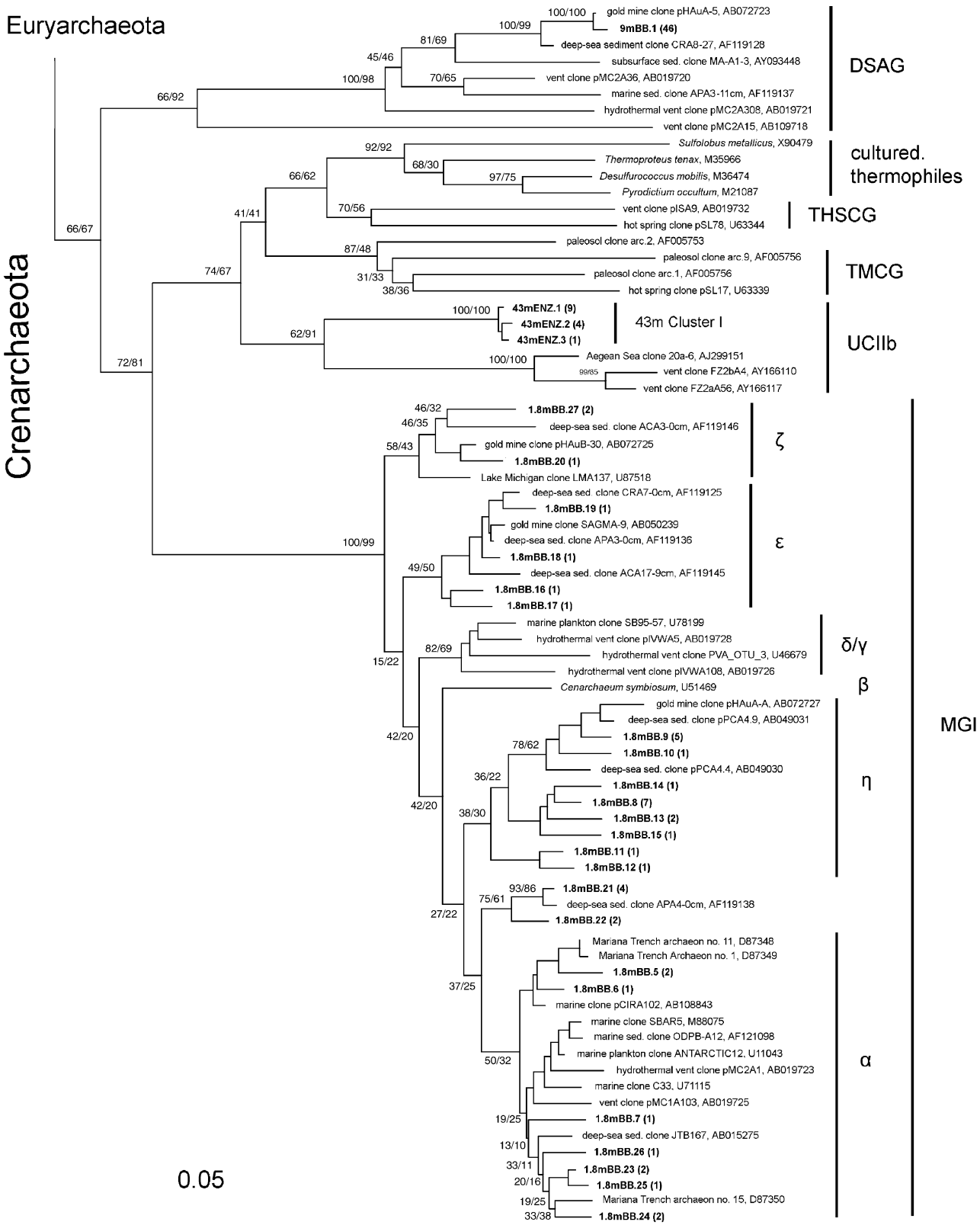

Fig. 2 *Continued*

a South African gold mine (Takai *et al.*, 2001b), and clone OHKA4.7 from volcanic ash and pumice layer sediments 18 mbsf in the Okhotsk Sea (Inagaki *et al.*, 2003a). Bootstrap analysis indicated that these clones form a monophyletic cluster termed as the Terrestrial Miscellaneous Euryarchaeotal Group (TMEG, Takai *et al.*, 2001b), which thus appears to include also freshwater and marine organisms (Fig. 2A).

Phylotypes affiliated with the Deep-sea Hydrothermal Vent Euryarchaeotal Group 6 (DHVE6) were obtained from the 1.8 mbsf sample using extraction protocol BB (Fig. 2A). This was one among at least five clusters comprising the Deep-sea Hydrothermal Vent Euryarchaeotal Group II (DHVE group II, Takai & Horikoshi, 1999; Nercessian *et al.*, 2003), found previously at hydrothermal vents only.

The most abundant and diverse group of phylotypes obtained using the BB extraction protocol on the 18 mbsf sample belonged to the Marine Group I Crenarchaeota (Fuhrmann *et al.*, 1992; Delong *et al.*, 1992). Recently, MGI has been divided into four clusters (clusters α – δ; Massana *et al.*, 2000; Takai *et al.*, 2004). In our analysis, several MGI clones from Site 1231, together with other deep-sea and subsurface clones, could not be accommodated into the previously defined four groups. Some of these formed two new groups (ε and ξ) at a basal position within the MGI lineage, while most of the MGI clones from Site 1231 formed a sister group (η) to the α cluster (Fig. 2B). As a note of caution, bootstrap support for all groups remains low (near or below 50%), with the exception of the combined gamma and delta cluster. However, we note that the MGI archaea contain more diversity than previously assumed, and that much of this intragroup diversity can be attributed to sediment or subsurface clones.

Organisms affiliated with the MGI group are ubiquitous in the marine water column and represent the numerically dominant prokaryotic picoplankton group in deep-sea water (Karner *et al.*, 2001). Contamination tests for Site 1231 sediments with chemical tracers indicated a potential seawater contamination of <0.1 $\mu L\ g^{-1}$ sediment, which would correspond to <50 contaminating cells per gram sediment (House *et al.*, 2003). Because 3 gram sediment was used to obtain 100 µL extraction product, 1 µL PCR template would represent at most 1–2 contaminating cells, which can not account for the large diversity of MG1 organisms observed in the sediment. Apart from pelagic water samples, MGI organisms have previously been detected in samples from marine sediments (Kato *et al.*, 1997; Li *et al.*, 1999; Vetriani *et al.*, 1999; Inagaki *et al.*, 2001; Inagaki *et al.*, 2003a; Inagaki *et al.* submitted), hydrothermal vents (Moyer *et al.*, 1998; Takai & Horikoshi, 1999; Takai *et al.*, 2001a; Huber *et al.*, 2002), as well as non-marine systems such as South African and Japanese gold mines (Takai *et al.*, 2001b; Inagaki *et al.*, 2003b) and Lake Michigan sediment (MacGregor *et al.*, 1997). So far, no consistent pattern has emerged linking specific clusters of MGI organisms with a particular habitat preference (Massana *et al.*, 2000; Takai *et al.*, 2004). Whether their abundance in the relatively shallow 1.8 mbsf sample reflects an actual ecological role for this group in the sediment or is caused by deposition of pelagic organisms from the water column above remains an open question.

Numerous phylotypes affiliated with the SAGMEG-1 cluster were also detected in the 1.8 mbsf sample using the BB protocol. Organisms from this group have been found to be abundant in anaerobic/microaerophilic water samples from a South African gold mine (Takai *et al.*, 2001b) as well as marine sediment from the Sea of Okhotsk (Inagaki *et al.*, 2003a) and hydrate-bearing sediment of the Nankai Through (Reed *et al.*, 2002). Importantly, the group is not widely distributed in marine water columns, and its presence in the sediment at Site 1231 probably reflects a native sedimentary population.

9.0 mbsf

Only protocol BB resulted in DNA extracts suitable for PCR amplification from the 9.0 mbsf sample. A total of 48 clones were analysed from this depth, yielding 46 sequences, all of the same phylotype (Table 1). This phylotype was affiliated with the Deep-Sea Archaeotal Group (DSAG, Inagaki *et al.*, 2003a) which has been found and reported under different names from numerous environments including deep sea sediments from the north-western Atlantic Ocean (Marine Benthic Group B, Vetriani *et al.*, 1999), hydrate-bearing sediments in the Nankai Through (NT-A2, Reed *et al.*, 2002), subseafloor sediments of the Sea of Okhotsk (Inagaki *et al.*, 2003a), deep-sea hydrothermal vents (Deep-Sea Hydrothermal Vent Crenarchaeotic Group, Takai & Horikoshi, 1999), and hydrothermal rock samples from a Japanese gold mine (Inagaki *et al.*, 2003b). Thus, this group seems to be widely associated with both terrestrial and marine subsurface and/or hydrothermal environments. The group is rooted near the branching point between the Cren- and Euryarchaeota, and has been ascribed to either of these two in different publications. (Takai & Horikoshi, 1999; Vetriani *et al.*, 1999; Takai *et al.*, 2001a; Reed *et al.*, 2002; Inagaki *et al.*, 2003a,b). Here, we included the group at the root of the Crenarchaeota (Fig. 2B).

43 mbsf

Archaeal DNA was only successfully extracted from the 43 mbsf sample using the ENZ protocol. A total of 34 successfully sequenced clones yielded 6 phylotypes that formed two tight clusters within the Crenarchaeota and the Euryarchaeota (43 m cluster I and II, respectively). The 43 m Cluster I was related to crenarchaeotal phylotypes including clones FZ2aA56 and FZ2bA4 from the outer layers of a metal-sulphide (pyrite, sphalerite, wurtzite) vent chimney from the Juan de Fuca Ridge (Schrenk *et al.*, 2003), and clone 20a-6 from sediments in the Aegean Sea (Brehmer, unpublished). Bootstrapping values supported the grouping of the 43 m Cluster I with these clones together forming of an expanded version of the Uncultured Crenarchaeotal group IIb (UCIIb, Schrenk *et al.*, 2003). The nearest relative of 43 m Cluster II

was a single euryarchaeotal phylotype, clone 33-P27A98, which has previously shown no clear affiliation to any other euryarchaeotal lineage. This clone originates from low-temperature hydrothermal fluids (30–55 °C) that emerged from a basalt fissure on Axial Seamount at Juan de Fuca (Huber *et al.*, 2002).

The archaeal community at Site 1231

Most of the archaeal phylotypes were affiliated with previously recognized deep-sea or deep subsurface clusters. Some of these, particularly the MG1 organisms, seem likely to be deposited from the pelagic water above, while others like the phylotypes affiliated with DSAG and SAGMEG-1 probably represent true subsurface lineages.

The broad peak of dissolved (reduced) iron and manganese concentrations in the upper portion of the Site 1231 sediment column were compatible with the activity of metal-reducing prokaryotes. Various cultured Eury- and Crenarchaeota are known to reduce metals including iron and/or manganese (Vargas *et al.*, 1998; Kashefi & Lovley, 2000), but these are hyperthermophilic species, and they are unrelated to the phylotypes from Site 1231. The phylogenetic affiliation of the 1.8 m cluster suggests a shared preference of these uncultured archaeal lineages for such anoxic or microoxic habitats with an abundance of dissolved or solid-phase reduced metals. This habitat preference may also apply to the 1.8 mbsf clones within the DHVE6 cluster, previously known from metal-sulphide chimneys (Takai & Horikoshi, 1999). However, the lack of close relatives with known metabolisms makes it impossible to plausibly extrapolate physiological information from the phylogenetic data.

The fact that the two extraction protocols seemed to select strongly for different groups of organisms in the sediment complicates the comparative evaluation of phylotypes from different samples. Nevertheless, the archaeal lineages detected in samples from 9.0 and 43 mbsf were phylogenetically different from the phylotypes from the 1.8 m sample, suggesting a vertical succession of organisms. This is significant, since the chemical regime of the sediment in these depths seems similar and is characterized by low methane concentrations and abundant electron acceptors like sulphate, oxidized iron, and manganese (Shipboard Scientific Party, 2003). The changes in the archaeal community component with depth may be caused by burial of different microbial assemblages during sedimentation or selection and vertical migration of microorganisms according to *in situ* sedimentary conditions. The absence of typical pelagic archaeal groups in the samples from 9 and 43 mbsf suggests that the archaeal populations in these depths are specialized and adapted to life in the sediment rather than to the remnants of sedimentation.

The finding that clones from hydrothermal environments are the closest sister taxa of many phylotypes retrieved from Site 1231 is remarkable given the differences in chemical and physical characteristics between these two types of environments. This is probably to some extent due to a disproportionate number archaeal sequences from hot vent environments in the database. Thus, clusters hitherto detected only in hydrothermal environments are likely to be less restricted in their habitat diversity than suggested by the current database. It is certainly also possible that some of the phylotypes detected in the hydrothermal fluid and in chimney sulphides may not be indigenous to the hot vent system, but originate from cooler sediments from which they are flushed out as seawater is entrained into the hydrothermal circulation system on mid-ocean ridges and ridge flanks.

ACKNOWLEDGEMENTS

This work was supported by the NASA Astrobiology Institute 'Subsurface Biospheres', 'Environmental Genomes' and by the Danish Natural Science Research Council. We thank all members of the Leg 201 shipboard scientific party for their help during the cruise, Lev Neretin for inspiration and help during protocol development, and Kai Finster for supplying the bacterial culture.

REFERENCES

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-blast: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389–3402.

Bale SJ, Goodman K, Rochelle PA, Marchesi JR, Fry JC, Weightman AJ, Parkes RJ (1997) *Desulfovobrio profundus* sp. nov., A novel barophilic sulphate-reducing bacterium from deep sediment layers in the Japan Sea. *International Journal of Systematic Bacteriology* **47**, 515–521.

Barnes SP, Bradbrook SD, Cragg BA, Marchesi JR, Weightman AJ, Fry JC, Parkes RJ (1998) Isolation of sulphate reducing bacteria from deep sediment layers of the Pacific Ocean. *Geomicrobiology Journal* **15**, 67–83.

Cragg BA, Harvey SM, Fry JC, Herbert RA, Parkes RJ (1992) Bacterial biomass and activity in the deep sediment layers of the Japan Sea, Hole 798B. *Proceedings of the Ocean Drilling Program, Scientific Results 127/128*. College Station, Texas, pp. 761–776.

D'Hondt SL, Rutherford S, Spivack AJ (2002) Metabolic activity of subsurface life in deep-sea sediments. *Science* **295**, 2067–2070.

Delong EF (1992) Archaea in coastal marine environments. *Proceedings of the National Academy of Sciences USA* **89**, 5685–5689.

Delong EF (1998) Everything in moderation: archaea as 'non-extremophiles'. *Current Opinion in Genetics and Development* **8**, 649–654.

Dojka MA, Hugenholtz P, Haack SK, Pace NR (1998) Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. *Applied and Environmental Microbiology* **64**, 3869–3877.

Dymond J, Corliss JB, Stillinger R (1976) Chemical composition and metal accumulation rates of metalliferous sediments from Sites 319, 320, and 321. In *Initial Reports DSDP 34*. (eds Yeats RS, Hart SR *et al.*) U.S. Government Printing Office, Washington, pp. 575–588.

Felsenstein J (1988) Phylogenies from molecular sequences: inference and reliability. *Annual Review of Genetics* **22**, 521–565.

Felsenstein J (1989) PHYLIP – phylogeny inference package. Version 3.2. *Cladistics* **5**, 164–166.

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

Gilbert DG (1996) Seqpop sequence, editor Version 0.6f. Biology Department. Indiana University, Bloomington.

House CH, Cragg BA, Teske A (2003) Drilling Contamination Tests on ODP Leg 201 Using Chemical and Particulate Tracers. *Proceedings of the Ocean Drilling Program, Initial Reports, Leg 201 [CD-ROM]*. Available from: Ocean Drilling Program, Texas and M University. College Station TX 77845–9547, USA.

Huber JA, Butterfield DA, Baross JA (2002) Temporal changes in Archaeal diversity and chemistry in a mid-ocean ridge subseafloor habitat. *Applied and Environmental Microbiology* **68**, 1585–1594.

Hugenholtz P, Huber T (2003) Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. *Interntional Journal of Systematic and Evolutionary Microbiology* **53**, 289–293.

Hurt RA, Qiu X, Wu L, Roh Y, Palumbo AV, Tiedje JM, Zhou J (2001) Simultaneous recovery of RNA and DNA from soils and sediments. *Applied and Environmental Microbiology* **67**, 4495–4503.

Inagaki F, Nunoura T, House C, Schippers A, Teske A, Hinrichs KU, Cragg B, Parkes J, Biddle JF, Kelly TJ, Brenchley JE, Neretin LN, Lauer A, Lever M, Suzuki M, Delwiche M, Takai K, Colwell FS, Nealson KH, Horikoshi K, D'Hondt SL, Jørgensen BB (Submitted to Science) Distribution of unidentified prokaryotes in deeply buried marine sediments.

Inagaki F, Suzuki M, Takai K, Oida H, Sakamoto T, Aoki K, Nealson KH, Horikoshi K (2003a) Microbial communities associated with geological horizons in coastal subseafloor sediments from the Sea of Okhotsk. *Applied and Environmental Microbiology* **69**, 7224–7235.

Inagaki F, Takai K, Hirayama H, Yamato Y, Nealson KH, Horikoshi K (2003b) Distribution and phylogenetic diversity of the subsurface microbial community in a Japanese epithermal gold mine. *Extremophiles* **7**, 301–317.

Inagaki F, Takai K, Komatsu T, Kanamatsu T, Fujioka K, Horikoshi K (2001) Archaeology of Archaea: geomicrobiological record of Pleistocene thermal events concealed in a deep-sea subseafloor environmentet. *Extremophiles* **5**, 385–392.

Juniper SK, Cambon M-A, Lesongeur F, Barbier G (2001) Extraction and purification of DNA from organic rich subsurface sediments (ODP Leg 169S). *Marine Geology* **174**, 241–247.

Karner MB, DeLong EF, Karl DM (2001) Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature* **409**, 507–510.

Kashefi K, Lovley DR (2000) Reduction of Fe (III), Mn (IV), and toxic metals at 100 degrees C by *Pyrobaculum islandicum*. *Applied and Environmental Microbiology* **66**, 1050–1056.

Kato C, Li L, Tamaoka J, Horikoski K (1997) Molecular analyses of the sediment of the 11 000 m deep Mariana Trench. *Extremophiles* **1**, 117–123.

Kormas KA, Smith DC, Edgcomb VE, Teske A (2003) Molecular analysis of deep subsurface microbial communities in Nankai Trough sediments (ODP Leg 190, Site 1176a). *FEMS Microbiology Ecology* **45**, 115–125.

Kvenvolden KA (1993) Gas hydrates – geological perspective and global change. *Reviews of Geophysics* **31**, 173–187.

Li L, Chiaki K, Horikoshi K (1999) Microbial diversity in sediments collected from the deepest cold-seep area, the Japan Trench. *Marine Biotechnology* **1**, 391–400.

MacGregor BJ, Moser DP, Alm EW, Nealson KH, Stahl D (1997) Crenarchaeota in Lake Michigan sediment. *Applied and Environmental Microbiology* **63**, 1178–1181.

Mancinelli RL (2000) Accessing the Martian deep subsurface to search for life. *Planetary and Space Science* **48**, 1035–1042.

Marchesi JR, Weightman AJ, Cragg BA, Parkes RJ, Fry JC (2001) Methanogen and bacterial diversity and distribution in deep gas hydrate sediments from the Cascadia Margin as revealed by 16S rRNA molecular analysis. *FEMS Microbiology Ecology* **34**, 221–228.

Massana R, Delong EF, Pedrós-Alió C (2000) A few cosmopolitan phylotypes dominate planktonic archaeal assemblages in widely different provinces. *Applied and Environmental Microbiology* **66**, 1777–1787.

Moyer CL, Tiedje JM, Dobbs FC, Karl DM (1998) Diversity of deep-sea hydrothermal vent archaea from Loihi Seamount, Hawaii. *Deep-Sea Research* **45**, 303–317.

Nercessian O, Reysenbach A-L, Prieur D, Jeanthon C (2003) Archaeal diversity associated with in situ samplers deployed on hydrothermal vents on the East Pacific Rise (13° N). *Environmental Microbiology* **5**, 492–502.

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.

Parkes RJ, Cragg BA, Bale SJ, Goodman K, Fry JC (1995) A combined ecological and physiological approach to studying sulphate reduction within deep marine sediment layers. *Journal of Microbiological Methods* **23**, 235–249.

Parkes R, Cragg B, Wellsbury P (2000) Recent studies on bacterial populations and processes in subseafloor sediments: a review. *Hydrogeology Journal* **8**, 11–28.

Reed DW, Fujita Y, Delwiche ME, Blackwelder DB, Sheridan PP, Uchida T, Colwell FS (2002) Microbial communities from methane hydrate-bearing deep marine sediments in a forearc basin. *Applied and Environmental Microbiology* **68**, 3759–3770.

Rochelle P, Cragg BA, Fry JC, Parkes RJ, Weightman AJ (1994) Effects of sample handling on estimation of bacterial diversity in marine sediments by 16S rRNA gene sequence analysis. *FEMS Microbiology Ecology* **15**, 215–225.

Rochelle P, Fry JC, Parkes RJ, Weightman AJ (1992) DNA extraction for 16S rRNA gene analysis to determine genetic diversity in deep sediment communities. *FEMS Microbiology Letters* **100**, 59–66.

Rogers DR, Santelli CM, Edwards KJ (2003) Geomicrobiology of deep-sea deposits: estimating community diversity from low-temperature seafloor rocks and minerals. *Geobiology* **1**, 109–117.

Schrenk MO, Kelley DS, Delaney JR, Baross JA (2003) Incidence and diversity of microorganisms within the walls of an active deep-sea sulfide chimney. *Applied and Environmental Microbiology* **69**, 3580–3592.

Shipboard Scientific Party (2003) Site 1231. In (eds D'Hondt SL, Jørgensen BB, Miller DJ et al.). *Proceedings of the Ocean Drilling Program, Initial Reports* **201**, 1–64 [CD-ROM]. Available from: Ocean Drilling Program, Texas A & M University, College Station TX 77845–9547, USA.

Stein LY, Jones G, Alexander B, Elmund K, Wright-Jones C, Nealson KH (2002) Intriguing microbial diversity associated with metal-rich particles from a freshwater reservoir. *FEMS Microbiology Ecology* **42**, 431–440.

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

Takai K, Komatsu T, Inagaki F, Horikoshi K (2001a) Distribution of archaea in a black smoker chimney structure. *Applied and Environmental Microbiology* **67**, 3618–3629.

Takai K, Moser DP, DeFlaun M, Onstott TC, Fredrickson JK (2001b) Archaeal diversity in waters from deep South African gold mines. *Applied and Environmental Microbiology* **67**, 5750–5760.

Takai K, Oida H, Suzuki Y, Hirayama H, Nakagawa S, Nunoura T, Inagaki F, Nealson KH, Horikoshi K (2004) Spatial distribution of marine Crenarchaeota group I in the vicinity of deep-sea hydrothermal systems. *Applied and Environmental Microbiology* **70**, 2404–2413.

Teske A, Hinrichs KU, Edgcomb V, de Vera-Gomez A, Kysela D, Sylva SP, Sogin ML (2002) Microbial diversity of hydrothermal sediments in the Guaymas Basin: evidence for anaerobic methanotrophic communities. *Applied and Environmental Microbiology* **68**, 1994–2007.

Vargas M, Kashefi K, Blunt-Harriset EL, Lovley DR (1998) Microbiological evidence for Fe (III) reduction on early Earth. *Nature* **395**, 65–67.

Vetriani C, Jannasch HW, MacGregor BJ, Stahl D, Reysenbach A-L (1999) Population structure and phylogenetic characterization of marine benthic Archaea in deep-sea sediments. *Applied and Environmental Microbiology* **65**, 4375–4384.

Watanabe K, Kodama Y, Hamamura N, Kaku N (2002) Diversity, abundance, and activity of archaeal populations in oil-contaminated groundwater accumulated at the bottom of an underground crude oil storage facility. *Applied and Environmental Microbiology* **68**, 3899–3907.

Webster G, Newberry CJ, Fry JC, Weightman AJ (2003) Assessment of bacterial community structure in the deep sub-seafloor biosphere by 16S rDNA-based techniques: a cautionary tale. *Journal of Microbiological Methods* **55**, 155–164.

Wellsbury P, Goodman K, Cragg BA, Parkes RJ (2000) The geomicrobiology of deep marine sediments from Blake Ridge containing methane hydrate (Sites 994, 995, and 997). *Proceedings of the Ocean Drilling Program, Scientific Results* **164**. College Station, Texas.

Wellsbury P, Mather I, Parkes RJ (2002) Geomicrobiology of deep, low organic carbon sediments in the Woodlark Basin, Pacific Ocean. *FEMS Microbiology Ecology* **42**, 59–70.

Whitman WB, Coleman DC, Wiebe WJ (1998) Prokaryotes: the unseen majority. *Proceedings of the National Academy of Sciences USA* **95**, 6578–6583.