APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2002, p. 3759–3770 Vol. 68, No. 8
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.8.3759–3770.2002

Microbial Communities from Methane Hydrate-Bearing Deep Marine Sediments in a Forearc Basin

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Received 20 December 2001/Accepted 3 May 2002

Microbial communities in cores obtained from methane hydrate-bearing deep marine sediments (down to more than 300 m below the seafloor) in the forearc basin of the Nankai Trough near Japan were characterized with cultivation-dependent and -independent techniques. Acridine orange direct count data indicated that cell numbers generally decreased with sediment depth. Lipid biomarker analyses indicated the presence of viable biomass at concentrations greater than previously reported for terrestrial subsurface environments at similar depths. Archaeal lipids were more abundant than bacterial lipids. Methane was produced from both acetate and hydrogen in enrichments inoculated with sediment from all depths evaluated, at both 10 and 35°C. Characterization of 16S rRNA genes amplified from the sediments indicated that archaeal clones could be discretely grouped within the *Euryarchaeota* and *Crenarchaeota* domains. The bacterial clones exhibited greater overall diversity than the archaeal clones, with sequences related to the *Bacteroidetes*, *Planctomycetes*, *Actinobacteria*, *Proteobacteria*, and green nonsulfur groups. The majority of the bacterial clones were either members of a novel lineage or most closely related to uncultured clones. The results of these analyses suggest that the microbial community in this environment is distinct from those in previously characterized methane hydrate-bearing sediments.

Gas hydrates are crystalline deposits consisting of a cage (clathrate) of water molecules surrounding gas molecules. Natural hydrates most commonly contain methane, but ethane, propane, isobutane, and branched-chained hydrocarbons may also be present. These structures are formed and remain stable under conditions of high pressure and low temperature (28). Consequently, gas hydrates are typically found hundreds of meters below the ground in circumpolar permafrost regions or in offshore marine sediments on continental margins (35, 46). They have attracted considerable interest recently because they represent a potentially vast energy reservoir and because of past and continuing impacts on global climate change and seafloor stability (27, 29).

It has been known since the late 1980s that isotopic signatures, as well as the ratio of methane to ethane plus propane (the $C_1/[C_2 + C_3]$ ratio), in natural gas hydrates indicate that the methane is predominantly of biological origin in marine deposits (see reference 30 for a review). However, until recently there have been few investigations of the microbial communities associated with methane hydrate-bearing sediments. Most of the reported studies have focused on shallow sediments obtained from seafloor hydrate deposits near methane seeps (6, 31). Such sites have excited keen interest because of the reported indirect evidence of active methane oxidation by sulfate-reducing consortia (7, 24). In contrast, in deep sediments (>100 m below the sea floor [mbsf]), sulfate concentrations are negligible (9), organic carbon is believed to be largely refractory, and overall biomass concentrations are significantly lower (42). For these reasons, as well as factors such as elevated pressure and temperature, the microbial communities in deep submarine sediments are expected to be quite different from those higher in the sedimentary column.

Investigations of microbial activity in deep hydrate-bearing sediments from the Blake Ridge and Cascadia Margin have suggested that the total bacterial numbers increase in the hydrate stability zone relative to the populations in the overlying sediments (13, 14, 53). In samples obtained from the Blake Ridge, offshore of southeastern North America, methanogenesis rates increased in samples from the hydrate zone, and acetoclastic methanogenesis continued to increase in samples from deeper layers (53). In sediments from the Cascadia Margin in the eastern Pacific, the methane oxidation rates peaked in the hydrate zone sediments, and the methanogenesis rates increased dramatically just below this zone (13, 14). These findings support the hypothesis that the methane in deep submarine hydrates can originate in the sediments below the hydrate stability zone and subsequently migrate upward to form hydrates (18, 53) and support methane-oxidizing populations.

Recently, Marchesi et al. reported on investigations of the microbial communities in Cascadia Margin deep sediments (9, 198, 222, and 234 mbsf) in which bacterium- and methanogen-specific PCR primers were used to amplify and clone 16S rRNA gene fragments (37). These authors found that samples above, within, and immediately below the hydrate stability zone yielded bacterial sequences that were primarily affiliated with the *Proteobacteria*, and overall bacterial diversity was relatively low. Methanogen diversity was also low, with all of the

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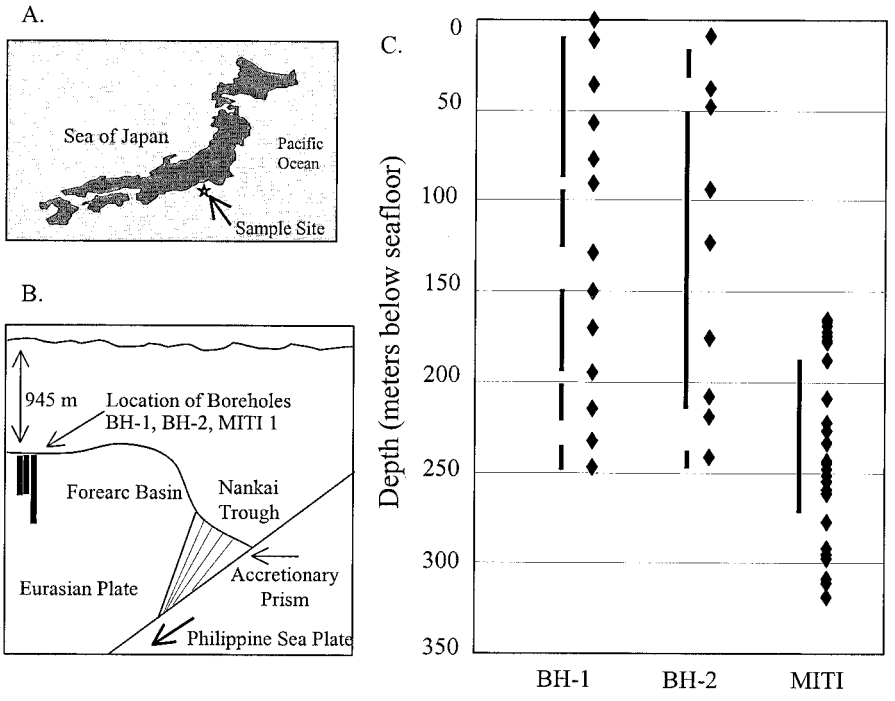

FIG. 1. Description of sampling site (see Materials and Methods). (A) Sampling site located 50 km east of Tokai, Japan. (B) Location of boreholes in the forearc basin of the Nankai Trough. The diagram is not to scale. (C) Sediment depths at which samples were obtained for microbial analyses (♦) and intervals where hydrates were detected (vertical lines). The data for hydrate presence were obtained from reference 38 and Takahashi et al. (Offshore Technol. Conf., 2001).

detected sequences associated with the *Methanosarcinales* and *Methanobacteriales* groups.

In this study, we also examined sediments above, within, and below a deep hydrate-bearing sediment zone cored during exploratory drilling conducted by the Japanese Ministry of International Trade and Industry (MITI) and the Japanese National Oil Corporation in the forearc basin of the Nankai Trough off the east coast of Japan. The forearc basin is landward of the accretionary prism where the Philippine Sea Plate subducts under the Eurasian Plate, and it represents a more tectonically stable environment than an accretionary prism, such as that at the Cascadia Margin. However, gas and fluid flow in the sediments would be expected to be more important in the forearc basin than at a passive margin, such as the Blake Ridge. Our study complements the findings cited above by describing microbial communities from geologically distinct hydrate-bearing sediments and also the application of additional tools for microbial community analysis. Our results indicate the existence of a unique microbial community in deep hydrate-bearing sediment.

MATERIALS AND METHODS

Site description. Marine sediment samples were obtained from three offshore boreholes (BH-1, BH-2, and MITI) located just west of the Nankai Trough (MITI located at 34°12′56.07″N, 137°45′02.63″E), approximately 50 km east of Tokai, Japan (Fig. 1A). Drilling occurred beneath 945 m of water in a flat-top deep-sea terrace; the three boreholes were aligned on a northwest-southeast axis and were separated by approximately 200 m (Fig. 1B).

The sediments consisted largely of clay and clayey silt siliciclastic materials with occasional volcanic ash layers and woody debris. The abundance of sandy layers and turbidite-containing sandstone increased with depth (38). All of the sediments that were sampled came from the Quaternary- to Pliocene-aged Soga and Kakegawa groups, and the deepest samples were estimated to be 1.7 million years old by planktonic foraminiferal dating techniques (26).

The locations of gas hydrates were inferred from seismic surveys and were verified during drilling by the occurrence of high torque exerted on the drilling apparatus while it was coring through the consolidated hydrate-rich formations (10; H. Takahashi, T. Yonezawa, and Y. Takedomi, Offshore Technol. Conf., 2001). The presence of hydrates in the actual retrieved core samples (Fig. 1C) was initially detected by visual monitoring of bubbling gas on the core surface and by observing core temperature depression (−2 to −3°C) due to hydrate dissociation (Takahashi et al., Offshore Technol. Conf., 2001). The methane was determined to be biogenic by carbon isotope analysis, and the hydrate concentration was estimated by examination of the chloride and oxygen concentrations in the extracted pore waters; anomalies in pore water chloride and oxygen concentrations are two of the key indicators of the presence of hydrate in marine sediments (Takahashi et al., Offshore Technol. Conf., 2001). Hydrates in samples taken from BH-1 and BH-2 were estimated to occupy between 3 and 7% of the pore space based on the chloride anomalies and between 5 and 25% of the pore space based on oxygen anomalies (38). At the MITI site most of the samples contained hydrates at levels similar to the levels at the BH-1 and BH-2 sites, although at depths of 236 and 259 mbsf the hydrates were calculated to occupy 80% of the pore space based on the chloride anomaly data (Takahashi et al., Offshore Technol. Conf., 2001).

Sample collection and preparation. Samples were collected from BH-1 and BH-2 between 0.02 and 245 mbsf by using conventional coring technology (Takahashi et al., Offshore Technol. Conf., 2001). MITI borehole samples were collected from between 160 and 320 mbsf. A specialized pressure and temperature core sampler system was deployed between 230 and 309 mbsf to maintain downhole pressure and temperature during sample retrieval and to enhance hydrate preservation (Takahashi et al., Offshore Technol. Conf., 2001). The pressure and temperature core sampler consists of an insulated barrel inside the drill pipe with a ball valve mechanism to maintain downhole pressure and a battery-powered thermo-electric cooler to maintain the temperature at less than 5°C. Only sections of core that appeared to be physically undamaged by handling were collected and used for microbiological analyses.

Onboard ship, retrieved core material was rapidly processed with strict aseptic methods to minimize drilling fluid intrusion and exogenous contamination. Cores were pared to a depth of 5 mm around the circumference and packaged in

doubled sterile sample bags. One portion was immediately frozen on dry ice and stored at −70°C. Another portion was flushed with N_2 gas, sealed in canning jars placed inside cans containing oxygen-scavenging catalyst packets (Gas Pak; BBL Inc., Franklin Lakes, N.J.), and stored at 4°C.

Quality control. To assay the potential for core contamination from drilling fluid or nonnative sediment, fluorescent 1.0-μm-diameter carboxylated latex YG microspheres (Polysciences, Inc., Warrington, Pa.) were added to fluid in the open drill pipe at the surface prior to coring. Coring was timed to ensure that the microspheres arrived at the drill bit as the cores for microbiological studies were being cut. Drilling fluid from freshly cut core was filtered onto 25-mm-diameter black polycarbonate filters (pore size, 0.2 μm), and core solids (3 g) were slurried with 2% formaldehyde prior to deposition on clean preweighed glass slides for epifluorescent microscopy. Control slides consisted of seawater and drilling fluid or of parings and core collected from intervals where microspheres were not deployed. The detection limit for the microspheres was 1 microsphere ml of $liquid^{-1}$ or 200 microspheres g of $sediment^{-1}$, as determined by serial dilution of a known concentration of microspheres in sediment slurry.

Direct microscopic counts. To determine the total numbers of cells in the sediments, triplicate frozen and formaldehyde-fixed subcore samples (2.5 g) were prepared and enumerated by the acridine orange direct count (AODC) method (22).

Biochemical analyses. To obtain information on community structure and to provide an additional indicator of microbial biomass, phospholipid fatty acid (PLFA) and diether lipid (DE) analyses were used to assay frozen (−70°C) core samples from the MITI borehole. Samples (ca. 50 g) were sent frozen overnight to Microbial Insights, Inc. (Rockford, Tenn.). Lipids were extracted and analyzed for PLFA and DE by using methods previously described (32, 56).

Methanogen enrichment. Anaerobic enrichments were prepared to determine whether methanogens could be cultured from the MITI sediments. The basal medium was carbonate-buffered MSH mineral medium, prepared by using 2 parts of MS and 1 part of MH mineral medium (8, 40). Surface layers of individual core samples were removed with sterilized tools, and newly exposed facies were aseptically subcored with sterile plastic syringes from which the needle ends had been removed (23). Three core samples from each sediment zone (above, within, and below the hydrate stability zone) were used for inoculation. Approximately 10 g of subcore was added to each medium tube. The controls included uninoculated tubes, tubes with sediment but no added substrate, tubes containing combusted (550°C for 18 h) or autoclaved core, and tubes inoculated with the known methanogen *Methanoculleus marisnigri* (D. R. Boone, Portland State University). Cultures were incubated at either 10 or 35°C and provided with either sodium acetate (10 mM) or hydrogen (1 atm). The tubes were maintained in canning jars sealed under N_2. The headspace was monitored for methane production by gas chromatography with atomic emission detection. The quantification limit was 5 ppm. Methane production rate data were analyzed with the statistical software package SYSTAT 10 (SPSS Science, Chicago, Ill.).

DNA isolation, amplification, and cloning. Genomic DNA was extracted from duplicate 0.5- to 0.7-g samples of frozen (−70°C) core from one interval within each zone by using an UltraClean soil DNA kit (MoBio, Solana Beach, Calif.). DNA was eluted in 10 mM Tris (pH 8.5). A negative control extraction (where no sediment was added to the extraction reagents) was also performed and carried through the entire PCR, cloning, and sequencing process. Archaeal 16S ribosomal DNA (rDNA) was amplified with primers 21Fa (5′TTCCGGTTGA TCCYGCCGGA) and 958Ra (5′YCCGGCGTTGAMTCCAATT) (15). Archaeal 16S rDNA PCR mixtures (25 μl) contained each deoxynucleoside triphosphate (dNTP) at a concentration of 200 μM, each primer at a concentration of 1 μM, 1.25 U of SureStart polymerase (Stratagene, La Jolla, Calif.), and 1 μl of DNA extract. The amplification reaction was activated by heating at 95°C for 10 min, and then thermal cycling was performed by using 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, followed by a final elongation step of 72°C for 15 min.

Bacterial 16S rDNA was amplified with primers 8F (5′AGAGTTTGATCCT GGCTCAG) and 1492R (5′GGTTACCTTGTTACGACTT). The bacterial 16S rDNA PCR mixtures (25 μl) contained each dNTP at a concentration of 200 μM, 0.8 μM primer, 1.25 U of AmpliTaq DNA polymerase LD (Applied Biosystems, Foster City, Calif.), 0.4 mg of bovine serum albumin per ml, and 2 μl of template. The dNTPs and bovine serum albumin were obtained from Roche Molecular Biochemicals (Indianapolis, Ind.). The thermal cycler program included a hot start at 94°C for 5 min, 33 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, and a final elongation step of 72°C for 15 min. The PCR products were observed with UV illumination following electrophoresis through 1% agarose and staining with ethidium bromide.

For each sediment extract and primer set, four independent PCR products were combined, and the PCR products were purified by agarose gel electrophoresis and gel extraction (Q1Aquick kit; Qiagen, Valencia, Calif.). Products from the negative control extract PCR amplifications were also purified; negative control gel fragments at distances corresponding to the migration distances for expected products were cut and processed like the sediment sample products. Individual archaeal and bacterial clone libraries for each sediment depth (a total of six sample libraries) and the negative controls were constructed from amplified DNA by using the pCR4-TOPO vector and TA cloning (Invitrogen, Carlsbad, Calif.). Recombinant plasmids were recovered in chemically competent *Escherichia coli* TOP10 cells following transformation and selection on Luria-Bertani agar with kanamycin (50 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (40 μg/ml).

RFLP analysis. To identify unique 16S rDNA, at least 50 bacterial and 50 archaeal clones from each sample depth were screened by restriction fragment length polymorphism (RFLP) analysis. Insert DNA was amplified in reaction mixtures similar to those described above by using 0.4 μM primer M13 Forward(−20) and 0.4 μM primer M13 Reverse. The thermal cycler conditions were as follows: hot start at 94°C for 4 min, 33 to 35 cycles of 94°C for 20 s, 53°C for 20 s, and 72°C for 75 to 105 s, and a final extension at 72°C for 7 to 10 min. The longer times were used for the longer bacterial DNA inserts. Amplified insert (15 μl) was digested with 0.4 U of restriction enzyme *Hin*PI (5′G/CGC) and 0.4 U of restriction enzyme *Msp*I (5′C/CGG) (New England Biolabs, Beverly, Mass.) with 0.1% Triton X-100 for 4.5 h at 37°C. Digested DNA was separated with 3% MetaPhor agarose (BioWhittaker Molecular Applications, Rockland, Maine) by using Tris-borate-EDTA buffer. Unique restriction patterns were identified, and representative clones from each library were selected for sequencing.

Sequence and phylogenetic analysis. Plasmids were purified with a QIAprep Spin Miniprep kit (Qiagen) and were sequenced by using BigDye terminator chemistry with an automated capillary sequencer (Applied Biosystems, Foster City, Calif.). Sequencing was carried out at the Idaho National Engineering and Environmental Laboratory or by Amplicon Express (Pullman, Wash.). Inserts were sequenced multiple times on each strand by using plasmid primers T3, T7, M13 Forward(−20), and M13 Reverse and additional primers as follows. For the archaeal sequences primers 514Fa (5′GGTGBCAGCCGCCGCGGTAA) and 514Ra (5′TTACCGCGGCGGCTGVCACC) were designed and used for sequencing. For the bacterial sequences, the following primers were used: 1100R (5′AGGGTTGCGCTCGTTG), 519R (20), 515F, and 906F (43).

Electropherograms were edited by using the Chromas freeware (version 1.45; School of Health Science, Griffith University, Gold Coast Campus, Southport, Queensland, Australia), and sequences were assembled, aligned, and analyzed with the DNAStar Lasergene software package (DNAStar, Madison, Wis.). Sequences were checked with Chimera Check from the Ribosomal Database Project II (RDP) (36). Closest relatives were initially identified from 16S rDNA sequences aligned against known sequences in the GenBank database by using the gapped BLAST tool and the RDP databases (1, 5, 54). Alignments against known taxonomic sequences obtained from the RDP were performed by using the BioEdit sequence alignment editor freeware (version 5.0.9; Department of Microbiology, North Carolina State University, Raleigh), and sequences were manually corrected by using the MacClade software (version 3.0; Sinauer Associates, Inc., Sunderland, Mass.) to ensure that only homologous nucleotides were compared between sequences.

The closest cultured relative was determined by the maximum-likelihood method by using the dnadist program in PHYLIP 3.5c (19). The edited alignments were evaluated with maximum-parsimony, maximum-likelihood, and distance methods by utilizing the PAUP package (version 4.0b10; Sinauer Associates, Inc.). Trees generated with all three methods were congruent, with only minor rearrangements in branching order. Phylogenetic inference and evolutionary distance calculations were made by using the distance Jukes-Cantor model (gamma parameter equal to 2.0). Bootstrap analysis (1,000 replicates) was used to obtain confidence estimates for the phylogenetic trees.

Nucleotide sequence accession numbers. The 16S rDNA sequences obtained have been deposited in the GenBank database under accession numbers AY093446 to AY093483 (see Fig. 3 and 4).

RESULTS

Sample integrity. To estimate the extent of drilling fluid intrusion into the core, fluorescent microspheres were counted in the outer layers of cores (which were pared away during processing), in the inner core material, and in drilling fluid in the pressure and temperature core sampler. The parings and

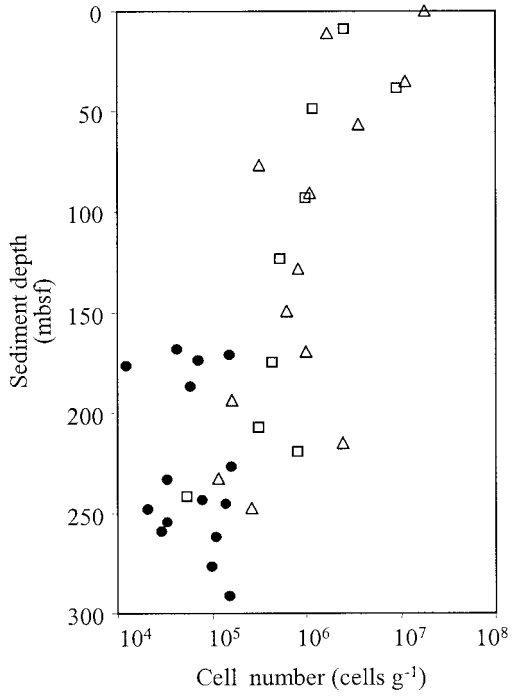

FIG. 2. Distribution of microorganisms in Nankai Trough sediment with respect to depth below the seafloor. The number of cells was determined by AODC. Symbols: △, borehole BH-1; □, borehole BH-2; ●, borehole MITI.

fluids contained averages of 60 spheres g^{-1} and 50 spheres ml^{-1}, respectively, while microspheres were not detected in any internal core material. This suggests that fluid intrusion during coring and contamination during processing were minimal. These results support the deployment of fluorescent tracers as an effective means of quality assurance for core collection in deep submarine sediment (47).

Quantitation of biomass by AODC and membrane lipid analyses. Total counts of microbial cells in the Nankai Trough sediment cores from the BH-1, BH-2, and MITI boreholes were determined by AODC. The data indicate that the numbers of cells decreased with sediment depth (Fig. 2), from approximately 10^7 cells g of $sediment^{-1}$ near the seafloor to between 10^4 and 10^5 cells g^{-1} at 300 mbsf.

Intact PLFA were detected in all of the MITI sediments examined (Table 1), suggesting that a viable microbial population was present throughout the cored interval (55, 56). The concentrations of DE were generally 1 order of magnitude greater than the PLFA concentrations, suggesting that archaea were prevalent in these sediments. However, because DE are more stable in sediments than PLFA due to the ether linkages (16), it is important to note that DE are not as strong an indicator of viable biomass as PLFA. For four samples from within the hydrate zone and two samples from below the hydrate zone, more detailed information on PLFA composition was obtained (Table 2). The majority (57 to 72%) of the PLFA from each sample consisted of normal saturated fatty acids containing 14 to 18 saturated carbon atoms. These fatty acids are associated with both bacterial and eukaryal organisms and thus are not particularly useful for community profiling. However, other fatty acid structures that were detected allowed postulation of specific members of the sediment community (56). The mid-chain-branched saturated acids, present at levels of 6 to 13% of the PLFA in the samples, are typical of the *Actinomycetes* and sulfate-reducing bacteria. Monoenoic fatty acids, indicating gram-negative bacteria, were present in all of the samples at levels ranging from 6 to 9%. Terminally branched saturated acids were present at low levels (1 to 4%) in all of the samples, suggesting the presence of gram-positive bacteria. No PLFA characteristic of anaerobic metal reducers (branched monoenoic fatty acids) or eukaryotes (polyenoic fatty acids) were detected. The PLFA concentrations in the MITI sediments showed no apparent correlation with depth, and all values were between 12 and 36 pmol g^{-1}. DE appeared to be less uniformly distributed throughout the strata; in two samples from within the hydrate zone (209 and 227 m) no DE were detected, although other samples from that zone contained very high concentrations (241 to 700 pmol g^{-1}).

TABLE 1. Concentrations of PLFA and DE in samples obtained from the MITI borehole sediments

Location[a]	Sampling depth (mbsf)	PLFA concn (pmol g^{-1} [dry wt])	DE concn (pmol g^{-1} [dry wt])
Above hydrate zone	166.1	22	158
	176.7	24	183
Within hydrate zone	208.9	28	ND[b]
	222.8	34	296
	226.9	35	ND
	243.3	36	700
	245.3	12	241
	251.9	20	276
Below hydrate zone	291.4	19	306
	294.8	30	629
	297.6	14	180
	309.0	17	197

[a] Relative to the sediment zone where hydrates were detected (192 to 267 mbsf).
[b] ND, not detected.

Detection of methanogenic microorganisms. Methane was produced in all of the inoculated microcosms, regardless of the substrate conditions or the incubation temperature (Table 3). Controls without sediment or with autoclaved or combusted sediment had negligible rates of methane production. Controls without added substrate produced low levels of methane; this production may have represented slow release of methane trapped in the sediment that was mobilized by disturbance or biological activity using endogenous substrates.

The enrichments inoculated with sediment from 251.9 mbsf, within the hydrate zone, consistently produced the most methane. However, analysis of the methane production rates for all samples by a three-factor analysis of variance showed no significant effect attributable to where the sample came from with respect to zone, as defined by hydrate presence ($P = 0.508$), nor was there a significant effect of the substrate across the zones ($P = 0.145$). Temperature did have a significant effect ($P < 0.0005$), and enrichments incubated at 35°C consistently produced more methane than enrichments incubated at 10°C, regardless of the substrate or the origin of the inoculum.

TABLE 2. PLFA compositions of samples from the MITI borehole

Sample depth (mbsf)	% of total PLFA						
	Terminally branched saturated	Monoenoic	Branched monoenoic	Mid-chain-branched saturated	Normal saturated	Other	Polyenoic
208.9	3.6	7.6	0.0	12.5	56.9	19.5	0.0
222.8	2.5	6.6	0.0	8.3	68.4	14.1	0.0
226.9	2.3	8.5	0.0	6.9	67.8	14.5	0.0
243.3	1.4	5.6	0.0	5.6	71.1	16.3	0.0
291.4	2.5	6.9	0.0	9.1	65.5	16.0	0.0
294.8	1.3	5.5	0.0	6.4	71.5	15.4	0.0

16S rDNA from the Nankai Trough. The low AODC numbers were reflected in the quantities of DNA extracted from the sediments. Five-microliter aliquots of the DNA extracts (total volume, 50 μl) could not be visualized with ethidium bromide staining, suggesting that the total amount of extracted DNA was in the low nanogram range (<1 ng μl^{-1}). Nevertheless, archaeal and bacterial 16S rRNA gene fragments were successfully amplified from the extracts. Because contaminating exogenous DNA is of particular concern when 16S rDNA is amplified from low-biomass samples, meticulous procedures were used for acquisition and analysis of sequence data. Stringent anticontamination controls (e.g., PCR-dedicated pipettors, barrier tips, UV irradiation of all surfaces) were utilized, and products from the negative controls (no template added) were cloned and sequenced. In addition, for the bacterial 16S rDNA PCR, AmpliTaq DNA polymerase LD (low DNA) was used.

Following PCR, no visible product was observed in the archaeal negative control after ethidium bromide staining, but a very faint band was seen in the bacterial negative control. Negative control visible and invisible products were cloned, and transformants that putatively contained 16S rDNA were obtained at levels that were about 1/10 or less the number of the sediment clone library colonies. Several of the clones from sediment archaeal and bacterial libraries were eliminated after comparison to the negative control RFLP and sequence analysis data. Although some of the sediment clones may have been prematurely removed prior to a comprehensive gene sequence analysis, we believe that this conservative approach helped ensure that the sequences which we report here are genuine representatives of microorganisms present in the Nankai Trough sediments.

Of the more than 300 clones analyzed, 246 were determined to contain amplified 16S rDNA representing prokaryotes from the Nankai Trough forearc basin sediment (Table 4). The remaining clones were removed from the analysis because a clone was determined to be a contaminant, the insert did not amplify or produce DNA fragments of the anticipated size (1 kb for archaea and 1.5 kb for bacteria), or the plasmid insert did not yield a reliable DNA sequence.

Archaeal domain sequence diversity. More than 40% of the archaeal clones were most closely related to the cultured organism *Desulfurococcus mobilis* or the cultured organism *Pyrodictium occultum* (Table 4). However, phylogenetic analyses indicated that these two groups (NT-A3 and NT-A4) formed unique clusters with environmental clones, such as JTA173 isolated from the Japan Trench seafloor (34), pSL123 obtained from Yellowstone's Obsidian Pool (4), and Arc clones isolated from 188 m below the ground surface in south-central Washington (11) (Fig. 3).

The cultured organism most closely related to the clones represented by MA-A1-3 from above the hydrate zone was *Methanothermobacter thermophilus*, a member of the *Euryarchaeota*; however, these clones were more closely related to clones originally classified as members of the *Crenarchaeota*, marine benthic group B (50). The marine benthic group B clones have been recently reclassified in the RDP with the *Euryarchaeota* thermophilic methanogens. Our analysis clus-

TABLE 3. Methane production rates in enrichments from MITI borehole sediments after 4 weeks, with acetate or hydrogen as the primary substrate[a]

Location[b]	Depth (mbsf)	Methane production (ppm g^{-1} day^{-1}) at 10°C		Methane production (ppm g^{-1} day^{-1}) at 35°C	
		Acetate	Hydrogen	Acetate	Hydrogen
Above hydrate zone	165.7	0.31	0.22	1.09	0.83
	165.8	0.33	0.27	1.53	1.19
	174.2	0.19	0.19	1.02	0.70
Within hydrate zone	245.3	0.43	0.33	1.86	1.37
	248.5	0.41	0.19	1.16	0.92
	251.9	0.60	0.50	3.80	2.39
Below hydrate zone	297.6	0.31	0.14	0.91	0.66
	310.4	0.25	0.16	1.18	0.79
	318.7	0.25	0.19	0.76	0.61

[a] Each value represents a single sample. The values were corrected for the methane that was observed in the corresponding controls with no added substrate.
[b] Relative to the sediment zone where hydrates were detected (192 to 267 mbsf).

TABLE 4. Closest cultured relatives of representative clones from the archaeal and bacterial 16S rRNA gene libraries

Type sequence from hydrate sediment[a]			No. of clones in library[b]	Closest cultured relative
Above HZ	Within HZ	Below HZ		
42 clones	35 clones	49 clones	126	*Archaea*
		MA-C1-2	31	*Thermococcus celer*
		MA-C1-4	3	*Thermococcus celer*
		MA-C1-50	1	*Thermococcus celer*
MA-A1-3			36	*Methanothermobacter thermophilus*
MA-A1-1			6	*Desulfurococcus mobilis*
	MA-B1-3		8	*Desulfurococcus mobilis*
	MA-B1-5		27	*Desulfurococcus mobilis*
		MA-C1-3	4	*Desulfurococcus mobilis*
		MA-C1-5	10	*Pyrodictium occultum*
37 clones	50 clones	34 clones	121	*Bacteria*
	MB-B2-105		2	Unknown
MB-A2-107			4	*Prevotella pallens*
MB-A2-118			1	*Prevotella tannerae*
		MB-C2-105	1	*Pirellula* sp.
		MB-C2-147	2	*Pirellula* sp.
		MB-C2-128	1	*Duganella zoogloeoides*
MB-A2-115			1	*Kingella denitrificans*
MB-A2-102			2	*Curacaobacter baltica*
MB-A2-149			1	*Idiomarina loihiensis*
MB-A2-106			4	*Pseudomonas fluorescens*
MB-A2-137			1	*Pelobacter acetylenicus*
	MB-B2-106		9	*Pelobacter acetylenicus*
		MB-C2-152	1	*Pelobacter acetylenicus*
MB-A2-101			2	*Dehalococcoides ethenogenes*
		MB-C2-127	4	*Dehalococcoides ethenogenes*
MB-A2-103			2	*Dehalococcoides ethenogenes*
		MB-C2-126	1	*Dehalococcoides ethenogenes*
MB-A2-110			1	*Dehalococcoides ethenogenes*
	MB-B2-113		2	*Dehalococcoides ethenogenes*
MB-A2-100			5	*Acidimicrobium ferrooxidans*
MB-A2-108			4	*Frankia* "*myricanod*"
	MB-B2-107		23	*Streptomyces thermodiastaticus*
		MB-C2-120	16	*Streptomyces thermodiastaticus*
MB-A2-104			6	*Moorella glycerini*
	MB-B2-103		11	*Moorella glycerini*
		MB-C2-106	1	*Moorella glycerini*
MB-A2-105			3	*Moorella glycerini*
	MB-B2-116		3	*Moorella glycerini*
		MB-C2-103	7	*Moorella glycerini*

[a] Type sequences are sequences from representative clones having unique RFLP patterns. Above HZ, above hydrate stability zone at 165.5 mbsf; within HZ, within the hydrate stability zone at 248.4 mbsf; below HZ, below the hydrate stability zone at 297.6 mbsf.
[b] Number of clones represented by sequence data.

tered the NT-A2 clones distant from any cultured archaea; however, high bootstrap values (90%) suggested that the NT-A2 group was in the *Euryarchaeota* domain.

Archaeal amplification of DNA extracted from sediment obtained from below the hydrate zone consistently resulted in two PCR products (1 and 1.2 kb). The larger fragment (MA-C1-2) had a 138-bp DNA insert. Considering that MA-C1-2 was >96% similar to clones MA-C1-4 and MA-C1-50, it was initially posited that the sequence might be a chimera or heteroduplex (48). However, independent efforts (D. Cummings, unpublished results) to generate a library from methanogenic enrichments of sediment from a similar core depth yielded the same sequence as MA-C1-2. The NT-A1 clones, evidently members of the *Euryarchaeota*, form a unique clade that groups closely with clone SAGMA-R, isolated from fissure water in a deep South African gold mine (49). SAGMA-R has been assigned to a novel phylogenetic group lacking any cultured representatives and is believed to contain rRNA intron-like intervening sequences, which suggests that the MA-C1-2 sequence may be similarly constructed.

Bacterial domain sequence diversity. From the bacterial clone libraries, 22 unique 16S rDNA sequences primarily representing five phylogenetic phyla and at least three unique clades without any cultured representatives were acquired (Fig. 4). The clone sequences most closely related to cultured organisms were in the *Bacteroidetes* and the β- and γ-*Proteobacteria* phyla. Clones from above the hydrate zone represented most of the diversity across the known phyla. Phylogenetic clusters lacking cultured closest relatives (NT-B2, NT-B5, and NT-B6) dominated the bacterial sequences, particularly in sediments obtained from within and below the hydrate zone (Fig. 5).

Similar to the archaeal clones, almost 90% of the bacterial clones formed unique clusters or were more closely related to environmental clones than they were to cultured organism

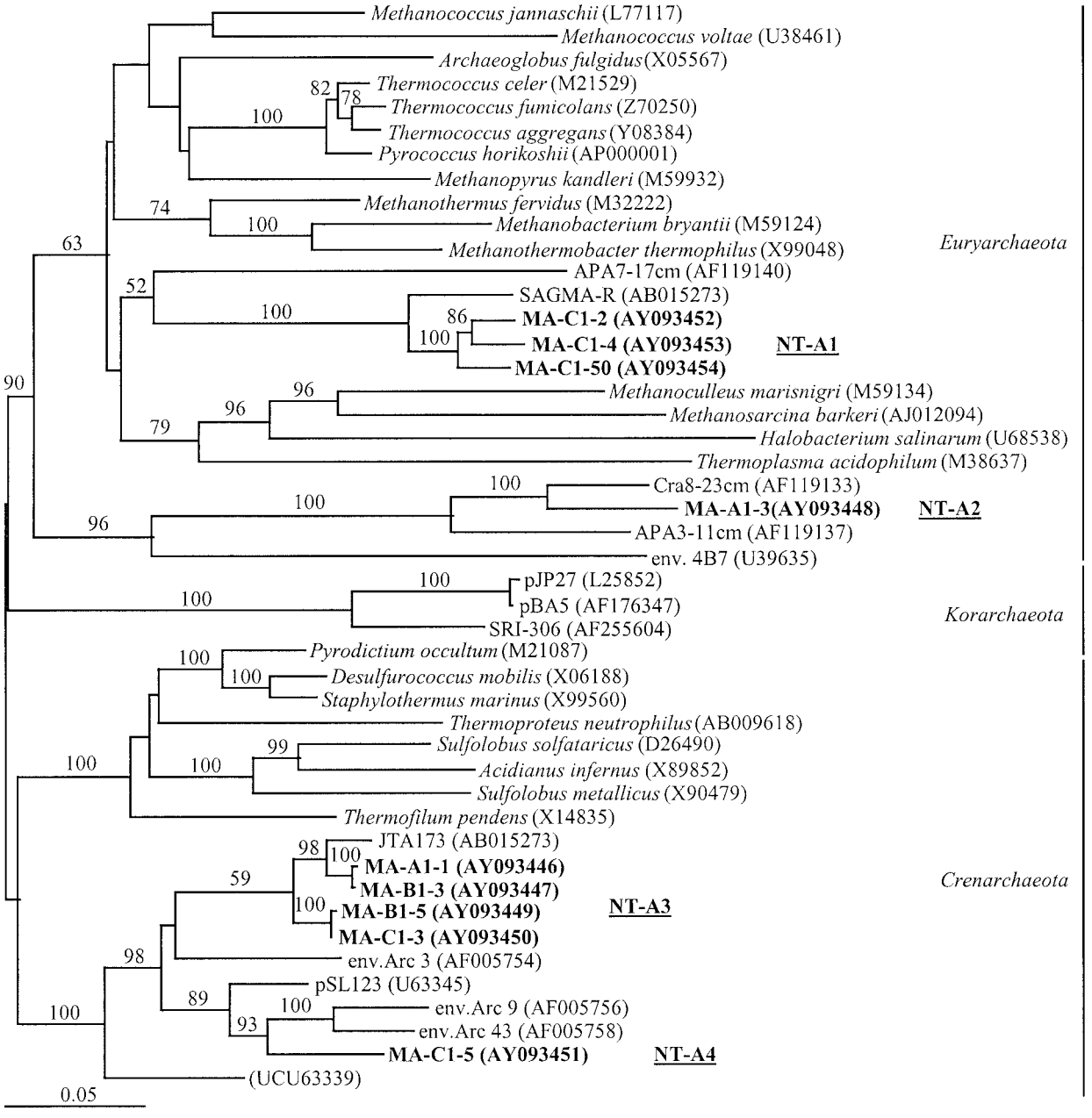

FIG. 3. Phylogenetic relationships of archaeal 16S rRNA sequences as determined by distance Jukes-Cantor analysis. The values at the nodes are bootstrap probabilities (percentages); only values greater than 50% are shown. Scale bar = 0.05 nucleotide substitution per site. The sequences from the Nankai Trough are indicated by boldface type, and unique clades lacking cultured organisms are indicated by boldface underlined type. The remaining sequences were obtained from the RDP or were obtained from GenBank and aligned with sequences from the RDP. Phyla were determined by using the classification in *Bergey's Manual of Systematic Bacteriology* (21). MA-A1 archaeal amplified sequences were from above the hydrate zone; MA-B1 archaeal amplified sequences were from within the hydrate stability zone; and MA-C1 archaeal amplified sequences were from below the hydrate zone. GenBank accession numbers are in parentheses.

sequences. Three unique clusters, NT-B9, NT-B10, and NT-B11, formed lineages within the *Proteobacteria*; however, the closest relatives were the environmental clones BPC076 (from a hydrocarbon seep), wr0798 (from a rape oilseed rhizosphere), and ZD0410 (from a sea algal bloom), respectively. Similarly, cluster NT-B1 was linked to the *Planctomycetes* phylum but greatly diverged from its closest cultured relative.

Clones representing about 40% of the bacterial library were most closely related to cultured organisms belonging to the *Actinobacteria* phylum. Clones MB-A2-100 and MB-A2-108 formed unique phylogenetic lineages (NT-B7 and NT-B8) within the *Actinobacteria*. However, phylogenetic analysis of the clones in the NT-B6 group indicated that they formed a unique cluster with clone 4-42 (obtained from coal tar waste-contaminated groundwater) and that this group was related to the novel clade consisting of the NT-B5 clones and environ-

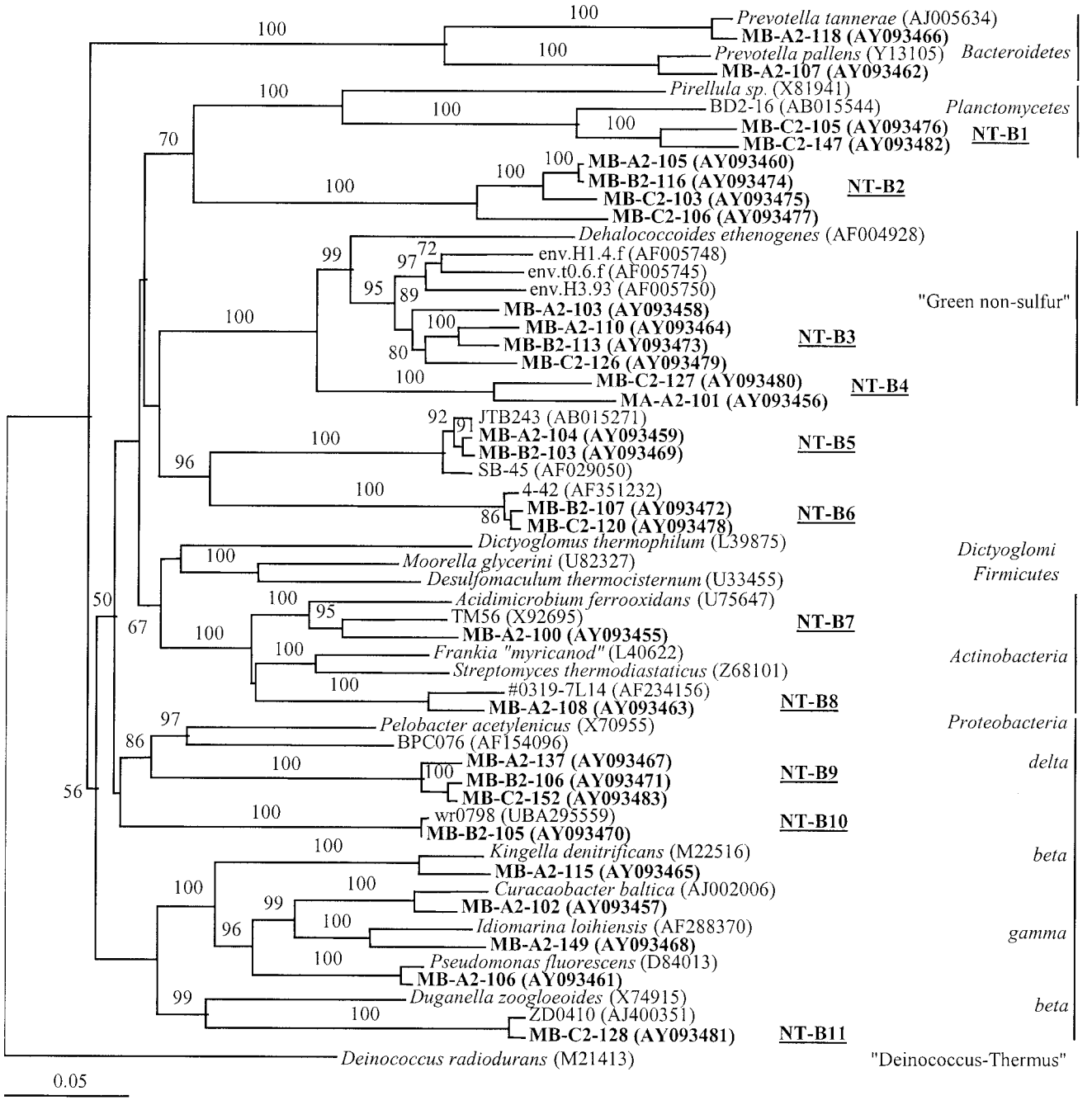

FIG. 4. Phylogenetic relationships of bacterial 16S rRNA sequences as determined by distance Jukes-Cantor analysis. The values at the nodes are bootstrap probabilities (percentages); only values greater than 50% are shown. Scale bar = 0.05 nucleotide substitution per site. Sequences from the Nankai Trough are indicated by boldface type, and unique or novel clades lacking cultured organisms are indicated by boldface underlined type. The remaining sequences were obtained from the RDP or were obtained from GenBank and aligned with sequences from the RDP. Phyla were determined by using the classification in *Bergey's Manual of Systematic Bacteriology* (21), except for the green nonsulfur bacteria (see text). MB-A2 bacterial amplified sequences were from above the hydrate zone; MB-B2 bacterial amplified sequences were from within the hydrate stability zone; and MB-C2 bacterial amplified sequences were from below the hydrate zone. GenBank accession numbers are in parentheses.

mental clones JTB243 (from the Japan Trench seafloor) and SB-45 (part of a sulfate-reducing consortium). The analysis also showed that the NT-B2 group was a novel clade, although it exhibited a distant relationship with the *Planctomycetes*.

Ten percent of the clones, some from each sediment depth, clustered near the obligately dehalorespiring organism *Dehalococcoides ethenogenes* (39). The NT-B3 group was most closely associated with clones T0.6.f, H1.4.f, and H3.93 (obtained from the terrestrial deep subsurface) and *D. ethenogenes* (11). Likewise, *D. ethenogenes* was the cultured organism most closely related to the clones in novel group NT-B4; however, NT-B4 clustered discretely from the clones in the NT-B3

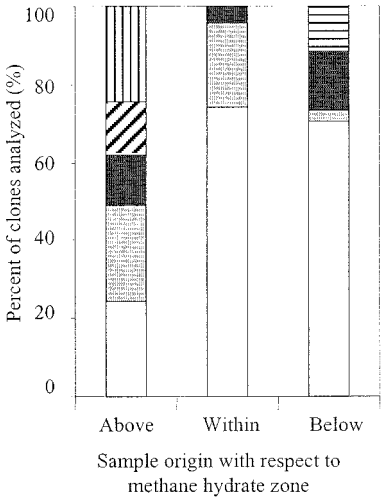

FIG. 5. Phylogenetic groups of bacterial clones with respect to sample origin in Nankai Trough sediment. Open bars, unclassified phyla; light shaded bars, *Proteobacteria*; dark shaded bars, green nonsulfur bacteria; bars with diagonal lines, *Bacteroidetes*; bars with vertical lines, *Actinobacteria*; bars with horizontal lines, *Planctomycetes*.

group. *D. ethenogenes* has an S-layer cell wall structure similar to that of the archaea and is located deep in the green nonsulfur lineage of bacteria (25).

DISCUSSION

The presence of biogenic methane in marine gas hydrate deposits suggests that microbiological activities are important for methane hydrate formation (13, 24, 53). However, information regarding microbial biomass and community distribution and activity associated with hydrate-bearing sediments is limited. This work focused on characterizing the indigenous microbial communities in hydrate-bearing deep marine sediments by combining culture-based and biochemical studies with molecular 16S rDNA analysis.

Microbial population data. The decrease in AODC with depth that we observed is consistent with the trend reported for marine sediments from around the world (42). However, at the Nankai Trough, the absolute cell numbers were slightly lower than the numbers at most other marine sites that have been studied, throughout the depths analyzed. For a 0.02-mbsf sample, we obtained an AODC value of 1.8×10^7 cells g^{-1}; typical values for seafloor sediments are on the order of 10^8 to 10^9 cells g^{-1} (42). By using a regression of AODC versus depth calculated with data from a number of different Ocean Drilling Program sites (42), a value of 3.8×10^5 cells g^{-1} is predicted for 250 mbsf. Our measured values at depths around this depth ranged from 2.1×10^4 to 2.6×10^5 cells g^{-1}. Lower cell numbers in the Nankai Trough than in other marine settings may reflect the low levels of total organic carbon (TOC). There is a significant correlation between TOC level and bacterial population for sediments containing 0 to 3% organic carbon (42). The average TOC contents in the Nankai Trough are approximately 0.4 to 0.5% (A. Waseda, 2000 Western Pacific Geophys. Meet., 2000). In contrast, the average TOC contents are estimated to be 0.8 to 1.4% for sediments deeper than 155 m at the Blake Ridge (the hydrate zone is between 190 and 445 m) (17) and 1.5% for the Cascadia Margin (52). If lower TOC contents are indicative of lower concentrations of available substrates for microorganisms, the lower TOC content could explain the lower cell densities.

At the Cascadia Margin the bacterial populations increased markedly at the base of the narrow (<25-m-thick) hydrate zone, with values up to 1 order of magnitude greater than the values at the top of the hydrate zone (13). At the Nankai Trough, the presumed hydrate zones are significantly thicker and not uniform, and we did not perceive such changes in the total microbial numbers. More discrete sampling would be necessary to determine whether microbial populations at the Nankai Trough follow the trend noted at the Cascadia Margin.

Lipid biomarker analyses. No apparent differences in lipid composition or abundance were noted between the defined sediment zones, but three major features were common to all of the lipid analyses: the relatively high concentrations of PLFA compared to previously published data for deep subsurface terrestrial sediments (PLFA data for other deep subsurface marine environments have not been reported); the high proportion of normal saturated PLFA (in all cases >57%); and the predominance of DE over PLFA. In general, PLFA concentrations in MITI sediments did not vary much with depth and were higher than the values reported for other deep subsurface sites. In samples obtained from deep boreholes in Idaho (69 to 183 m below the land surface [mbls]), South Carolina (34 to 300 mbls), and Washington (69 to 183 mbls), the PLFA concentrations ranged from approximately 1 to 16 pmol g of sediment^{-1} (56). Similar values were reported for a deep borehole in New Mexico (down to 300 mbls) (44). The intact PLFA concentrations in the MITI sediments were generally 1.5 to 2 times greater than these concentrations, supporting the recent proposition that subseafloor sediments comprise an active deep bacterial biosphere (53).

The high proportion of normal saturated PLFA is an indicator of simple populations (G. Davis, Microbial Insights, personal communication). Normal saturated PLFA are found in both bacteria and eukaryotes, but the absence of detectable polyenoic fatty acids suggests that eukaryotes are not important members of the sediment communities. The unique environmental conditions in these sediments (i.e., largely refractory and limited organic carbon, high methane concentrations, and high pressures) may restrict the types of organisms that can survive there.

The high concentrations of DE relative to the concentrations of PLFA were unusual, and this degree of predominance of DE over PLFA has not been reported for other environmental samples (D. C. White, personal communication). As noted above, however, DE are more apt to be preserved in the environment than PLFA, so it is uncertain whether viable archaea were predominant over viable bacteria. Nevertheless, the presence of methane production in enrichments from the Nankai Trough sediment samples provides evidence that at least some of the biomass represented by the DE was viable. However, archaeal distribution was apparently heterogeneous within the sediments, as no DE at all were detected in two samples from within the hydrate zone, whereas other samples from that zone contained high concentrations of DE.

DNA recovery and authentication. Cores were maintained under anoxic conditions prior to freezing and were frozen prior to DNA extraction to help ensure that the intact cores contained a representative microbial population (45). When examining sediments with cell numbers comparable to the cell numbers in our samples, Chandler et al. estimated that the extracted DNA concentrations were at low picogram levels (12). If this is indeed the case, it was not surprising that the concentration of extracted chromosomal DNA from the MITI sediment was low (estimated concentration, <10 ng g of sediment^{-1}). Nevertheless, 16S rDNA sequences were successfully amplified from each sediment extract. It is interesting, however, that amplification of bacterial 16S rDNA required addition of more template than archaeal amplification required, a finding which supports the dominance of archaeal cells inferred from the lipid biomarker data. The diversity of archaeal and bacterial sequences in the clone libraries suggests that the DNA extraction process was adequate to assess a wide range of cell types.

Because of the high potential for contaminant DNA originating in the laboratory to coamplify with our target environmental sequences in the low-biomass samples, we carried negative controls through the DNA extraction process, PCR amplification, cloning, and sequencing. Although cloning of invisible bands from sample preparations has been described (12), cloning of negative controls is not commonly reported. Several sequences were removed from this analysis after we noted their presence in negative control clone libraries. Based on our experience, we suggest that investigators seeking to amplify minute quantities of environmental DNA should routinely carry negative controls through their DNA extraction, cloning, and sequencing protocol to help authenticate environmental sequences.

Prokaryotic diversity in Nankai Trough sediments. We compared 16S rDNA sequences from only one depth in each of the three operationally defined zones, so it is difficult to say how well each sample represented the zone from which it originated. However, we can say that there did not seem to be marked differences in sequence diversity among the clone libraries except for the bacterial library from above the hydrate zone, where the majority of the clone sequences were closely related to cultured organisms and the overall diversity was at least twofold greater. Nonetheless, it is interesting to compare several features of the 16S rDNA data with other data collected in this study and with previously reported findings related to marine sediments bearing methane hydrates.

In our study none of the archaeal clones and less than 10% of the bacterial clones identified were >95% similar to cultured organisms. In contrast, at the Cascadia Margin most of the bacterial clone sequences were >95% similar to cultured organisms (37). In a similar study, 72% of the sequences recovered from Gulf of Mexico seafloor hydrates were >95% similar to cultured organisms (31). Finding novel sequences is likely a consequence of examining a relatively unknown natural environment (2, 41).

Clones forming unique phylogenetic groups were recovered in the *Euryarchaeota* and *Crenarchaeota* phyla in the archaeal domain. Other workers who have examined marine sediments associated with methane hydrates have also reported limited diversity among archaea (24, 31). Twenty-one unique methanogen clones related to the *Methanobacteriales* and *Methanosarcinales* were found above, within, and below the hydrate zone in the Cascadia Margin sediment (37). In our samples only one unique ribotype (MA-A1-3) related to a cultured methanogen was identified; however, the phylogenetic position of this sequence was more tenuous. However, in the enrichment studies methane was produced in cultures from all three sediment zones, and there was no statistically significant difference between zones with respect to the methane production rate. Moreover, a *Methanoculleus marisnigri* strain (Nankai-1) has been isolated from sediment in the hydrate zone (J. Mikucki, unpublished results). The discrepancies in results between the culture-based and non-culture-based analyses likely reflect the biases associated with each of these general approaches (2, 51).

The bacterial sequences in our study included unique ribotypes representing the major phyla *Actinobacteria*, *Bacteroidetes*, *Planctomycetes*, and *Proteobacteria*, as well as the green nonsulfur group. Several of the clones seemed to group in novel lineages within the known phyla often associated most closely only with previously identified environmental clones. We also detected at least three major unique clades (NT-B2, NT-B5, and NT-B6) not currently classified in known phyla. Our data represent more bacterial diversity than the diversity reported for the Cascadia Margin, where all of the bacterial phylotypes were in the *Proteobacteria* and *Flexibacter-Cytophaga-Bacteroides* groups (37).

Our results were similar to those from a study of Gulf of Mexico seafloor hydrates (31) in that we found that the clones related to the *Actinobacteria* were an important population. Although the clones obtained from within and below the hydrate zone (in group NT-B6) were most closely related to the cultured organism *Streptomyces thermodiastaticus*, these clones were clustered, following phylogenetic analysis, in a unique clade distinct from the *Actinobacteria*. Only sequences from above the hydrate zone grouped closely with the *Actinobacteria*. However, PLFA results indicated that mid-chain-branched saturated phospholipids, typical of *Actinomycetes*, were a significant component (6 to 13%) of the phospholipids within and below the hydrate zone. Likewise, terminally branched saturated phospholipids, indicative of gram-positive microorganisms, were also detected, although the levels were lower (1 to 4%).

PLFA data suggested that gram-negative bacteria were present, and sequence analyses confirmed their presence. Although representative *Proteobacteria* clones comprised less than one-fifth of the total bacterial clones, they were comparatively the most diverse of the bacterial phylogenetic groups, and the greatest diversity was exhibited in the library from the sample obtained above the hydrate zone.

One of our most interesting findings was the indication that growth occurred at temperatures higher than the in situ temperatures (approximately 4 to 12°C at the depths cored at the MITI hole). Methane production was enhanced in enrichments incubated at 35°C compared to enrichments incubated at 10°C. We also found that thermophiles and hyperthermophiles were the closest cultured relatives of many (>60%) of the clones in our 16S rDNA libraries. However, phylogenetic analyses classified these clones in novel clades devoid of cultured representatives (groups NT-B2, NT-B5, NT-A1, NT-A2,

NT-A3, and NT-A4). Previous studies of microorganisms associated with gas hydrates have not indicated such a marked presence of sequences having thermophiles as the closest cultured relatives. Analysis of seafloor hydrates in the Gulf of Mexico resulted in a few clones associated with *Thermus aquaticus* (31), but 16S rDNA sequences from the shallow and deep hydrate sediments obtained from the Cascadia Margin did not exhibit a pronounced relationship to thermophiles (6, 37). Inferences from uncultured phylotypes must of course be viewed with caution. However, an understanding of the relatedness of intact 16S rDNA sequences to cultured microorganisms is a step towards interpreting the physiological capabilities of the indigenous cells in an environment and inferring the possible origin of the cells (25, 41).

When the origin of the microorganisms found in the deep subsurface sediments is considered, the microbial communities in the sediments that we sampled may represent cells deposited during normal sedimentation of particulates from the overlying seawater. Sequences closely related to our sequences have been found in seafloor sediments of the nearby Japan Trench (34). It is conceivable that microorganisms can be transported over considerable distances by ocean currents and can survive to be deposited in the sediments. Another potential origin for the cells is transport through the sediments themselves. Strata that contain gas hydrates are believed to exhibit considerable fluid flux (57, 58). Although the Blake Ridge is a passive margin environment, stable isotope studies of iodine in its porewaters suggest that dissolved species are much older than the sediments in which they are found and that these solutes were transported upward through the sediments from deeper strata (18). Wellsbury et al. argued for the presence of a deep, hot biosphere when they considered the activity of microbial communities within and below the hydrate zones of the Blake Ridge (53). In the region of the Nankai Trough, gas venting at the sediment-seawater interface is characteristic of the area (33; S. Kuramoto, J. Hiramura, M. Joshima, and Y. Okuda, Int. Symp. Methane Hydrates, Resour. Near Future, 1998) and indicates relatively rapid efflux of gases from deeper in the formation. The available TOC in the Nankai Trough hydrate sediments is believed to be insufficient for in situ production of the methane in the hydrates, suggesting that the methane likely originated from deeper sediments and migrated up to the hydrate zone (3, 52). The evidence for large-scale movement of liquids and gases in these geologic materials presents the possibility that microorganisms have also been advected from below and then maintained in the strata near the hydrates.

When the 16S rDNA sequence data are discussed, it is important to remember that the diversity obtained from the data is not necessarily representative of the in situ community of microorganisms in the sediments but rather is representative of the libraries constructed. The biases inherent in DNA extraction, PCR, cloning, and RFLP processes prevent a full accounting of sequences in the environment and limit our ability to characterize total microbial diversity with these methods (25, 51). In addition, the heterogeneity of natural environments precludes complete representative sampling. These limitations notwithstanding, we have extended the database of known prokaryotic 16S rDNA sequences in deep marine sediments associated with methane hydrates.

In summary, using combined culture, biochemical, and molecular methods to examine deep marine sediments bearing gas hydrates, we determined the presence of viable methanogens, detected abundant archaeal DE, and amplified 16S rRNA genes by using both archaeal and bacterial primers. When 16S rRNA genes were used as a measure, all three sediment zones studied (above, within, and below gas hydrates) contained diverse communities that included novel and unique lineages. Inferences of relatedness to cultured organisms, such as thermophiles, may help guide cultivation efforts in future studies. Future research that accesses even deeper marine sediments may help explain the presence of these communities and their relationships to natural gas hydrates.

ACKNOWLEDGMENTS

We thank the Japanese Ministry of International Trade and Industry and the Japan National Oil Corporation for providing access to the samples. We also thank R. M. Lehman, D. T. Newby, A. Miller, and F. F. Roberto at the Idaho National Engineering and Environmental Laboratory for assistance with the statistical and sequence analyses and for useful discussions.

The research at the Idaho National Engineering and Environmental Laboratory was funded through contract DE-AC07-991D13727. The support of the INEEL Laboratory Directed Research and Development Programs, the U.S. Department of Energy Fossil Energy Office, and the Japanese Petroleum Exploration Company is gratefully acknowledged.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
3. **Aoki, Y., S. Shimizu, T. Yamane, T. Tanaka, K. Nakayama, T. Hayashi, and Y. Okuda.** 2000. Methane hydrate accumulation along the Western Nankai Trough. Ann. N. Y. Acad. Sci. **912:**136–145.
4. **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.
5. **Benson, D. A., I. Karsch-Mizrachi, D. J. Lipman, J. Ostell, B. A. Rapp, and D. L. Wheeler.** 2000. GenBank. Nucleic Acids Res. **28:**15–18.
6. **Bidle, K. A., M. Kastner, and D. H. Bartlett.** 1999. A phylogenetic analysis of microbial communities associated with methane hydrate containing marine fluids and sediments in the Cascadia Margin (ODP site 892B). FEMS Microbiol. Lett. **177:**101–108.
7. **Boetius, A., K. Ravenschlag, C. J. Schubert, D. Rickert, F. Widdel, A. Gieseke, R. Amann, B. B. Jorgensen, U. Witte, and O. Pfannkuche.** 2000. A marine microbial consortium apparently mediating anaerobic oxidation of methane. Nature **407:**623–626.
8. **Boone, D. R., R. L. Johnson, and Y. Liu.** 1989. Diffusion of the interspecies electron carriers H_2 and formate in methanogenic ecosystems and its implications in the measurement of K_m for H_2 or formate uptake. Appl. Environ. Microbiol. **55:**1735–1741.
9. **Borowski, W. S., C. K. Paull, and W. Ussler.** 1999. Global and local variations of interstitial sulfate gradients in deep-water, continental margin sediments: sensitivity to underlying methane and gas hydrates. Mar. Geol. **159:** 131–154.
10. **Bryan, G. M.** 1974. *In situ* indications of gas hydrate, p. 299–308. *In* I. R. Kaplan (ed.), Natural gases in marine sediments. Plenum Press, New York, N.Y.
11. **Chandler, D. P., F. J. Brockman, T. J. Bailey, and J. K. Fredrickson.** 1998. Phylogenetic diversity of archaea and bacteria in a deep subsurface paleosol. Microb. Ecol. **36:**37–50.
12. **Chandler, D. P., J. K. Fredrickson, and F. J. Brockman.** 1997. Effect of PCR template concentration on the composition and distribution of total community 16S rDNA clone libraries. Mol. Ecol. **6:**475–482.
13. **Cragg, B. A., R. J. Parkes, J. C. Fry, A. J. Weightman, P. A. Rochelle, and J. R. Maxwell.** 1996. Bacterial populations and processes in sediments containing gas hydrates (ODP leg 146: Cascadia Margin). Earth Planet. Sci. Lett. **139:**497–507.
14. **Cragg, B. A., R. J. Parkes, J. C. Fry, A. J. Weightman, P. A. Rochelle, J. R. Maxwell, M. Kastner, M. Hovland, M. J. Whiticar, and J. C. Sample.** 1995.

The impact of fluid and gas venting on bacterial populations and processes in sediments from the Cascadia Margin accretionary system (sites 888–892) and the geochemical consequences, p. 399–411. *In* B. Carson, G. K. Westbrook, R. J. Musgrave, and E. Suess (ed.), Proceedings of the Ocean Drilling Program Scientific Results, vol. 146. Oceanic Drilling Program, College Station, Tex.

15. **DeLong, E. F.** 1992. Archaea in coastal marine environments. Proc. Natl. Acad. Sci. USA **89:**5685–5689.
16. **De Rosa, M., A. Gambacorta, and A. Gliozzi.** 1986. Structure, biosynthesis, and physicochemical properties of archaebacterial lipids. Microbiol. Rev. **50:**70–80.
17. **Dickens, G. R., C. K. Paull, and P. Wallace.** 1997. Direct measurement of *in situ* methane quantities in a large gas-hydrate reservoir. Nature **385:**426–428.
18. **Fehn, U., G. Snyder, and P. K. Egeberg.** 2000. Dating of pore waters with I-129: relevance for the origin of marine gas hydrates. Science **289:**2332–2335.
19. **Felsenstein, J.** 1989. PHYLIP—phylogeny inference package. Cladistics **5:**164–166.
20. **Fujita, Y., F. G. Ferris, D. L. Lawson, and F. S. Colwell.** 2000. Calcium carbonate precipitation by ureolytic subsurface bacteria. Geomicrobiol. J. **17:**305–318.
21. **Garrity, G. M. (ed.).** 2001. Bergey's manual of systematic bacteriology, 2nd ed., vol. 1. Springer-Verlag, New York, N.Y.
22. **Ghiorse, W. C., and D. L. Balkwill.** 1983. Enumeration and morphological characterization of bacteria indigenous to subsurface sediments. Dev. Ind. Microbiol. **24:**213–224.
23. **Griffin, W. T., T. J. Phelps, F. S. Colwell, and J. K. Fredrickson.** 1997. Methods for obtaining deep subsurface microbiological samples by drilling, p. 23–44. *In* P. S. Amy and D. L. Haldeman (ed.), The microbiology of the terrestrial deep subsurface. CRC Press, New York, N.Y.
24. **Hinrichs, K. U., J. M. Hayes, S. P. Sylva, P. G. Brewer, and E. F. DeLong.** 1999. Methane-consuming archaebacteria in marine sediments. Nature **398:**802–805.
25. **Hugenholtz, P., B. M. Goebel, and N. R. Pace.** 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. **180:**4765–4774.
26. **Ibaraki, M.** 1986. Planktonic foraminiferal datum levels recognized in the neogene sequence of the Kakegawa area, and their relationship with lithostratigraphy. J. Geol. Soc. Jpn. **92:**119–134.
27. **Katz, M. E., D. K. Pak, G. R. Dickens, and K. G. Miller.** 1999. The source and fate of massive carbon input during the latest Paleocene thermal maximum. Science **286:**1531–1533.
28. **Kvenvolden, K. A.** 1988. Methane hydrate—a major reservoir of carbon in the shallow geosphere. Chem. Geol. **71:**41–51.
29. **Kvenvolden, K. A.** 1999. Potential effects of gas hydrate on human welfare. Proc. Natl. Acad. Sci. USA **96:**3420–3426.
30. **Kvenvolden, K. A.** 1995. A review of the geochemistry of methane in natural gas hydrate. Org. Geochem. **23:**997–1008.
31. **Lanoil, B. D., R. Sassen, M. T. La Duc, S. T. Sweet, and K. H. Nealson.** 2001. Bacteria and archaea physically associated with Gulf of Mexico gas hydrates. Appl. Environ. Microbiol. **67:**5143–5153.
32. **Lehman, R. M., F. S. Colwell, D. Ringelberg, and D. C. White.** 1995. Combined microbial community-level analyses for quality assurance of terrestrial subsurface cores. J. Microbiol. Methods **22:**263–281.
33. **Le-Pichon, X., K. Kobayashi, and K.-N. S. Crew.** 1992. Fluid venting activity within the Eastern Nankai Trough accretionary wedge: a summary of the 1989 Kaiko-Nankai results. Earth Planet. Sci. Lett. **109:**303–318.
34. **Li, L., C. Kato, and K. Horikoshi.** 1999. Microbial diversity in sediments collected from the deepest cold-seep area, the Japan Trench. Mar. Biotechnol. **1:**391–400.
35. **MacDonald, I. R., N. L. Guinasso, R. Sassen, J. M. Brooks, L. Lee, and K. T. Scott.** 1994. Gas hydrate that breaches the sea-floor on the continental-slope of the Gulf of Mexico. Geology **22:**699–702.
36. **Maidak, B. L., J. R. Cole, T. G. Lilburn, C. T. Parker, Jr., P. R. Saxman, J. M. Stredwick, G. M. Garrity, B. Li, G. J. Olsen, S. Pramanik, T. M. Schmidt, and J. M. Tiedje.** 2000. The RDP (Ribosomal Database Project) continues. Nucleic Acids Res. **28:**173–174.
37. **Marchesi, J. R., A. J. Weightman, B. A. Cragg, R. J. Parkes, and J. C. Fry.** 2001. Methanogen and bacterial diversity and distribution in deep gas hydrate sediments from the Cascadia Margin as revealed by 16S rRNA molecular analysis. FEMS Microbiol. Ecol. **34:**221–228.
38. **Matsumoto, R.** 2000. Methane hydrate estimates from the chloride and oxygen isotopic anomalies. Examples from the Blake Ridge and Nankai Trough sediments. Ann. N. Y. Acad. Sci. **912:**39–50.
39. **Maymo-Gatell, X., Y.-T. Chien, J. M. Gossett, and S. H. Zinder.** 1997. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Science **276:**1568–1571.
40. **Ni, S. S., and D. R. Boone.** 1991. Isolation and characterization of a dimethyl sulfide-degrading methanogen, *Methanolobus siciliae* Hi350, from an oil well, characterization of *M. siciliae* T4/Mt, and emendation of *M. siciliae*. Int. J. Syst. Bacteriol. **41:**410–416.
41. **Pace, N. R.** 1997. A molecular view of microbial diversity and the biosphere. Science **276:**734–740.
42. **Parkes, R. J., B. A. Cragg, and P. Wellsbury.** 2000. Recent studies on bacterial populations and processes in subseafloor sediments: a review. Hydrogeol. J. **8:**11–28.
43. **Reysenbach, A. L., K. Longnecker, and J. Kirshtein.** 2000. Novel bacterial and archaeal lineages from an in situ growth chamber deployed at a Mid-Atlantic Ridge hydrothermal vent. Appl. Environ. Microbiol. **66:**3798–3806.
44. **Ringelberg, D. B., S. Sutton, and D. C. White.** 1997. Biomass, bioactivity and biodiversity: microbial ecology of the deep subsurface. Analysis of ester-linked phospholipid fatty acids. FEMS Microbiol. Rev. **20:**371–377.
45. **Rochelle, P. A., B. A. Cragg, J. C. Fry, R. J. Parkes, and A. J. Weightman.** 1994. Effect of sample handling on estimation of bacterial diversity in marine sediments by 16S ribosomal RNA gene sequence analysis. FEMS Microbiol. Ecol. **15:**215–225.
46. **Sassen, R., S. Joye, S. T. Sweet, D. A. DeFreitas, A. V. Milkov, and I. R. MacDonald.** 1999. Thermogenic gas hydrates and hydrocarbon gases in complex chemosynthetic communities, Gulf of Mexico continental slope. Org. Geochem. **30:**485–497.
47. **Smith, D. C., A. J. Spivack, M. R. Fisk, S. A. Haveman, and H. Staudigel.** 2000. Tracer-based estimates of drilling-induced microbial contamination of deep sea crust. Geomicrobiol. J. **17:**207–219.
48. **Speksnijder, A., G. A. Kowalchuk, S. De Jong, E. Kline, J. R. Stephen, and H. J. Laanbroek.** 2001. Microvariation artifacts introduced by PCR and cloning of closely related 16S rRNA gene sequences. Appl. Environ. Microbiol. **67:**469–472.
49. **Takai, K., D. P. Moser, M. DeFlaun, T. C. Onstott, and J. K. Fredrickson.** 2001. Archaeal diversity in waters from deep South African gold mines. Appl. Environ. Microbiol. **67:**5750–5760.
50. **Vetriani, C., H. W. Jannasch, B. J. MacGregor, D. A. Stahl, and A. L. Reysenbach.** 1999. Population structure and phylogenetic characterization of marine benthic archaea in deep-sea sediments. Appl. Environ. Microbiol. **65:**4375–4384.
51. **von Wintzingerode, F., U. B. Gobel, and E. Stackebrandt.** 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. **21:**213–229.
52. **Waseda, A.** 1998. Organic carbon content, bacterial methanogenesis, and accumulation processes of gas hydrates in marine sediments. Geochem. J. **32:**143–157.
53. **Wellsbury, P., K. Goodman, T. Barth, B. A. Cragg, S. P. Barnes, and R. J. Parkes.** 1997. Deep marine biosphere fueled by increasing organic matter availability during burial and heating. Nature **388:**573–576.
54. **Wheeler, D. L., C. Chappey, A. E. Lash, D. D. Leipe, T. L. Madden, G. D. Schuler, T. A. Tatusova, and B. A. Rapp.** 2000. Database resources of the National Center for Bio/Technology Information. Nucleic Acids Res. **28:**10–14.
55. **White, D. C., W. M. Davis, J. S. Nickels, J. D. King, and R. J. Bobbie.** 1979. Determination of the sedimentary microbial biomass by extractable lipid phosphate. Oecologia **40:**51.
56. **White, D. C., and D. B. Ringelberg.** 1997. Utility of the signature lipid biomarker analysis in determining the *in situ* viable biomass, community structure, and nutritional/physiologic status of deep subsurface microbiota, p. 119–136. *In* P. S. Amy and D. L. Haldeman (ed.), The microbiology of the terrestrial deep subsurface. CRC Press, New York, N.Y.
57. **Wood, W. T., and J. F. Gettrust.** 2001. Deep-tow seismic investigations of methane hydrates. Geophys. Monogr. **124:**165–178.
58. **Xu, W., and C. Ruppel.** 1999. Predicting the occurrence, distribution, and evolution of methane gas hydrate in porous marine sediments. J. Geophys. Res. **104:**5081–5095.