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3. Enrichment and Cultivation of Microorganisms from Sediment from the Slope of the Peru Trench (ODP Site 1230)[1]

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ABSTRACT

The deep biosphere is estimated to hold a significant percentage of the Earth's prokaryotic biomass; however, little is known about the organisms in this environment. Here, we describe investigations of the diversity of microorganisms enriched from surface and subsurface sediment collected during Leg 201 of the Ocean Drilling Program at Site 1230 on the slope of the Peru Trench. This site contains methane hydrates, high levels of organic matter, and high direct cell counts, all of which indicate the potential for thriving microbial populations. To investigate these populations, we examined prokaryotes in samples from seafloor to 258 meters below seafloor (mbsf) using both cultivation and molecular methods. From seafloor samples, we cultivated isolates representing the genera *Photobacterium, Shewanella,* and *Halomonas*. The population found in an enrichment cultivated at low temperatures, 0.67 mbsf, contained many cell morphologies and deoxyribonucleic acid (DNA) signatures, but this population, except for a *Vibrio* sp., was difficult to separate and grow as pure cultures. Most isolates produced extracellular lytic enzymes that were active at low temperatures. Methanogens have been expected to play a large role in the creation of methane hydrates in the sediment; therefore, we also attempted to enrich for psychrophilic methanogens. No methane was found above background levels in anaerobic enrichments incubated for 2 yr, nor was any 16S ribosomal DNA detected following amplification using archaeal primers with DNA extracted from these incubated cultures. These results illus-

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trate the need for further extensive microbiological studies in order to understand the biogeochemistry of this important subseafloor environment.

INTRODUCTION

Marine sediments constitute a large global carbon reservoir and an environment with numerous microorganisms (Whitman et al., 1998). Although there have been a number of studies on this environment, there are still microbial ecosystems to explore, especially in regions of deep sediment environments. With a few exceptions (Wang et al., 2004), analyses of deep sediment column samples have studied either environmental deoxyribonucleic acid (DNA) (Bidle et al., 1999; Inagaki et al., 2003; Kormas et al., 2003; Newberry et al., 2004) or have cultivated organisms at temperatures higher than those in situ (Bale et al., 1997; Mikucki et al., 2003; Toffin et al., 2004).

Samples from Leg 201 of the Ocean Drilling Program (ODP) presented a unique opportunity for microbiological studies of deep-sea sediments that could be compared with the biogeochemical data. During this cruise, cores were collected from the equatorial Pacific and Peru margin, covering a wide range of marine sediment conditions: organic carbon-rich margin sites to more organic-poor open-ocean sites (D'Hondt, Jørgensen, Miller, et al., 2003). The most organic-rich core from Leg 201 was from Site 1230 on the slope of the Peru Trench (D'Hondt, Jørgensen, Miller, et al., 2003). This site also contained the highest microbial populations, with direct counts of $>10^8$ cells/cm^3 sediment at the seafloor (D'Hondt, Jørgensen, Miller, et al., 2003). This site is also of special interest because it contains abundant biologically produced methane hydrates throughout the sediment column (D'Hondt, Jørgensen, Miller, et al., 2003).

For these reasons, Site 1230 core serves as an excellent resource for comparing microbial populations at different depths in the same borehole. Although only a small percentage of the total microbial population in an environmental sample can be cultivated, obtaining isolates is an important way to study physiological reactions that may be occurring in situ. In addition, the cultivation of microbes in conjunction with molecular studies of diversity provides a more complete composite of the total population (molecular studies from Leg 201 will be performed by other researchers). With this in mind, we initiated the cultivation of organisms starting with the region of most aerobic heterotrophic activity and abundant carbon source, the seafloor. We were interested in whether psychrophilic organisms could be isolated with extracellular enzymatic activities at seafloor temperatures (2°–6°C). We also examined populations existing in deeper sediments with continued cultivation combined with molecular analyses.

In addition, because of the presence of methane hydrates at Site 1230, we specifically enriched for methanogens that might be within the sediment column. Previous studies have examined archaeal populations of subseafloor sediment (Bidle et al, 1999; Marchesi et al., 2001; Mikucki et al., 2003; Newberry et al., 2004) and have usually not found 16S ribosomal ribonucleic acid (rRNA) genes of methanogens as part of their libraries. Evidence for methanogens has been found using functional gene (*mcrA*) or more specific 16S rRNA gene primers (Marchesi et al., 2001; Newberry et al. 2004), and recently a mesophilic methanogen species designated *Methanoculleus submarinus* was isolated from 247

meters below seafloor (mbsf) at the Nankai Trough (Mikucki et al., 2003). The difficulty in finding evidence for methanogens at many sites suggests that they are absent, present in low numbers, or cannot be detected by current methods. Although a few psychrophilic methanogens are in pure culture, none are from a deep marine sediment column (Chong et al., 2002; Franzmann et al., 1997). Additional studies are required to provide information on methanogens in deep sediment columns and clarify their relationship with methane hydrates. Here, we describe our attempts to cultivate methanogens from Site 1230 core samples. We also describe bacterial isolates obtained from seafloor and 0.67 mbsf samples and the studies of mixed populations of *Bacteria* and Archaea detected by molecular methods in cultures incubated through multiple enrichments at low temperatures.

MATERIAL AND METHODS

Site Description and Sampling

Sediment cores from Site 1230 (9°6.75′S, 80°35.0′W) from the Peru margin were obtained using the advanced piston corer (APC). Site 1230 is located on the lower slope of the Peru Trench in a water depth of 5098 m and contains several horizons of methane hydrate below 70 mbsf. Sediment methane levels were tested on board the ship and ranged from 10 to 1000 µM (D'Hondt, Jørgensen, Miller, et al., 2003). Acetate levels at Site 1230 were particularly high at 5–20 µM in the sulfate reduction zone (0–8 mbsf) and reached 230 µM in the methane zone (8–250 mbsf) at 145 mbsf. Sediment temperature ranged from 2°C at the seafloor and increased to 12°C by 300 mbsf (D'Hondt, Jørgensen, Miller, et al., 2003). Contamination from drilling fluid and seawater in the drill cores was determined to be low or nonexistent (D'Hondt, Jørgensen, Miller, et al., 2003). The uppermost sediment from core 201-1230C-1H, the seafloor sediment, was placed into a mylar bag in the ship's cold room. The bag was flushed with filter-sterilized N_2, sealed, and then stored under refrigeration and shipped as described below. Cores of deeper sediment were subsampled as whole round cores, stored under N_2 in the dark at 4°C, and shipped cold to The Pennsylvania State University (USA), where they were stored in a cold room at 4°C. Whole-round cores were sampled for microbiological study by removing the outermost 1 cm of sediment and sampling the inner core with sterile syringes under an N_2 atmosphere.

Heterotroph Enrichments

Seafloor Enrichments

Marine broth 2216 (Difco) (DMB) medium was prepared according to manufacturer's instructions, and 15 g/L agar was added when plates were used. Aerobic liquid cultures were incubated with shaking at 200 rpm. Seafloor sediment was suspended in liquid media (1 cm^3 sediment/5 mL media) on ice, and 100 µL of this slurry was immediately plated in duplicate for incubation at both 10° and 2°C. Selected isolated colonies were restreaked several times to homogeneity. Isolates were maintained on agar at 10° or 2°C. Genomic DNA was extracted from pure cultures grown in liquid media using the Puregene DNA isolation kit (Gentra Systems) according to the manufacturer's instructions for

gram-negative cells. The 16S ribosomal DNA (rDNA) genes were amplified using the general bacterial primer set 8F-1492R and Ready to Go polymerase chain reaction (PCR) beads (Amersham) (Pace et al., 1986; Weisburg, et al., 1991). The amplified products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced at the Pennsylvania State Nucleic Acid Facility on an ABI 3100 sequencer. Sequences are available in the GenBank nucleotide sequence database under accession numbers AY849798–AY849802.

Isolates were examined for their ability to grow at different temperatures on agar media. Optimal growth temperature was estimated by measuring colony size after equivalent incubation times at different temperatures (results from liquid cultures were similar). Growth rates were determined by Klett meter readings of liquid cultures incubated at various temperatures. Four distinct extracellular enzyme activities were assayed, between 2° and 37°C, as follows: (1) protein hydrolysis was detected by clearing zones on DMB agar with skim milk added (15 g/L) (Lichenstein et al., 1992); (2) esterase activity was detected by precipitation zones on DMB agar supplemented with 1% Tween-20 (ICN Biomedicals) (Jensen, 1983); (3) chitin hydrolysis was detected by clearing zones on DMB agar containing colloidal chitin (15g/L) (Lingappa and Lockwood, 1961; Morgavi et al., 1994); and (4) starch hydrolysis was indicated by clear zones on iodine-stained DMB agar with starch (15g/L).

Enrichment of Mixed Populations

Sediment from 0.67 mbsf (Section 201-1230C-1H-1) was inoculated (2 cm^3/50 mL) into aerobic liquid DMB and incubated at 10°C. Mixed populations were then transferred to both aerobic DMB and anaerobic DMB (N_2 atmosphere). After incubating for 3 weeks, the mixed population was passed through the following enrichment and dilution steps to encourage growth of possible facultative anaerobes. Each step included inoculation of 1 mL culture into 50 mL of media and alternated between aerobic and anaerobic media. All enrichments were incubated for 1 month at 2°C prior to further transfer. At the sixth month, the enrichment culture was diluted 1:10^8 into anaerobic DMB and sampled after 1 month in an attempt to achieve a pure culture. Microscopic observations were made using wet mounts and viewed at 1000× under oil immersion on a MicroMaster phase contrast microscope.

DNA was extracted from the mixed populations using the UltraClean Microbial DNA isolation kit (MoBio) according to manufacturer's instructions. Ribosomal intergenic spacer analysis (RISA) was performed with bacterial primers 16S 1406F–23S 115R or archaeal primers 16S 1214F–23S 46R and amplified by Ready-to-Go beads (Amersham) (annealing temperature = 59°C) (Lane, 1991). The RISA products were analyzed on 3% TAE-agarose gels. The 16S rDNA genes were amplified from total extracted DNA purified as described above. The 16S rDNA libraries were prepared using the PCR-script Amp cloning kit (Stratagene) and were transformed into Z-competent *Escherichia coli* DH5α cells (Zymo, Inc.). Transformants containing inserts were identified by blue/white screening and the plasmids extracted using the Wizard Plus SV Miniprep kit (Promega). Plasmid inserts were screened using amplified ribosomal DNA restriction analysis (ARDRA) and grouped by digestion pattern by *Rsa*I (Promega). At least one representative of each pattern was sequenced as described above. Comparable sequences from GenBank (**www.ncbi.nlm.nih.gov**) and RDPII (**rdp.cme.msu.edu**) (Cole et al., 2003) databases were gathered. Phylogenetic trees were prepared by

aligning these by Clustal X found in the BioEdit platform (www.mbio.ncsu.edu/BioEdit/bioedit.html) and tree preparation by PAUP (version 4.0b10; paup.csit.fsu.edu). Bootstrap values represent 100 replicates.

Methanogen Enrichments

A basal anaerobic marine salts media, DGH (D'Hondt, Jørgensen, Miller et al., 2003), modified from *Desulfotomaculum geothermicum* media (Daumas et al., 1988), was prepared with the addition of one of four carbon sources: 5 g/L trimethylamine, 8 ml/L methanol, 8 g/L acetate, or 2-atm CO_2. Media were reduced with sodium sulfide, degassed under N_2/CO_2, and aliquoted under an N_2/H_2 glovebag atmosphere; the headspace was then exchanged for either 1.5-atm N_2/CO_2 or 2-atm H_2/CO_2. Resazurin was used as an oxygen indicator. Sediment was added to make an anaerobic slurry preparation of 1 cm^3 of sediment in 9 mL DGH media with no carbon source; 1 mL of this was added to 10 mL media for incubation. Enrichment cultures were incubated at 4°C without shaking. Growth was monitored by analysis of headspace gas on a HP 5890 Series II gas chromatograph. DNA analysis was performed as described above for mixed populations.

RESULTS

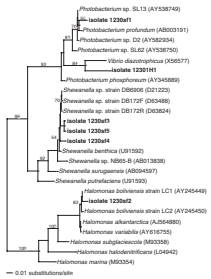
Cultivations from Seafloor Samples

Isolations from seafloor sediment were performed as described above prior to attempting subseafloor enrichments. Seafloor sediment was plated in duplicate, and one plate of each set was incubated at either 2° or 10°C. Colonies appeared within 3 days at 10°C but required up to 17 days at 2°C, and similar morphologies were seen on media incubated at each temperature. Three colony morphologies were seen, and samples of these were restreaked from plates at 2°C to homogeneity to yield five pure cultures.

Analysis of 16S rRNA gene sequences from these five cultures revealed five distinct sequences (Fig. F1). Isolate 1230sf1, which had formed large colonies within the first 3 days of incubation at 2°C during the initial plating, was most related to the deep-sea benthic bacterium *Photobacterium profundum*. Isolate 1230sf2 was most closely related to *Halomonas boliviensis*. Isolates 1230sf3, 1230sf4, and 1230sf5, which had very similar colony morphologies, were all closely related to previously cultivated deep-sea strains of *Shewanella benthica* (Fig. F1).

The abilities of these five isolates to grow at different temperatures and produce extracellular enzymes is summarized in Table T1. All isolates grew well between 2° and 20°C. Only isolate 1230sf2, which had an optimal growth temperature between 20° and 35°C, was able to grow at 37°C. In addition to its high 16S rDNA similarity to the two other described isolates of *P. profundum*, isolate 1230sf1 also had similar growth characteristics with a doubling time of 11 hr at 2°C and 2.5 hr. at 15°C (Nogi et al., 1998). Isolate 1230sf1 also had a genome size and piezotolerance (tolerant to 40 MPa, the highest pressure tested) (F. Lauro, pers. comm., 2003) that is consistent with its identification as a *P. profundum* strain (Bidle and Bartlett 1999; Nogi et al., 1998). Similar to isolate 1230sf1, isolates 1230sf3, 1230sf4, and 1230sf5 also did not grow at temperatures above 30°C and had optimal growth between 18° and

F1. Phylogenetic tree of isolates, p. 13.



T1. Isolates and their characteristics, p. 18.

25°C. All isolates can therefore be characterized as psychrophilic according to the definition of Neidhardt et al. (1990). All isolates grew slowly anaerobically at low temperatures, taking 6 months to form isolated colonies on anaerobic plates at 10°C (Table T1).

All isolates, except for 1230sf2, produced extracellular degradative enzymes that were active on the screening media at temperatures between 2° and 18°C (Table T1). The *Shewanella* related species produced proteases and esterases that continued to be active at temperatures up to 30°C. Chitinase activity was not seen for any of the isolates tested. The frequency of protease and esterase production may reflect the substrate availability of deep seafloor areas where proteins and lipids are present as substrates and possible nutrients (Boetius and Lochte, 1994; Luna et al., 2004).

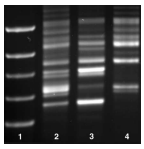
Cultivations from Subsurface Samples

Based on results from the seafloor isolation experiments, we attempted to cultivate similar microbes farther down the sediment column at 0.67 mbsf (Section 201-1230C-1H-1; as reported, the contamination in this sediment was nonexistent or low) (D'Hondt, Jørgensen, Miller, et al., 2003). DMB media were inoculated with sediment and incubated aerobically at 10°C. After 3 days of incubation, the culture was examined microscopically. Numerous cell morphologies were observed, with the most striking being numerous spirochetes and spirilla-like cells. Plating of this culture revealed a single colony morphology, so molecular methods were used to explore the additional population members. Genomic DNA was extracted from the culture, and the intergenic spacer regions between rRNA genes were amplified using both bacterial and archaeal primers. RISA showed a diverse bacterial population in this sample (Fig. F2). Surprisingly, archaeal primers amplified a single band at 500 bp from this enrichment. Purification and sequencing of this archaeal PCR product of RISA fingerprinting showed that it was from a group classified as uncultivated benthic Crenarchaea from low-temperature environments (Fig. F3) (Bowman and McCuaig, 2003; Vetriani et al., 1998). Additional research showed that this archaeal fingerprint was only detected in the enrichment culture until day 10, after which it could not be detected.

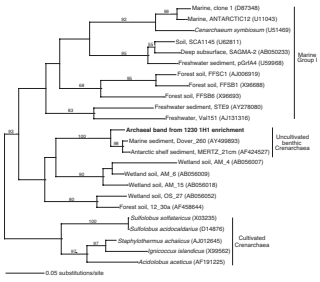
The diverse bacterial populations indicated by these RISA fingerprints were also examined through the construction of 16S rDNA libraries (Fig. F4). Following enrichment for 3 days, the majority of ARDRA-determined ribotypes in the16S rDNA library were related to *Vibrio* spp. and *Halomonas* spp., with the *Halomonas* spp. being represented by three separate ribotypes after digestion with *Rsa*I. This high representation of up to 70% of the ribotypes by possible *Halomonas* spp., which are rod-shaped cells, suggests that the cells observed microscopically with the distinctive morphologies of spirochete and spirilla-like cells were either not lysed or their rRNA genes were poorly amplified by general bacterial primers.

Incubation of these cultures was continued and observed microscopically after 15 days. The 15-day cultures appeared similar to the 3-day cultures both microscopically and in the banding patterns of the bacterial RISA (Fig. F2). Because many of the members of the population, such as spirilla, spirochetes, *Vibrio* spp., and *Halomonas* spp., are known to be facultative anaerobes, the culture was cycled through aerobic and anaerobic conditions, at 2°C, in an attempt to enrich for facultative anaerobes. The final culture examined had been incubated anaerobi-

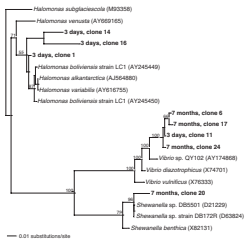
F2. Fingerprint of RISA on DNA, p. 14.



F3. Phylogenetic tree of archaeal sequence, p. 15.



F4. Phylogenetic tree of bacterial 16S rRNA gene sequences, p. 16.



cally for a month, making the total liquid cultivation time 7 months. Microscopic observation of the population showed a large number of rod-shaped cells, and analyses by both RISA fingerprinting (Fig. F2) and 16S rDNA libraries (Fig. F4) showed that it was populated by *Vibrio* spp. and a *Shewanella* sp.

Further subcultivation of this mixed population was performed on agar media and yielded isolated colonies similar to the dominant colony morphology observed initially, designated 12301H1, related to *Vibrio diazotrophicus* (Fig. F1). This isolate had an optimal growth temperature at 37°C, grew well at colder temperatures, and produced amylases for starch hydrolysis at all temperatures tested (Table T1). The 16S rDNA sequences of this *Vibrio*-like sp. and the *Vibrio*-like sp. previously detected in the mixed culture were identical. Other colonies contained mixed cell types that could not be subcultured to homogeneity and were not identified. Additional undescribed isolates have been obtained through similar methods of aerobic plating of samples from 12, 30, and 100 mbsf (data not shown).

Because spirochetes have been previously reported in enrichment cultures from 4.15 mbsf at the Nankai Trough (ODP Site 1173) (Toffin et al., 2004), the spirochetes observed after the 3-day incubation were particularly interesting. We attempted to isolate these by enriching for their growth through traditional methods such as adding antibiotics to the media, incubating anaerobically, serially diluting the sample to decrease the number of competitors, incubating in soft agar with gradient conditions, and filtering cultures. We also used a more nontraditional method of flow cytometry to separate individual cells from the population. However, none of these methods increased the number of spirilla or spirochete-like morphologies seen in enrichment.

Enrichments for Methanogens from Subsurface Samples

Several enrichments were designed to cultivate methanogens from samples taken throughout the sediment column at Site 1230 (1, 30, 102, and 258 mbsf) (D'Hondt, Jørgensen, Miller, et al., 2003). Four different potential carbon sources were added individually (H_2/CO_2, methanol, acetate, and trimethylamine) to anaerobic marine salts media, DGH. After sediment addition, the enrichments were incubated anaerobically at 4°C. Because growth of a psychrophilic methanogen could be slow and might not yield turbidity that would be detectable in the presence of the sediment, the headspace was analyzed for the presence of methane as an indication of methanogenic activity. Methane levels were measured by gas chromatography over a period of 2 yr (Table T2); however, no increase above background levels was found.

T2. Methanogen enrichments and headspace methane, p. 19.

In situ levels of methane in the sediment were determined on board the ship (D'Hondt, Jørgensen, Miller, et al., 2003) and would have yielded an 8 to 10-ppm background level in our experiments after methane was desorbed from the sediment. We also confirmed these in situ background levels in selected enrichments by adding 1 mL of 3-M sodium hydroxide to 10 mL of enrichment culture to release the methane absorbed on the sediment (D'Hondt, Jørgensen, Miller, et al., 2003). In these tests, background methane levels from sediment added to each bottle equaled ~7 ppm, which is in reasonable agreement with shipboard measurements considering the loss of some methane during storage. In this cultivation experiment, growth of methanogens could have

been detected by a signal above background (even if some of the gas had absorbed onto the sediment used as inoculum). As a control to determine whether the media could support growth of methanogens if they had been present in the samples, *Methanosarcina acetivorans* cells were inoculated into the media and incubated. The culture became turbid within 2 days and produced gas, demonstrating that the medium could support the growth of a known methanogen.

In addition to examining the cultures for methane production, these anaerobic enrichments were examined under phase contrast microscopy after 1 yr of incubation, and extracted DNA was analyzed by PCR amplification using archaeal primers for both the 16S rDNA and RISA. No evidence for methanogens was found by any of these methods. In an additional experiment, we attempted to detect a possible extreme minority population of methanogens by using the GenomiPhi kit (Amersham), which amplifies total DNA nonspecifically, to increase the overall amount of extracted DNA. No archaeal RISA signatures were detected even after PCR amplification of the GenomiPhi-treated DNA extracted from the methanogen enrichments. During the course of these enrichments, the cultivation of a deep-sea methanogen, *Methanoculleus submarinus*, was reported from Nankai Trough sediment (Mikucki et al., 2003). At this time, we replicated the conditions under which *M. submarinus* was isolated using sediment from 1.02, 30.4, 65.9, and 102.3 mbsf because our original enrichments would not have supported growth of this organism. In these new enrichments, incubated at 37°C, no evidence for methanogenic cells was found, although bacterial growth occurred in cultures from 30.4 and 102.3 mbsf.

DISCUSSION

The deep sea and its sediments represent the largest permanently cold environment on Earth. We incubated cultures at low temperatures (2°–10°C) in order to increase the number of psychrophilic microorganisms and to explore their potential enzyme activities in situ. We isolated and characterized organisms that are phylogenetically related to *Photobacterium, Halomonas, Shewanella,* and *Vibrio* species. All isolates are closely related (by >98% 16S rRNA similarity) to previously isolated deep-sea strains, consistent with their being from the core sample rather than contaminants. These genera are also commonly found in deep sediment studies, especially those in the Pacific (Wang et al., 2004). The trend in extracellular degradative enzyme production agrees with previously published results of deep-sea sediment isolates and corresponds to available nutrient sources, suggesting a possible adaptation to this environment or competitive advantage within this ecosystem (Boetius and Lochte, 1994; Luna et al., 2004; Wang et al., 2004). The one *Vibrio* sp. isolate from the sediment column (0.67 mbsf) adds to the database of other sediment-dwelling microbes isolated from deeper than 0.5 mbsf (Bale et al., 1997; Toffin et al., 2004). Further, the characterization of these isolates increases the numbers of described species for these genera and provides information on their production of cold-active enzymes of possible industrial interest.

Our mixed population studies of cultures from the 0.67-mbsf sample also detected 16S rRNA genes from *Halomonas* spp. and *Shewanella* sp., and microscopic examinations suggested the presence of numerous spirochetes and spirilla. This population differs from the *Marinilactibacillus* and *Acetobacter* type community described from 4.15 mbsf in the Nan-

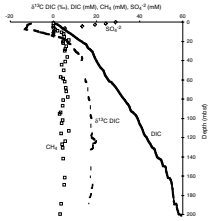
kai Trough (Toffin et al., 2004). In other ways, our cultivated mixed population from 0.67 mbsf appears similar to other cultivated populations from below the seafloor (Toffin et al., 2004). Spirochetes have often been found in seafloor and hydrothermal vent environments (Bowman et al., 2000; Campbell and Cary, 2001), and have recently been identified in DNA libraries from the Nankai Trough (Newberry et al., 2004), but their cultivation from deep sediment was a surprise (Toffin et al., 2004). In both studies described here, spirochetes were seen in culture for short periods in media containing yeast extract. It is possible that this provides important nutrients for these organisms. However, because the spirochete population has not been maintained for longer incubations, their growth requirements remain unknown.

The presence of a number of facultative anaerobic microorganisms in our cultivations suggests that the capability for anaerobic growth may allow cell survival after burial by the accumulation of the sediment column over time. However, because facultative organisms grow more rapidly aerobically, especially at low temperatures, we used aerobic cultivation to examine this population. In such an aerobic enrichment culture, it was surprising to find crenarchaeal signatures. *Crenarchaea* have been shown to be members of the seafloor community (Bowman and McCuaig, 2003; Vetriani et al., 1998, 1999) and have been detected deeper in sediment columns (Bidle et al., 1999). However, to our knowledge, they have not yet been reported as members of a cultivated community from marine sediment. Here we report the existence of a marine benthic Crenarchaeon in a bacteria-dominated enrichment culture at 10°C for more than a week. The conditions needed to prolong the existence of these *Crenarchaea* in liquid culture are being investigated.

In addition to the numerous crenarchaeal and euryarchaeal sequences retrieved from the subsurface by other researchers during Leg 201 (F. Inagaki, unpubl. data; A. Teske, unpubl. data), methanogens are expected to exist throughout the sediment column. The difficulties, reported here and elsewhere, in detecting subseafloor psychrophilic methanogens are especially puzzling. The light isotopic values of methane in the sediment column (K. Hinrichs et al., unpubl. data) show that the methane is of biological origin. In addition, the dissolved inorganic carbon (DIC) isotopes of Site 1230 sediment (D. Shrag, unpubl. data) show that methanogenesis is occurring within the sediment column, due to the heavy swing of the DIC isotopes in the methane zone (Fig. F5).

Methanogens, however, have been difficult to demonstrate in the marine subsurface where 16S rRNA gene diversity (Bidle et al., 1999; Inagaki et al., 2003; Marchesi et al., 2001) or functional gene diversity (Marchesi et al., 2001; Newberry et al., 2004) studies have encountered patchy and unequally distributed detection of methanogen DNA. Whereas the methanogen population may be limited near the sulfate-rich and oxygenated seafloor, the abundant biogenic methane present suggests that methanogens exist in the subsurface. Based on the assumption that previous inconsistent methanogen detections were the result of their being a minority population, our culturing attempts were designed to measure very small levels of methanogenesis or to encourage growth that could be detected by molecular methods. These enrichments, however, were unsuccessful in increasing numbers of any cold-loving microorganisms, methanogenic or nonmethanogenic, to detectable levels in the time allowed. Because of the long incubation times needed for anaerobic cultivations at low temperatures for seafloor

F5. Geochemistry at Site 1230, p. 17.



isolates, longer incubations may be required to truly examine anaerobic psychrophilic microorganisms from the deep subsurface.

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Figure F1. Phylogenetic tree of isolates, showing the relationship of the 16S rDNA of isolates from ODP Site 1230 seafloor (sf) and 0.67 mbsf (1H1) to known species. Isolate designations are shown in bold text and the GenBank accession numbers for sequences obtained from the NCBI database are shown in parentheses. Phylogram was constructed using maximum likelihood method, with 100 bootstrap replicates for 1517 nucleotide positions.

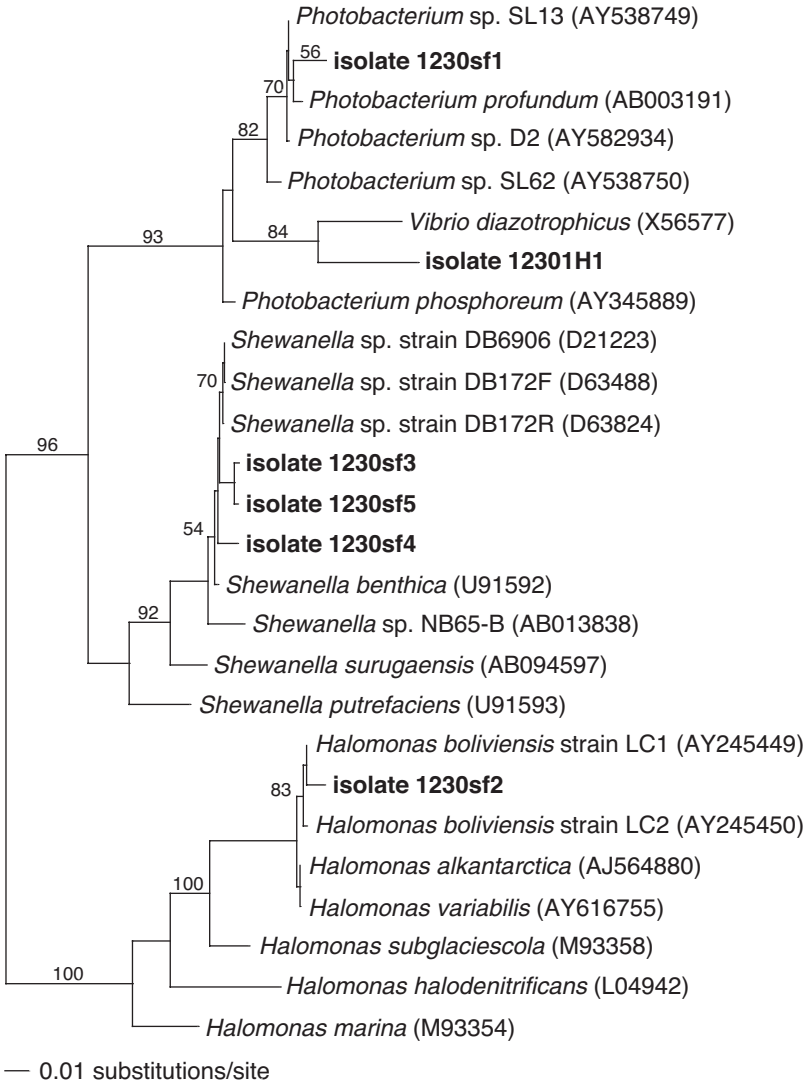

Figure F2. Fingerprint of RISA on DNA extracted from mixed populations from 0.67 mbsf and amplified with bacterial primers. Lane 1 is a molecular weight ladder. Lane 2 is the bacterial population fingerprint on day 3 of enrichment. Lane 3 is from day 15 of the same aerobic enrichment. Lane 4 is the population after numerous transfers and month-long anaerobic incubation. The progression of enrichment can be seen in the changing band patterns in lanes 2–4. Similar bands indicate that the organism is present at both times. Differing bands show the changes in population members. Band intensity can be used as a guide to the relative abundance of different organisms.

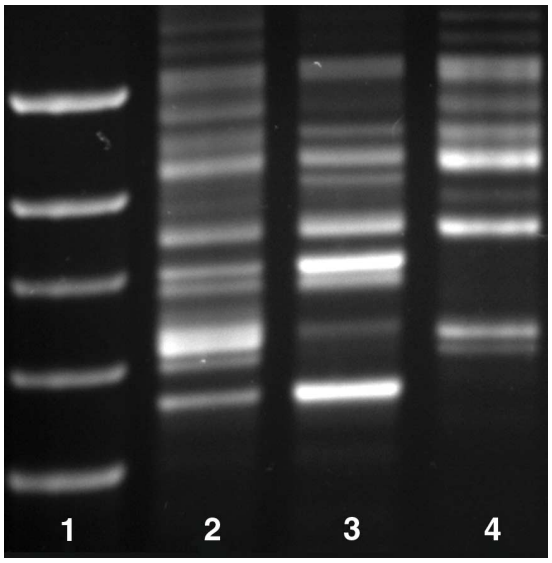

Figure F3. Phylogenetic tree of archaeal sequence seen in RISA fingerprint of mixed culture from days 0 to 10, shown in bold text. Cultured Archaea are shown in italics, uncultured clone sequences are denoted as the environment from which they were retrieved, and their clone description. The sequence from the 0.67-mbsf enrichment groups with other sequences retrieved from marine benthic environments. Phylogram was made using maximum likelihood method, with 100 bootstrap replicates. GenBank accession numbers for sequences obtained from the NCBI database are shown in parentheses.

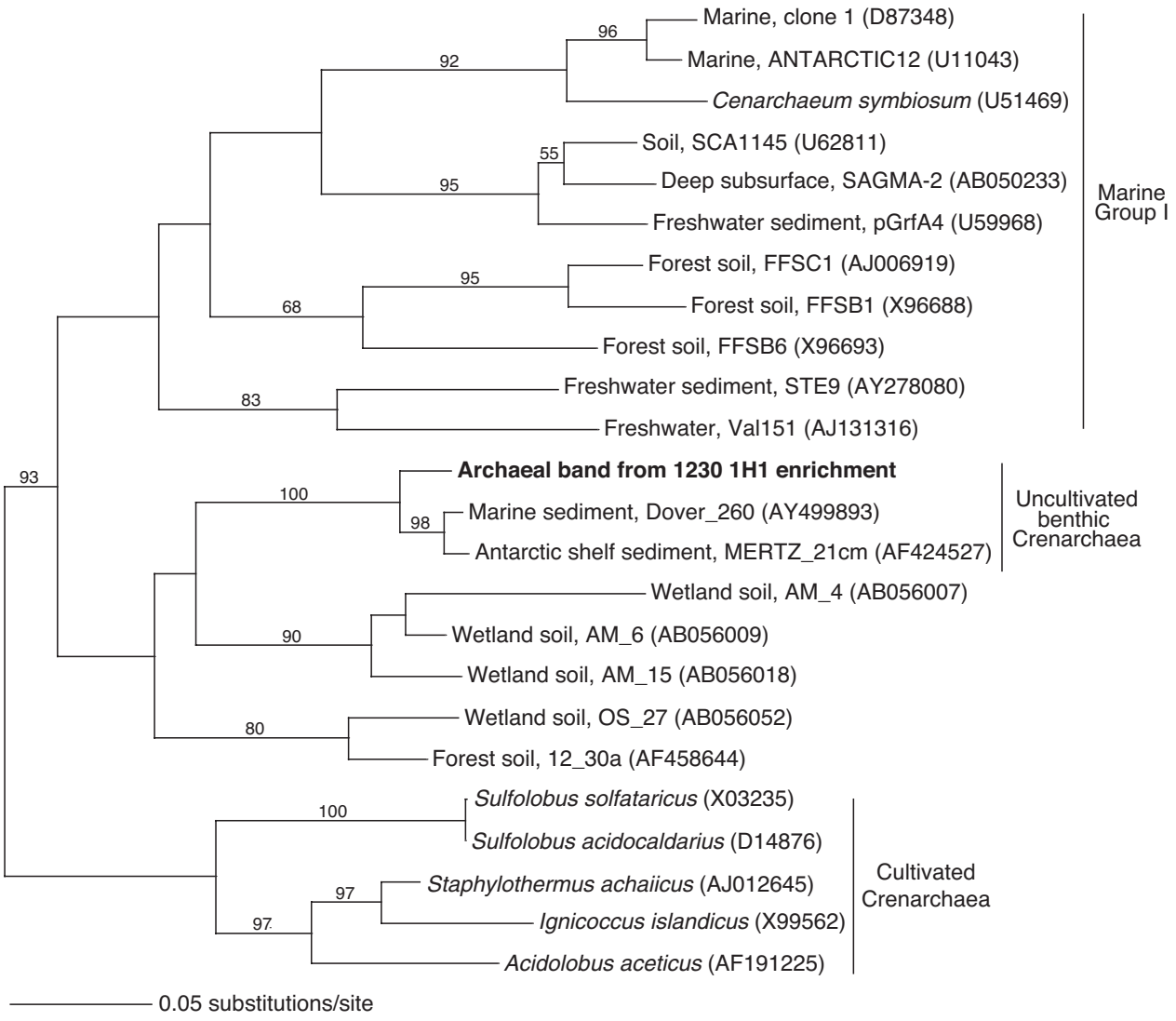

Figure F4. Phylogenetic tree of bacterial 16S rRNA gene sequences obtained from libraries prepared after amplification of DNA extracted from mixed cultures incubated for either 3 days or 7 months. Cloned 16S rDNA sequences are shown in bold text and the GenBank accession numbers for sequences obtained from the NCBI database are shown in parentheses. At day 3, most sequences are from *Halomonas* spp. and *Vibrio* spp. At 7 months, most clones belonged to the *Vibrio* spp.; however, some were found from *Shewanella* spp. Phylogram was made using neighbor joining method, with 100 bootstrap replicates.

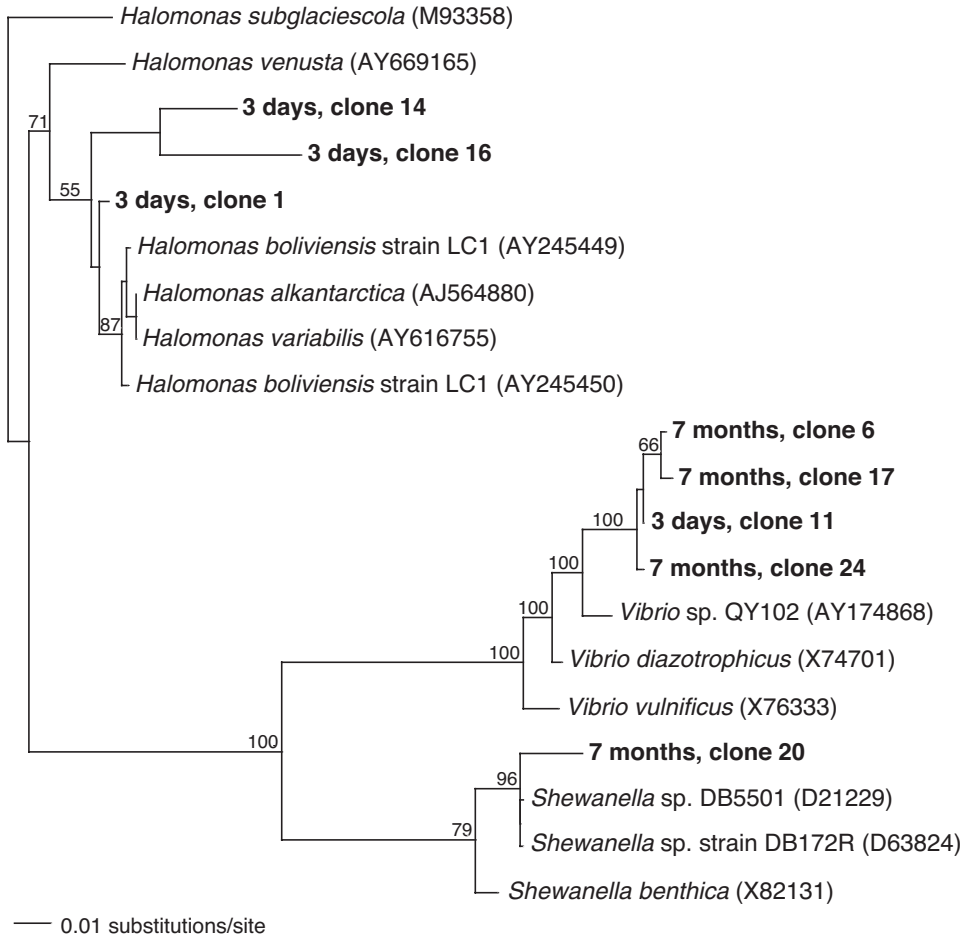

Figure F5. Geochemistry at Site 1230. Shown are methane, sulfate, dissolved inorganic carbon (DIC), and $\delta^{13}C$ DIC. Data taken from D'Hondt, Jørgensen, Miller, et al. (2003) and D. Shrag (pers. comm., 2004).

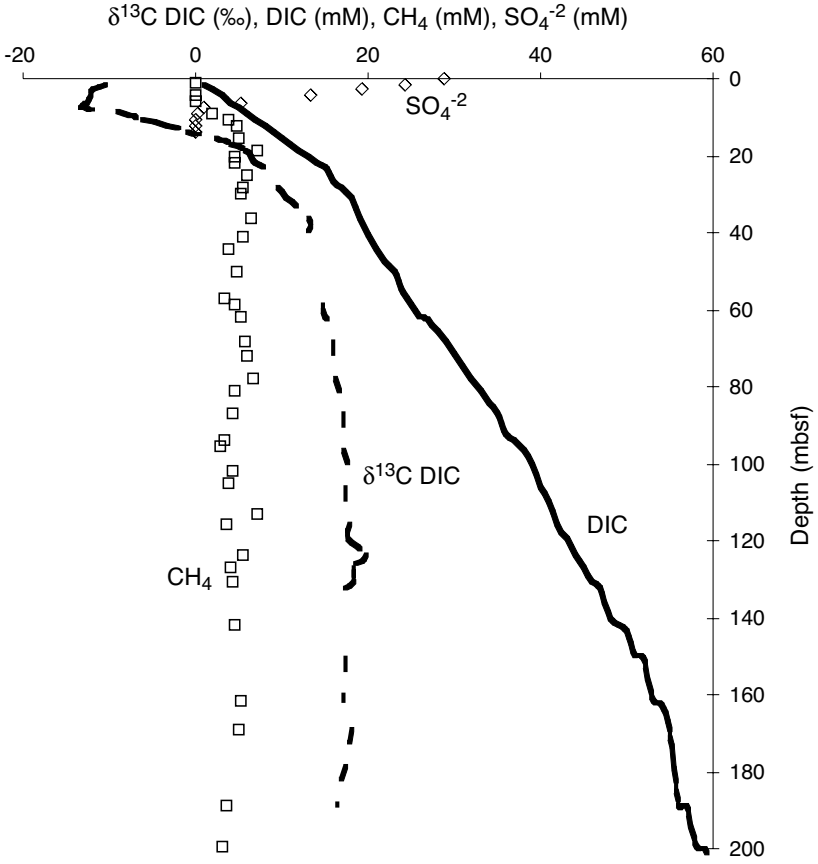

Table T1. Isolates and their characteristics.

Isolate	Closest relative based on 16S rRNA	Similarity (%)	Optimal temperature (°C)	Growth range (°C)	Anaerobic growth	Protease	Esterase	Chitinase	Amylase
1230sf1	*Photobacterium profundum*	98	18	2–22	+	+	+	–	+
1230sf2	*Halomonas boliviensis*	99	35	2–37	+	–	–	–	–
1230sf3*	*Shewanella* sp. D21223	98	22	2–25	+	+	+	–	–
1230sf4	*Shewanella* sp. DB172R	98	22	2–25	+	+	+	–	–
1230sf5*	*Shewanella* sp. D21223	98	22	2–25	+	+	+	–	–
12301H1	*Vibrio diazotrophicus*	98	37	2–37	+	–	–	–	+

Notes: Phylogenetic comparison was made from sequenced 16S rDNA amplified from extracted genomic DNA of isolates. Growth and enzyme characterization was on agar media incubated between 2° and 37°C. * = These isolates have different 16S sequences and different colony morphologies and as such are listed as two separate isolates.

Table T2. Methanogen enrichments and headspace methane.

Core, section	Depth (mbsf)	DGH media supplement	Headspace methane (ppm) measured over time of incubation				
			1 month	2 months	3 months	16 months	2 yr
201-1230A-							
1H-1	1.02	Acetate	1.26	–	1.38	–	–
		Methanol	0.93	1.25	–	1.38	1.36
		Trimethylamine	–	1.49	1.26	1.25	1.29
		CO_2	0.61	0.81	0.89	1.02	–
4H-5	30.4	Acetate	–	1.35	1.43	2.53	2.76
		Methanol	–	1.41	2.06	2.02	–
		Trimethylamine	–	1.03	1.22	1.25	0.98
		CO_2	–	0.74	1.07	–	–
13H-3	102.3	Acetate	–	2.88	–	–	3.54
		Methanol	–		–	–	–
		Trimethylamine	–	1.86	–	–	3.33
		CO_2	–	3.95	–	–	5.32
37X-1	258.3	Acetate	1.02	–	–	1.45	1.61
		Methanol	0.7	–	–	1.27	1.36
		Trimethylamine	0.75	–	–	1.16	1.26
		CO_2	–	–	–	0.75	0.88

Notes: Headspace methane was measured by gas chromatography. Measurements were made in duplicate and averaged. All values were below the concentration of methane released from sediment. DGH = basal anaerobic marine salts media. – = none detected.