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2. Microbial Community Composition in Deep Marine Subsurface Sediments of ODP Leg 201: Sequencing Surveys and Cultivations[1]

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ABSTRACT

Recent molecular analyses of Leg 201 subsurface sediments show that microbial communities of deep marine sediments harbor members of distinct, uncultured bacterial and archaeal lineages, in addition to Gram-positive bacteria (Firmicutes and Actinobacteria), members of the Cytophaga-Flavobacterium-Bacteroides phylum, and Proteobacteria that are detected by cultivation surveys. Several of these subsurface lineages show cosmopolitan occurrence patterns; they can be found in cold marine sediments and in hydrothermal habitats, suggesting a continuous deep subsurface and hydrothermal biosphere. Some archaeal and bacterial lineages appear to be well-diversified generalists that occur in subsurface sediments as well as in a wide range of terrestrial and aquatic habitats.

INTRODUCTION

Marine sediments cover more than two-thirds of Earth. Microbial cells and prokaryotic activity appear to be widespread in those sediments. Intact cells (Parkes et al., 2000) and intact membrane lipids (Zink et al., 2003; Sturt et al., 2004) provide evidence of prokaryotic populations in sediments as deep as 800 meters below seafloor (mbsf). Prokaryotic activity, in the form of sulfate reduction and/or methanogenesis, occurs in sediments throughout the world's oceans (D'Hondt et

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al., 2002). The prokaryotes of subseafloor sediments have been estimated to constitute one-half to five-sixths of Earth's prokaryotic biomass (Whitman et al.,1998) and one-tenth to one-third of Earth's total living biomass (Parkes et al., 2000; Whitman et al., 1998). Fundamental aspects of this subseafloor ecosystem are poorly known. What is the phylogenetic composition of subseafloor prokaryotic communities? What are their functional genes and their metabolic activities that allow these prokaryotes to grow and survive in the subsurface?

Leg 201 was the first Ocean Drilling Program (ODP) expedition dedicated to the study of life deep beneath the seafloor (D'Hondt, Jørgensen, Miller, et al., 2003). Leg 201 combined detailed geochemical analyses, cell counts, cultivations, and molecular screening of subsurface microbial communities. The objective of this multidisciplinary approach was a comprehensive census of subsurface microbial life (diversity, density, and activity) in the context of geochemical controls that shape microbial community composition and activity. Sampling sites were selected that represented low-activity sediments from central oceanic basins in the Pacific, as well as organic-rich sediments on the Peru margin and in the Peru Trench influenced by the Peruvian upwelling system.

Subsurface sediment cores of 120–420 m depth were obtained from three deepwater, open-ocean sites with organic-poor sediments in the Eastern eastern equatorial Pacific (Sites 1225 and 1226) and the Peru Basin (Site 1231), from three shallow sites with organic-rich sediments on the Peruvian continental shelf (Sites 1227, 1228, and 1229), and from an organic-rich deep-sea sediment site in the Peru Trench (Site 1230) (D'Hondt, Jørgensen, Miller, et al., 2003). Sites 1227–1230 had a total organic carbon (TOC) content in the range of 2–8 wt% (2–4 wt% for Site 1230), with outliers near 10 wt% (**Meister et al.,** this volume), cell densities in the upper range of previously recorded acridine orange direct count (AODC) profiles for ODP sediments (Parkes et al., 2000; D'Hondt, Jørgensen, Miller, et al., 2003), and steep sulfate and methane gradients indicating downhole sulfate depletion and methane buildup caused by organic carbon decomposition by sulfate reduction and methanogenesis (D'Hondt et al., 2004). The open-ocean Sites 1225 and 1231 had cell densities near or below the average of AODC profiles for ODP sediments (Parkes et al. 2000; D'Hondt, Jørgensen, Miller, et al., 2003). Their organic C content was at least an order of magnitude lower than at Peru margin sites (0.05–0.2 wt%; some datapoints at Site 1231 as much as 0.7 wt%) (**Meister et al.,** this volume). Their near-linear profiles of sulfate and methane indicate little sulfate depletion or methane accumulation; instead, mutually overlapping broad zones with high concentrations of dissolved metals (Mn and Fe) indicate active microbial metal respiration (D'Hondt et al., 2004). All these characteristics contrast strongly with those of Peru margin sites (D'Hondt et al., 2004). Therefore, Leg 201 offered the opportunity to examine subsurface microbial communities from a wide range of geochemically distinct marine subsurface sediments. This paper reviews and summarizes the available data sets for microbial community composition in Leg 201 sediments.

MICROBIAL COMMUNITY STRUCTURE

Methodological Background of 16S rRNA Analyses

Because of its high degree of conservation, high information content, and good agreement with most physiological and genetic markers, the 16S ribosomal ribonucleic acid (rRNA) gene is the most widely used molecular marker to infer phylogenetic relationships in the living world (Woese, 1987) and provides the basis for the three-domain tree of life, with bacteria, archaea, and eukaryotes as the largest phylogenetic units (domains) of life (Woese et al., 1990). The current census of the microbial diversity of life, based on 16S rRNA genes of pure cultures and natural mixed populations in environmental samples, includes at least 52 phylum-level bacterial and ~20 phylum-level archaeal phylogenetic lineages, most of them environmental populations not available in pure culture (Rappe and Giovannoni, 2003; Hugenholtz et al., 1998; Hugenholtz, 2002). In other words, only a small portion of the microbial world has been brought into pure culture and studied biochemically or physiologically. These phylum-level lineages go back to the deep, early radiations of the bacterial and archaeal domains where the phylogenetic resolution of the 16S rRNA molecule breaks down, in the sense that a hierarchical branching pattern cannot be obtained. The number of these mutually exclusive phylogenetic lineages may increase in the near future, as a function of more comprehensive sequencing surveys.

For microbial community analyses of deep subsurface environments, deoxyribonucleic acid (DNA) recovery is usually the critical factor. DNA has to be extracted from deep subsurface sediments, usually by enzymatic, mechanical, or freeze-thawing lysis of cells, followed by removal of lipids and proteins by extraction with organic solvents (phenol and chloroform) and precipitation of DNA in the aqueous phase with salts and alcohols at cold temperatures. Because of low DNA content and recovery, standard methods must be fine-tuned empirically for optimized DNA recovery from deep subsurface samples (for detailed discussion of empirically optimized protocols, see Sørensen et al., 2004, and Webster et al., 2003). DNA extraction, amplification of 16S rRNA genes by polymerase chain reaction (PCR), cloning, and sequencing of selected clones yield individual 16S rRNA gene sequences of uncultured bacteria and archaea that occur in a specific sediment sample (Sørensen et al., 2004; Newberry et al., 2004; Webster et al., 2003). Sequence alignments and phylogenetic analyses of the 16S rRNA gene sequences are performed with several software packages as detailed in the original references of Tables **T1** and **T2,** which summarize the results in terms of percent representation of archaeal and bacterial lineages in clone libraries. A necessary caveat is that because of potential biases in nucleic acid extraction, PCR amplification, and cloning efficiency, the percent representation of a bacterial or archaeal phylogenetic lineage in a clone library cannot be equated with the relative abundance of these cells in the environment. So far, clone libraries are used as a first approximation to describe the microbial community; group-specific fluorescent in situ hybridization (FISH) counts give direct, quantitative information on community composition (Amann et al., 1995; Pernthaler and Amann, 2005).

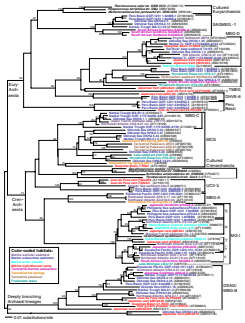
Subsurface Archaea

16S rDNA clone libraries from all Leg 201 sites were dominated by diverse uncultured lineages of bacteria and archaea. Several phylum-level archaeal lineages recur consistently in deep subsurface environments and Leg 201 subsurface sediments. The phylogenetic tree (Fig. F1) shows these archaeal lineages, illustrated with selected sequences from Sites 1225 and 1231. Table T1 lists the percent representation of archaeal subsurface lineages in 16S rDNA clone libraries from Leg 201 sediment samples. For comparison, clone library data from other marine subsurface sediments are included.

Members of the Deep-Sea Archaeal Group (DSAG) are conspicuously well represented in clone libraries of archaeal 16S rRNA genes from diverse sampling sites and sediment types. DSAG archaea were originally found at hydrothermal vent sites (Takai and Horikoshi, 1999) and appear in a growing number of molecular surveys of deep subsurface and hydrothermal vent sites. In addition to the examples shown in Table T1, the deep-sea archaeal group represents >50% of all archaeal clones in 16S rDNA clone libraries at ODP Leg 204 Sites 1245 and 1251 on Hydrate Ridge (Inagaki et al., 2006). Further, DSAG archaeal clones were the second largest archaeal group (13%) recovered in clone libraries from surficial Atlantic deep-sea sediments (Vetriani et al., 1999). Thus, DSAG archaea show a conspicuously cosmopolitan occurrence pattern in a wide spectrum of sediments and vents. For Leg 201, DSAG archaea occur from organic-poor sediments of the central oceanic basins (Sites 1225 and 1231) (Fig. F1) to predominantly organic-rich, methane- or methane hydrate–containing sediments near continental margins (Sites 1230, 1245, and 1251). As an exception to this pattern, Peru Margin Sites 1227 and 1229 appear to be dominated by archaeal groups other than DSAG, specifically members of the Miscellaneous Crenarchaeotal Group (MCG) and of the South African Goldmine Euryarchaeotal Group (SAGMEG) archaea (Inagaki et al., 2006; Parkes et al., 2005; Sørensen and Teske, in press).

The second major archaeal lineage that is frequently found in subsurface sediments are the Marine Group I (MG-I) archaea (Table T1). Members of this group were originally identified by sequencing of environmental rRNA from seawater (DeLong, 1992; Fuhrman et al., 1992). MG-I archaea account for a major portion of all prokaryotic picoplankton in seawater (DeLong et al., 1994; Fuhrman and Ouverney 1998; Karner et al., 2001). In the deep-sea water column below ~3000 m depth, MG-I archaea constitute the majority of prokaryotic picoplankton (Karner et al., 2001). Although pure culture studies are not available, members of MG-I have been shown to take up amino acids, indicating potential for heterotrophic nutrition (Ouverney and Fuhrman, 2000). However, stable C isotope analyses of MG-I lipids suggested that these archaea utilize inorganic carbon (Pearson et al., 2001), which matches the recent finding that Marine Group archaea are capable of HCO_3^- uptake, suggesting autotrophic CO_2 fixation (Wuchter et al., 2003). Thus, MG-I archaea might be facultative autotrophs, or there is broad metabolic diversity within this group. Recently, the first isolate of this group was described as an aerobic ammonia oxidizer with the capability for autotrophic growth (Könneke et al., 2005). Since MG-I archaea are abundant in seawater, retrieving their 16S rRNA genes from subsurface clone libraries poses the question whether (1) they could be seawater contaminants that are introduced into the sediments during the drilling process; (2) seawater archaea that permeate the subsurface natu-

F1. Phylogenetic tree, p. 17.



T1. Uncultured archaeal lineages, p. 18.

rally, for example by entrainment in subsurface flow through basement basalt, and tolerate the subsurface conditions well enough to persist in this environment; or (3) native archaea of the subsurface that originate and grow in this environment. The second and third possibility are not unreasonable, since the number of 16S rRNA phylotypes of MG-I archaea in subsurface samples (Sites 1225 and 1231) exceeds the number of prokaryotic cells that could have contaminated the samples based on perfluorocarbon tracer (PFT) assays (Sørensen et al., 2004). Also, some phylogenetic clusters within the MG-I archaea (clusters ε, η, and ξ; see Fig. F1) appear to consist of sediment and subsurface phylotypes, suggesting evolutionary diversification of these MG-I subgroups in marine sediments and subsurface environments (Sørensen et al., 2004; A. Lauer, unpubl. data).

In contrast to DSAG and MG-I, archaea of the Marine Benthic Groups A (MBG-A) and D (MBG-D) have been detected in fewer samples and sites (Table T1) and usually do not dominate deep subsurface clone libraries. Originally, they were found in 16S rDNA surveys of push cores retrieved from surficial sediments (upper 30 cm) of the Atlantic continental slope and abyssal plain offshore New England (Vetriani et al., 1999). Clones of these groups occur in deep subsurface sediments of Leg 201 (MBG-A at Site 1225, see Figure F1; MBG-D at Sites 1227 and 1230). In contrast to MG-I archaea, they are not detected in the water column and they appear to be benthic, sediment-dwelling archaea. Interestingly, a clone of MBG-A was found in enrichment cultures inoculated with Site 1230 sediment and incubated under aerobic conditions at 10°C (**Biddle et al.,** this volume).

Several uncultured lineages appear in molecular studies of marine, as well as terrestrial, deep subsurface environments. For example, members of the MCG and SAGMEG lineages (Fig. F1) have been found in Leg 201 sediments, especially at Sites 1227 (Inagaki et al., 2006; Sørensen and Teske, in press) and 1229 (Parkes et al., 2005), but also in Mediterranean sapropel sediments (Coolen et al., 2002), and in the deep terrestrial subsurface, such as South African Goldmines (Takai et al., 2001). A similarly mixed habitat range applies to archaea of the Terrestrial Miscellaneous Euryarchaeotal Group (TMEG) lineage (Fig. F1), which have been found in a wide range of terrestrial and freshwater environments and marine subsurface sediments, including Site 1231 (Takai et al., 2001; Sørensen et al., 2004). None of these archaea are cultured, and their physiology remains unknown. It is hoped that the designation "Miscellaneous" in the original names of these archaeal lineages (Takai et al., 2001) will change to a more informative label that reflects the very wide environmental occurrence of these interesting but elusive generalists.

In a few cases, archaeal phylotypes from Leg 201 sediments are specifically related to archaea from hydrothermal vents. Examples include Methanocaldococcus-related 16S rDNA clones from Site 1230 (Inagaki et al., 2006) and archaeal phylotypes from Site 1231 (members of the "Peru Basin cluster") (Fig. F1) that form a monophyletic lineage with an archaeal clone from hydrothermal vent fluids at Juan de Fuca (Huber et al., 2002). These observations pose interesting questions about links between nonhydrothermal subsurface sediments and hydrothermal habitats. As general working hypotheses, hydrothermal archaea may reach deep nonhydrothermal subsurface sediments by subsurface fluid flow, potentially through conduits in basement basalt, following the same flow paths as chemical oxidants (D'Hondt et al., 2004). Conversely, nonhydrothermal sediment archaea could become entrained in hydro-

thermal circulation and are then identified in vent fluids (Sørensen et al., 2004).

Subsurface Bacteria

Among the bacteria, the recently identified candidate division JS-1 (Webster et al., 2004) and the Chloroflexi division (divided into four subphyla) (Hugenholtz et al., 1998; Rappe and Giovannoni, 2003) are well represented in 16S rDNA clone libraries at Leg 201 sites and other subsurface locations (Table T2). The only cultured members of the Chloroflexi Subphyla I, II, and IV include the anaerobic, H_2-dependent dehalogenating bacterium *Dehalococcoides ethenogenes* (Maymó-Gatell et al., 1997) and thermophilic filamentous bacteria that grow chemoheterotrophically on diverse carbohydrates (Sekiguchi et al., 2001, 2003). No cultured member of the JS-1 group is known at present. Of all bacterial phyla found in the deep subsurface, only proteobacterial subsurface clones are sometimes closely related to cultured species, allowing physiological inferences.

T2. Uncultured bacterial divisions, p. 19.

The JS-1 division and the Chloroflexi division are consistently found in diverse subsurface environments, using different methodologies. In addition to the examples in Table T2, members of the Chloroflexi accounted for as much as 69% of total DNA detected by quantitative PCR testing in subsurface sapropel layers of the eastern Mediterranean (Coolen et al., 2002). The JS-1 candidate division was detected with group-specific primers in marine sediments worldwide; deep subsurface sediments as well as in coastal surficial sediments (Webster et al., 2004, and references therein). The uncultured members of the Chloroflexi and JS-1 candidate divisions also predominate at the Peru margin sites.

Sulfate-Reducing and Methanogenic Prokaryotes

The conspicuous methane/sulfate gradients in Leg 201 subsurface sediments have led to working hypotheses that guided initial molecular community surveys. In brief, these large-scale subsurface gradients were thought to be dominated by sulfate-reducing, methanogenic, and methane-oxidizing microbial communities with a clear stratification. Sulfate-reducing prokaryotes should dominate in the sulfate-containing upper sediment layers (Bale et al., 1997; Barnes et al., 1998); methanogenic archaea were expected in the methane-enriched deeper sediment layers, as in some previous subsurface surveys (Marchesi et al., 2001); and sulfate-dependent, methanotrophic consortia analogous to those found at methane seeps and vents (Hinrichs et al., 1999; Boetius et al., 2000; Orphan et al., 2001, 2002; Teske et al., 2002; Michaelis et al., 2002) were expected to dominate the sulfate–methane transition zones. Consequently, 16S rDNA surveys were complemented by functional key gene surveys targeting key genes of sulfate reduction and methanogenesis, dissimilatory sulfate reductase (dsrAB) (Wagner et al., 1998; Klein et al., 2001; Zverlov et al., 2005) and methyl-coenzyme M reductase (mcrA) (Springer et al., 1995; Hales et al., 1996; Luton et al., 2002). Partially modified mcrA primers also cover the mcrA genes of anaerobic, sulfate-dependent, methane-oxidizing archaea (Hallam et al., 2003).

Interestingly, 16S rRNA genes of sulfate-reducing prokaryotes and methanogenic archaea were found only in relatively few cases and suggested that these populations constitute only a minor component in subsurface microbial ecosystems dominated by novel, uncultured archaeal and bacterial phylum-level lineages. Identifiable methanogens

included *Methanocaldococcus*-related phylotypes in the deepest sediment layers (below 200 mbsf) at Site 1230 (Inagaki et al., 2006). Evidence for sulfate-reducing bacteria was similarly spotty. Small numbers of delta-Proteobacterial clones that potentially represent sulfate reducers were found by 16S rRNA gene amplification at Sites 1227 and 1230 but with no apparent depth stratification that mirrored sulfate gradients. At Site 1230, deep, sulfate-free sediment layers (below 150 mbsf) yielded more delta-Proteobacterial clones than the surface layers (Inagaki et al., 2006).

The detection of methanogens and sulfate reducers required the use of primers for key genes of sulfate-reducing and methanogenic pathways. The key gene of methanogenesis, mcrA, was detected with specific primers in nested PCR assays at a few depth horizons at Sites 1229 and 1230. The mcrA phylotypes were related to members of the genera *Methanobrevibacter* and *Methanosarcina* at Site 1229 (Parkes et al., 2005) or formed a sister group to the genus *Methanosaeta* at Site 1230 (Lever and Teske, 2005; Inagaki et al., 2006). As at other ODP sites, the detection of methanogens required the use of group-specific or selective PCR primers, either for methanogen 16S rRNA genes (Marchesi et al., 2001) or for mcrA genes (Newberry et al., 2004). Interestingly, the mcrA phylotypes at sites from ODP Leg 190 (Newberry et al., 2004) were most closely related to *Methanosarcina* and *Methanobrevibacter* genera, as at Site 1229 (Parkes et al., 2005). *Methanosarcina* species use acetate, methylated compounds, and H_2/CO_2 as substrates of methanogenesis (Boone and Mah, 2001); *Methanosaeta* species grow strictly by acetoclastic methanogensis (Patel, 2001); and *Methanobrevibacter* species use H_2 and occasionally formate as methanogenic substrates (Miller, 2001).

Given the dominance of novel, uncultured bacterial and archaeal 16S rRNA gene lineages in all clone libraries recovered from Leg 201 samples, the working hypothesis that deep subsurface sediments are dominated by classical sulfate-reducing, methanogenic, and methane-oxidizing communities appears to be problematic. The most parsimonious explanation is that these classic anaerobic communities exist in low population densities and activities in deep subsurface sediments and create the conspicuous methane–sulfate gradients that have guided the sampling schemes for Leg 201. Very low rates of methanogenesis and sulfate reduction are sufficient to maintain these sulfate and methane profiles (D'Hondt et al., 2004). The dominant populations of novel, uncultured bacteria and archaea in the Leg 201 sediments may not be involved in these processes at all and could rely on other metabolic processes, such as fermentation (Biddle et al., 2006). Alternatively, at least some of the uncultured bacterial and archaeal lineages in Leg 201 sediments could be sulfate reducers, methanogens, or methane oxidizers, perhaps with highly altered key genes that escape PCR detection.

QUANTIFICATIONS OF BACTERIA AND ARCHAEA

Previous quantifications of microbial cells in deep subsurface sediments used generic DNA stain acridine orange and did not allow a separate quantification of bacterial and archaeal cells. In Leg 201 sediments, quantifications of bacteria and archaea were performed by quantitative PCR and by catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH). Quantitative PCR of bacterial and archaeal 16S rRNA genes at Sites 1227 and 1230 indicated that bacterial 16S rRNA genes outnumber archaeal 16S rRNA genes by one to three orders of

magnitude, with considerable site-to-site variability (Schippers et al., 2005). Quantifications with quantitative PCR by another team showed that the archaeal 16S rRNA genes constituted maximally 5%–10% of the total prokaryotic 16S rRNA gene population in the upper 10–20 m of the sediment column (Sites 1227 and 1230); the archaeal proportion dropped below detection limit at greater depths (Inagaki et al., 2006).

Direct counts of bacterial and archaeal cells with the highly sensitive CARD-FISH approach targeting rRNA within metabolically active cells (Pernthaler et al., 2002; Teira et al., 2004) indicated that bacterial cells outnumber archaeal cells in deep marine sediments (Schippers et al., 2005). Bacterial cells constituted ~10%–30% of the total AODC of prokaryotic cells; archaeal cells remained below the statistical detection limit of CARD-FISH counts (Schippers et al., 2005). However, other groups that quantified bacterial and archaeal populations in Leg 201 sediments using CARD-FISH found substantially higher archaeal numbers (Mauclaire et al., 2005; Biddle et al., 2006). Systematic methodological comparisons and experimental standardizations seem to be necessary to resolve these discrepancies.

The observation that CARD-FISH counts account only for 10%–30% of AODC indicates that only a minority of cells harbor detectable amounts of 16S rRNA, the molecule that is the target for CARD-FISH detection. Cellular ribosomal RNA content is roughly proportional to metabolic activity and nutritional state of microbial cells (Poulsen et al., 1993; Fukui et al., 1996; Molin and Givskov, 1999). In other words, the CARD-FISH counts quantify metabolically active, living cells, whereas AODC quantifies living, inactive, and even dead cells, as long as their cellular morphology and integrity remains intact. Thus, CARD-FISH counts give solid evidence for presently active subsurface bacterial and archaeal communities in the deep subsurface, in contrast to the "palaeome" concept that the subsurface accumulates inactive or fossil cells (or their DNA) as a record of past microbial activity (Inagaki et al., 2005).

PHYLOGENETIC AND PHENOTYPIC DIVERSITY OF CULTURED ISOLATES

Cultivations from Leg 201 sediments yielded a wide diversity of cultivable bacteria, predominantly members of two phyla with a Gram-positive cell wall, Firmicutes and Actinobacteria, a range of alpha and gamma-Proteobacteria, and a novel member of the Bacteroidetes phylum that was only distantly related to any other cultured isolate within this phylum (D'Hondt et al., 2004; **Biddle et al.,** this volume; Lee et al., 2005).

The most frequently obtained cultured strains were members of the spore-forming genus *Bacillus* within the Firmicutes, isolated from open-ocean Sites 1231 and 1226 and Peru margin Sites 1227 and 1229. Actinobacteria were found in Pacific open-ocean Sites 1231, 1225, and 1226 and in Peru margin Site 1227 (D'Hondt et al., 2004). Also, species of the newly described thermophilic genus *Thermosediminibacter,* within the phylum Firmicutes, were isolated from the upper 10 m of Sites 1227, 1228, and 1230. The isolates grow anaerobically on a variety of sugars (hexoses and pentoses) and other heterotrophic substrates at a temperature optimum of 64°–68°C (Lee et al., 2005). This wide distribution of Gram-positive, spore-forming bacteria in terrestrial soils and marine sediments may be a consequence of broad dispersal of the highly dura-

ble resting stages of these bacteria, endospores that are resistant against nutrient depletion, desiccation, salinity and temperature fluctuations, radiation, and changing redox conditions. Spores can remain dormant over extremely long time periods; 34 yr for the revival of a historical bacterial culture of known age (Braun et al., 1981), several 1000 yr for spores from archaeological samples and ancient lake sediments (Gest and Mandelstam, 1987), and 25–40 m.y. for spore-forming bacteria that appear to have been revived from the guts of a bee encased in fossil amber (Cano and Borucki, 1995). Thus, the possibility cannot be ruled out that endospore-forming Gram-positive bacteria have been deposited as spores in deep subsurface sediments and have remained dormant until sample retrieval, resuscitation, and isolation. Thermophilic, spore-forming, Gram-positive, sulfate-reducing bacteria of the genus *Desulfotomaculum* (Isaksen et al., 1994) and thermophilic, spore-forming, alkane- and aromatic-degrading *Geobacillus* strains (Marchant et al., 2002) have been found in high numbers in cool marine sediments and cool soils that are too cold for them to grow, indicating effective environmental dispersal.

The most frequently isolated Proteobacteria from Leg 201 sediments include strains closely related to the alpha-Proteobacterium *Rhizobium radiobacter* and to the gamma-Proteobacterium *Vibrio mediterranei* (D'Hondt et al., 2004), although *R. radiobacter* (synonymous with *Agrobacterium tumefaciens*) is a soil bacterium and plant nodule symbiont, therefore an unlikely inhabitant of deep marine subsurface sediments. *R. radiobacter*-related strains have been isolated frequently from subseafloor sediments in the Mediterranean and other deep subsurface environments (Suess et al., 2004). By quantitative PCR, as much as 5% of the total bacterial 16S rDNA gene sequences in Mediterranean sediments were shown to be *R. radiobacter* (Suess et al., 2005). A cultivation survey of surficial and near-surface sediments from Site 1230 yielded gamma-Proteobacteria of the genera *Photobacterium*, *Vibrio*, *Shewanella*, and *Halomonas* (**Biddle et al.,** this volume). Gamma-Proteobacteria appear to be a major group of frequently cultured subsurface bacteria in Leg 201 samples and other subsurface studies. For example, *Vibrio* spp. have been isolated from deep Mediterranean sapropel sediments (Suess et al., 2004).

In general, the spectrum of cultured subsurface isolates (Proteobacteria, Actinobacteria, Firmicutes, and the Cytophaga-Flavobacterium-Bacteroides phylum) is narrower than the diverse lineages of bacteria and archaea that were detected using DNA-based molecular methods. The cultured bacteria appear to constitute only a small fraction of the total cell counts in each sample (D'Hondt et al., 2004). Cultivating representatives of the bacterial and archaeal phyla that dominate subsurface clone libraries remains a continuing challenge.

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Figure F1. Phylogenetic tree (maximum likelihood distance) of marine subsurface archaea and related phylotypes, based on ~900 positions of the 16S rRNA gene, based on PCR amplicons obtained with primers 8F and 915R (Sørensen et al., 2004). For each archaeal lineage discussed in this review and listed in Table T1, p. 18, representative published sequences were obtained from GenBank (Benson et al., 2005). When available, sequences from Sites 1225 and 1231 were used for tree inference. The source habitats of 16S rRNA sequences are color-coded as shown.

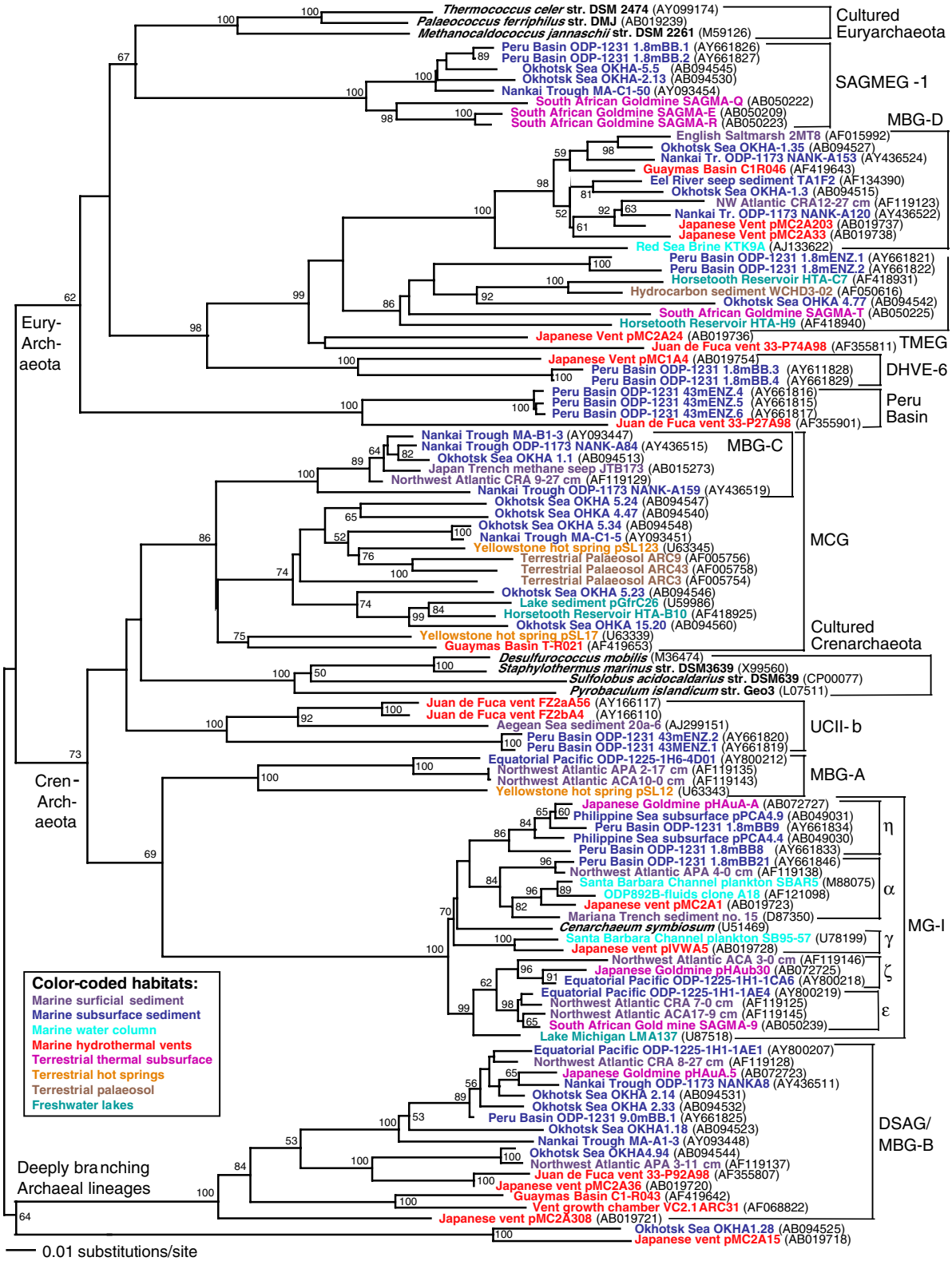

Table T1. Uncultured archaeal lineages in marine sediments.

Location	Leg-site	Water depth (m)	Depth (mbsf)	Archaeal lineages (clone library %)								References
				DSAG/MBG-B	MG-I	MGB-A	MBG-D	MCG	SAGMEG	TMEG	UC-IIb	
Peru margin	201-1229	150	6.7	4			4	88	4			Parkes et al., 2005
			30.2					100				
			42					90	7			
			86.7					93	6			
	201-1227	427	1–151	6*	2*		3*	48*	38.6*			Inagaki et al., 2006
Peru Trench	201-1230	5086	1–278	41.6*	50.7*		1*	1*				Inagaki et al., 2006
Eastern equatorial Pacific	201-1225	3760	1.5	44.4	37	14.8						A. Lauer, unpubl. data
			7.8		15.4	84.6						
Peru Basin	201-1231	4927	1.8		50				11	37		Sørensen et al., 2004
			9	100								
			43								41	
Okhotsk Sea†	NA	1225	1–58	77 (in pelagic clay)			6 (in pelagic clay)	71 (in ash layers)	12 (in ash layers)			Inagaki et al., 2003
Mediterranean sapropel‡	NA	2155	0.13–3.68	2.1*	4.2*			83.3* (58.3 in sapropel, plus 25.0 in sediment)	8.3	2.1*		Coolen et al., 2002
Nankai Trough	NA	945	165	86				14				Reed et al., 2002
			248					100				
			298					28.5	71			
	190-1173	4791	4.15	44.1	14.7	2.9	17.7	20.6				Newberry et al., 2004

Notes: DSAG/MBG-B = Deep-Sea Archaeal Group, synonymous with Marine Benthic Group B (Vetriani et al., 1999) and Deep-Sea Hydrothermal Vent Crenarchaeotic Group (Takai and Horikoshi, 1999), MG-I = Marine Group I (DeLong, 1992, 1998), MBG-A = Marine Benthic Group A (Vetriani et al., 1999), MBG-D = Marine Benthic Group D (Vetriani et al., 1999), MCG = Miscellaneous Crenarchaeotal Group (Takai et al., 2001), SAGMEG = South African Goldmine Euryarchaeotal Groups 1 and 2 (Takai et al., 2001), TMEG = Terrestrial Miscellaneous Euryarchaeotal Group (Takai et al., 2001), UC-IIb = Uncultured Chimney Group II (Schrenk et al., 2003). * = average of all depths. † = alternate layers of pelagic clay and volcanic ash, ‡ = alternate sediment and sapropel layers; see supplementary information in Coolen et al. (2002).

Table T2. Uncultured bacterial divisions in marine sediments.

Location	Leg, site	Water depth (m)	Depth (mbsf)	Bacterial division (clone library %)			Reference
				JS-1	Chloroflexi phylum	Proteobacteria	
Peru margin	201-1229	150	6.7, 30, 42, 86.7	<1*	55*	30*	Parkes et al., 2005
	201-1227	427	1–151	12.5*	64.5*	6*	Inagaki et al., 2006
Peru Trench	201-1230	5086	1–278	60*	4*	7*	Inagaki et al., 2006
Nankai Trough	190-1176	3017	1, 51, 98, 194	18*	13*	36*	Kormas et al., 2003
	190-1173	4791	4.15	0.53	Not found	0.25	Newberry et al., 2004
	NA	945	165	16	13.5	26.5	Reed et al., 2002
			248	22	4	22	
			298		15	6	
Okhotsk Sea†	NA	1225	1–58	58 (average in pelagic clay)	22 (average in pelagic clay)	83 (average in ash) 4 (average in clay)	Inagaki et al., 2003

Notes: JS-1 = Japan Sea-1 Candidate Division (Webster et al., 2004). Chloroflexi phylum synonymous with Green Non-Sulfur (GNS) bacterial division (Hugenholtz et al., 1998; Hugenholtz, 2002). In contrast to JS-1 and Chloroflexi clones, Proteobacterial clones are sometimes closely related to cultured species and strains. * = average of all depths. For Leg 190 Site 1176 Nankai Trough values, contaminant clones (*E. coli, Acinetobacter, Aquaspirillum* spp.) were excluded from analysis. † = alternate layers of pelagic clay and volcanic ash.