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6. Microbial Populations in Ocean Floor Basalt: Results from ODP Leg 187[1]

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ABSTRACT

The microbial population in samples of basalt drilled from the north of the Australian Antarctic Discordance (AAD) during Ocean Drilling Program Leg 187 were studied using deoxyribonucleic acid (DNA)-based methods and culturing techniques. The results showed the presence of a microbial population characteristic for the basalt environment. DNA sequence analysis revealed that microbes grouping within the *Actinobacteria*, green nonsulfur bacteria, the *Cytophaga*/*Flavobacterium*/*Bacteroides* (CFB) group, the *Bacillus*/*Clostridium* group, and the beta and gamma subclasses of the *Proteobacteria* were present in the basalt samples collected. The most dominant phylogenetic group, both in terms of the number of sequences retrieved and the intensities of the DNA bands obtained with the denaturing gradient gel electrophoresis analysis, was the gamma *Proteobacteria*. Enrichment cultures showed phylogenetic affiliation with the *Actinobacteria*, the CFB group, the *Bacillus*/*Clostridium* group, and the alpha, beta, gamma, and epsilon subclasses of the *Proteobacteria*. Comparison of native and enriched samples showed that few of the microbes found in native basalt samples grew in the enrichment cultures. Only seven clusters, two clusters within each of the CFB and *Bacillus*/*Clostridium* groups and five clusters within the gamma *Proteobacteria*, contained sequences from both native and enriched basalt samples with significant similarity. Results from cultivation experiments showed the presence of the physiological groups of iron reducers and methane producers. The presence of the iron/manganese-reducing bacterium *Shewanella* was confirmed with DNA analysis.

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The results indicate that iron reducers and lithotrophic methanogenic *Archaea* are indigenous to the ocean crust basalt and that the methanogenic *Archaea* may be important primary producers in this basaltic environment.

INTRODUCTION

Over the past 10 yr, geological and microbial investigations have provided textural, chemical, and molecular evidence for the existence of a microbial community capable of weathering ocean floor basalt (e.g., Thorseth et al., 1992, 1995, 2001; Giovannoni et al., 1996; Fisk et al., 1998; Torsvik et al., 1998; Furnes and Staudigel, 1999). Information about microbial communities, especially their requirements for carbon and energy sources and their phylogenetic and physiological properties, is important to develop further understanding of subsurface biogeochemical processes.

The primary microbial objective for Leg 187 was to identify microbes living in the basalt. This was accomplished by a combination of cultivation strategies and 16S ribosomal deoxyribonucleic acid (rDNA)-based methods.

Stevens and McKinley (1995) showed that microcosms containing crushed basalt and groundwater from the Columbia River Basalt Group supported microbial growth and proposed that microbial communities could be maintained on geochemical energy obtained from igneous rock. Lithoautotrophic microorganisms have been proposed as the primary producers in this subsurface ecosystem (Stevens, 1997). Hydrogen, either produced by serpentinization (Berndt et al., 1996) or by oxidation of ferromagnesian silicates (Stevens and McKinley, 1995), and reduced iron, manganese, and sulfur might be available as electron donors. However, a publication by Anderson et al. (1998) found that no hydrogen was produced from basalt-groundwater interactions at an environmentally relevant pH to support microbial growth in the subsurface. Oxidized iron and manganese are available as electron acceptors. The basaltic glass contains additional small amounts of phosphorus and metals that can be used as mineral nutrients by microorganisms.

Enrichment approaches are useful for understanding the physiology of isolated organisms, but this approach alone does not provide comprehensive information about the microbial community (Amann et al., 1995). Suzuki et al. (1997) investigated a marine environment and concluded that most abundant members of this microbial community were not readily culturable. Consequently, most work on microbial community structure involves methods based on deoxyribonucleic acid (DNA) analysis as a means to assess the diversity and distribution of microorganisms (e.g., Muyzer et al., 1993; Øvreås et al., 1997). These methods rely upon the sequence information of genes that are universally conserved, yet sufficiently different to reflect the phylogeny of the microbes. Denaturing gradient gel electrophoresis (DGGE) is used to resolve polymerase chain reaction (PCR)-amplified regions of gene encoding for 16S rDNA. This method is based solely on differences in the nucleotide sequence (Muyzer et al., 1993). The DNA sequences retrieved during this study were the V3 region of the 16S rDNA. The V3 region is a highly variable region of the 16S rDNA gene and gives a good indication of phylogenetic affiliation (Muyzer et al., 1993).

Interpretation of the results is complicated by possible contamination of samples with microbes from seawater, sediment, drilling mud,

and from postcollection processing of samples. Textural evidence of microbial activity and observations of cell morphologies is found on the surface of the pillow lava, mainly in alteration zones of fractures in the volcanic glass (e.g., Thorseth et al., 1995; Fisk et al., 1998; Torsvik et al., 1998). Smith et al. (2000) investigated the drilling-induced microbial contamination during Ocean Drilling Program (ODP) Leg 185, using both a chemical and a particulate tracer, and found that the particulate tracer was never detected in the interior of sediment cores, nor was it found in the interior of rock samples, and concluded that the contamination of cores by the fluorescent microspheres was minimal. This study involves a similar tracer test with fluorescent microspheres. It should be noted that although the microspheres mimic the size of microorganisms, their surface properties are different, which may cause a difference in migration into pores and cracks and attachment to mineral surfaces.

MATERIALS AND METHODS

Sample Collection and Preparation

Samples were collected during ODP Leg 187 to the north of the Australian Antarctic Discordance (AAD), Southeast Indian Ridge (Christie, Pedersen, Miller, et al., 2001; Thorseth et al., 2003). Samples of basalt, crystalline or breccia, with or without glassy margins were collected from the 18- to 28-Ma crust (Table T1). The water depth at the sites was 4000–5000 m, and the thickness of the sediment layer was up to 233 m. The maximum penetration was 374.2 meters below seafloor (mbsf). Igneous rock and sediment were recovered by using a rotary core barrel (RCB). The sediment cores were called "wash cores" because the RCB was pushed into the sediment layer and an unknown quantity of sample material passed through the core. Polycarbonate core liners were inserted into the core barrels, and after the core arrived on the deck the core liner was split longitudinally. The sample collection and preparation is described further in Christie, Pedersen, Miller, et al. (2001). The samples were placed under anaerobic and cold conditions within 30 min after the core arrived on deck. Sediment samples, surface seawater, collected by lowering a sterile bottle from the ship, and sepiolite (drilling mud) were collected for comparison. Surface seawater was used as drilling fluid. The seawater and the sepiolite samples were used for molecular analysis only. A list of all samples taken for microbial studies is given in Tables T1 and T2.

T1. Igneous rock collected for microbial studies, p. 20.

T2. Sediment, seawater, and drilling mud collected for controls of microbial studies, p. 22.

The samples for DNA analysis were transported frozen to Bergen, Norway, on dry ice. The enrichment cultures were transported at ~0°C, but were unfortunately stored at ~19°C for 1 week during transportation from Fremantle, Australia, to Bergen. This may have led to a loss of the original microbial diversity in the enrichments.

Primary Enrichment Cultures

Seventeen types of bacterial culture media, twelve anaerobic and five aerobic, were used to enrich viable microbial populations from the rock and sediment samples. Eight different media based on filtered (0.2-µm pore size) and autoclaved natural seawater (anoxic or oxic) either without additions or with the addition of methanol, lactate, succinate, glucose, or yeast extract as carbon sources, were used. In addition, the

following media were used: for iron reducers (iron basal culture medium: Lovley and Phillips, 1988, and Fe-TSB [iron-tryptone soya broth] medium: Küsel et al., 1999), manganese oxidizers (PYGV medium: Staley, 1968), sulfate reducers (W20: Widdel and Bak, 1992), methanogenic *Archaea* (methanogenic medium 2: Jones et al., 1983), and methanotrophic bacteria (NMS medium: Hanson et al., 1992). Approximately 1 g of crushed rock sample was added to each 10-mL bottle of microbial enrichment medium.

In addition, 4–5 g of crushed rock was added to two different types of microcosms, aimed at enriching microbes participating in the cycling of iron and manganese, in 20-mL tubes filled with anaerobic seawater. One contained Mn^{4+} as an electron acceptor, and the other contained Fe^{3+}. Chitin, pectin, and acetate were added as carbon sources. The setup was designed to establish a gradient from anaerobic conditions at the bottom of the tube to aerobic conditions at the top. Small amounts of oxygen were added at the top of this gradient.

Sediment enrichment cultures, used as controls, were set up on the same microbial enrichment media as basalt, except that no microcosms were started with sediment inoculum.

All enrichment cultures were incubated at 4°C. Growth was determined by phase-contrast microscopy after ~1 month of incubation.

Analysis of Metabolic Products

Sulfide was measured in sulfate-reducing media (W20) according to the quick colorimetric method described by Cord-Ruwisch (1985). Methane was measured in cultures of methanogenic medium by gas chromatography. Reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) in cultures for iron-reducing bacteria (IRB) were observed as a color change from reddish brown to light gray. All analyses of metabolic products were performed after ~1 month of incubation.

16S rDNA Amplification

PCR amplification (95°C for 15 min, 30–36 cycles of denaturation at 92°C for 1 min; annealing at 55°C for 30 s; extension at 72°C for 1 min; and a final extension at 72°C for 10 min) of the V3-region of 16S rDNA using HotStar Taq DNA polymerase (Qiagen) was performed in a GeneAmp2400 thermal cycler (PerkinElmer Applied Biosystems, USA). The bacterial primers PRUN518r and PRBA338f (Øvreås et al., 1997) were used. This primer set amplifies a 236 base-pair DNA segment.

Aliquots of 5 µL of PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. No DNA analysis was performed for *Archaea*.

DGGE Analysis of 16S rDNA Fragments

PCR products were further analyzed by DGGE. For DGGE analysis, 1-mm-thick polyacrylamide gels (8% (weight/volume) acrylamide-bis-acrylamide; Bio-Rad) were prepared with and electrophoresed in 0.5× TAE (0.04-M Tris base, 0.02-M sodium acetate, 1-mM EDTA [pH 7.4]). Next, 15 µL of PCR product mixed with 3 µL of loading buffer (blue-orange loading buffer; Promega) was added to the DGGE wells and a prerun (20 V for 10 min) was performed to concentrate the DNA on the bottom of the wells. The DGGE conditions were 25%–65% urea gradient, 18 hr, 70 V, and 65°C.

The gels were stained with a 1:10,000 dilution of SYBR Gold (Molecular Probes) in 1× TAE for 30 min and examined under ultraviolet light.

Extraction of PCR Products from DGGE Gels and 16S rDNA Sequencing

Samples of individual DGGE bands were obtained by excising small cores of the gel with sterile 1000-µL pipette tips. These gel cores were added to sterile 1.5-mL microcentrifuge tubes with 20 µL of sterile double-distilled water for elution of the DNA from the gel (diffusion of DNA into the water overnight at 4°C). Subsequently, these samples were reamplified and the DNA was sequenced.

Reamplified PCR products of excised DGGE bands were purified with the QIAquick PCR Purification kit (Qiagen). The BigDye Terminator Cycle Sequencing kit (PerkinElmer Applied Biosystems, USA) was used for direct sequencing in the PCR machine (25 cycles of denaturation at 96°C for 15 s, annealing at 50°C for 10 s, and extension at 60°C for 2 min). Sequencing reactions were analyzed with an ABI 377 DNA sequencer (PerkinElmer), and the obtained DNA sequences were analyzed using the BLAST tool (Karlin and Altschul, 1990) at the National Center for Biotechnology Information (NCBI) World Wide Web site (**www.ncbi.nlm.nih.gov**).

Evolutionary distances of the DNA sequences were calculated using Clustal_X software (Thompson et al., 1997), and phylogenetic trees were constructed using the neighbor-joining algorithm (Saitou and Nei, 1987). The phylogenetic trees were bootstrapped (1000 bootstrap replicates) using the Clustal_X program. For simplifying reasons, identical or near-identical (>97% similarity) sequences are presented as one operational taxonomic unit (OTU).

The PCR-DGGE analysis of partial 16S rDNA was chosen because it is practical to use with a large number of samples, even though phylogenetic information is lost because of a short fragment length. The DGGE fingerprinting method also provides useful information on community structure and can be used to view the similarities and differences between samples.

Nucleotide Sequence Accession Numbers

The sequences reported in this paper have been deposited into GenBank (Benson et al., 2000) under accession numbers AY129833 through AY129946.

Contamination Tracer Test

To examine possible drilling-induced contamination, yellow-green fluorescent carboxylate microspheres (Polysciences, Inc.) 0.518 µm (±0.021 µm) in diameter were used as a particulate tracer (Smith et al., 2000). The microsphere suspension was diluted to a concentration of ~10^{10} mL^{-1}, which was then poured into a plastic bag and secured inside the core barrel. The first core to enter the barrel ruptured the bag and dispersed the microspheres into the core barrel. After splitting the core liner, rock surfaces were washed with distilled water and the water was filtered onto polycarbonate filters. To check for microspheres within rocks, thin sections were prepared without any special precautions. For sediment cores, smear slides were prepared from both the outer part

and the interior of the cores. Filters, thin sections, and smear slides were examined under an epifluorescence microscope with a blue filter set. The contamination tracer test is further described in Smith et al. (2000) and in Christie, Pedersen, Miller, et al. (2001).

RESULTS

Primary Enrichment Cultures and Analysis of Metabolic Products

Enrichment cultures were set up on various carbon and energy sources as shown in Table T3. A total of 461 basalt enrichment cultures and 25 sediment cultures were started on board, and after ~1 month, growth was observed in 366 of the basalt enrichments and 17 of the sediment cultures (Table T3).

Methane was produced in 12 out of 25 basalt enrichment cultures with a methanogenic medium containing $H_2 + CO_2$ as energy and carbon sources (Table T4). No methane was detected in sediment enrichment cultures or in basalt enrichment cultures to which acetate or trimethylamine was added as energy and carbon sources. Reduction of ferric iron to ferrous iron was observed in four of the 78 media designed for IRB and inoculated with basalt; all of these were grown on the Fe-TSB medium. Iron reduction was not detected in IRB media inoculated with sediment (Table T4). Sulfide production was not observed in any of the enrichment cultures set up for sulfate reducing bacteria, either with basalt or with sediment as inoculum (Table T4).

Phylogenetic Affiliation

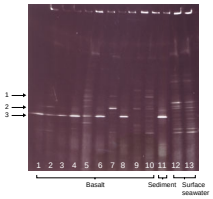
The DNA band profiles of bacterial populations from native samples of basalt, sediment, and seawater differed (Fig. F1). Figure F1 shows a representative selection of samples of basalt, sediment, and seawater from different sites and depths to illustrate similarities and differences in the bacterial community structures. Results from sequencing analysis also confirmed that the dominant bacterial population in basalt was different from that of sediment and seawater (Figs. F2, F3, F4, F5, F6).

The DGGE analysis showed a relatively high diversity of bacteria in native basalt samples, which was visualized as several DNA bands on the gel (Fig. F1). However, as seen in Figure F1, each basalt sample had only one or a few dominant bands, indicating a limited number of dominant members of the population in these samples. Three dominant populations (DNA bands 1–3 in Fig. F1) were common to most of the basalt samples. The sequencing results of the common DNA bands found in the basalt samples in lanes 7, 9, and 10 (DNA band 1 in Fig. F1), corresponding to the OTU sequence named ODP-1161A-1162A-1163A in Figure F2, were closely related (99% similarity) to the uncultured gamma *Proteobacterium* clone 33-PA57B00 (accession number AF469304), previously isolated from subseafloor habitats associated with a deep-sea volcanic eruption (B. Huber et al., unpubl. data). The common DNA bands found in basalt samples in lanes 2, 3, 4, 7, 9, and 10 (band 2 in Fig. F1), corresponding to the OTU sequences named ODP-1162B-1163A and ODP-1160B-1161A-1162A in Figure F2, were related (97%–99% similarity) to *Acinetobacter junii,* a gamma *Proteobacterium* associated with both bacterial infections (Higgins et al., 2001) and aquatic environments (Guardabassi et al., 1999). One dominant band

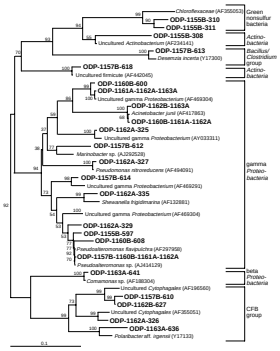
T3. Growth in enrichment cultures after ~1 month of incubation, p. 23.

T4. Results from analysis of the metabolic products, p. 25.

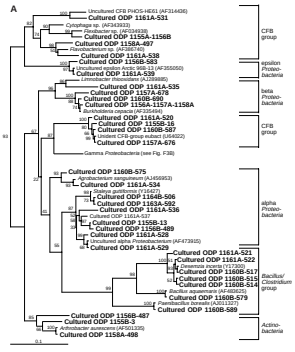
F1. DGGE analysis of 16S rDNA gene fragments, p. 13.



F2. Native basalt and reference strains, p. 14.



F3. Enriched basalt and reference strains, p. 15.



(band 3 in Fig. F1) was present in most basalt samples and was also found in the sediment sample. The sequencing results (Figs. F2, F5) showed that the bands in lanes 3, 4, 5, 6, 8, and 11, corresponding to OTU ODP-1157B-1160B-1161A-1162A in Figure F2 and the sequence ODP-sediment-1154B-644 in Figure F5, were 99% similar to the gamma *Proteobacterium Pseudoalteromonas flavipulchra,* previously isolated from seawater (Ivanova et al., 2002). The DNA band in lane 2, named ODP-1160B-600 in Figure F2, seemed to be in the same position on the gel but was only 87% similar to *P. flavipulchra*. Two DNA bands in lanes 1 and 10 were also in the same position on the DGGE gel, but these bands were not sequenced.

The 16S rDNA sequences retrieved from microbes in native basalt samples indicated phylogenetic affiliation with six main groups of the domain *Bacteria: Actinobacteria*, green nonsulfur bacteria, the *Cytophaga/Flavobacterium/Bacteroides* (CFB) group, the *Bacillus/Clostridium* group, and the beta and gamma subclasses of *Proteobacteria* (Fig. F2). The most abundant phylogenetic group present in the basalt samples, based on the number of partial DNA sequences retrieved, was the gamma *Proteobacteria*. The closest known relatives to these sample sequences were *P. flavipulchra* (Ivanova et al., 2002), the metal reducer *Shewanella* (Bowman et al., 1997), the marine bacterium *Marinobacter* (Coates et al., 2002), and representatives of several unidentified gamma *Proteobacteria* isolated from subseafloor habitats associated with a deep-sea volcanic eruption (B. Huber et al., unpubl. data).

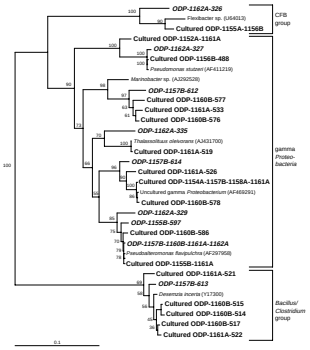
Results from the DNA analysis of microbes from enrichment cultures inoculated with basalt showed phylogenetic affiliation with seven main groups of *Bacteria: Actinobacteria*, the CFB group, the *Bacillus/Clostridium* group, and the alpha, beta, gamma, and epsilon subclasses of the *Proteobacteria* (Fig. F3A, F3B). As in the case of native basalt, the majority of these partial sequences are grouped with the gamma *Proteobacteria* (Fig. F3B). The DNA analysis revealed that few of the microbes from native basalt samples could be cultured (Fig. F4). The sequences affiliated with the green nonsulfur bacteria, found in native basalt samples, were not found in basalt enrichment cultures. Sequences grouping within the alpha and epsilon subclass of the *Proteobacteria* were found in basalt enrichment cultures but not in native basalt samples.

The sequences obtained from both basalt and seawater with the highest similarity were ODP-1157B-614 (Fig. F2) and ODP-seawater-1162-651 (Fig. F5), which were 96% similar. All other sequences, when aligned, showed 92% or less similarity. The sequences from microbes from native basalt and sediment, with the exception of the sequences clustering with *P. flavipulchra,* showed 93% or less similarity (Figs. F2, F5).

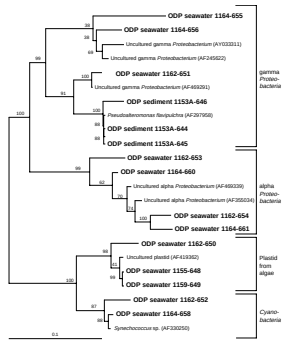
DNA analysis of microbes from sediment enrichment cultures showed phylogenetic affiliation with four main groups of bacteria: the CFB group, the *Bacillus/Clostridium* group, and the alpha and gamma subclasses of the *Proteobacteria* (Fig. F6). The sequences obtained from enriched basalt and sediment with the highest similarity were Cultured-ODP-1155B-18 (Fig. F3B) and Cultured-ODP-sediment-1154B-663 (Fig. F6), which were 100% similar, and the sequences clustering with the uncultured alpha *Proteobacterium* (Figs. F3A, F6), with 95%–96% similarity. All other sequences showed 93% or less similarity.

The sepiolite (drilling mud) sample did not yield a PCR product, and DNA sequences have therefore not been obtained from this sample.

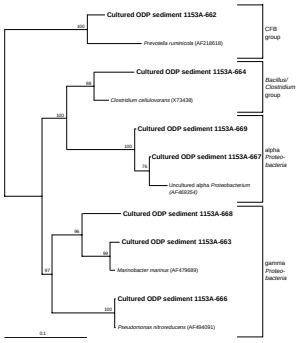
F4. Native basalt vs. cultured basalt and reference strains, p. 17.



F5. Native sediment and seawater and reference strains, p. 18.



F6. Cultured sediment and reference strains, p. 19.



Contamination Tracer Test

The fluorescent microsphere data for the igneous rock samples showed that microspheres were detected in wash water from the exterior of all rock samples examined (Table T5). In many of the thin sections, microspheres were found located both inside fractures and on the thin section surfaces. However, six of the thin sections lacked microspheres. In the two sediment cores examined, fluorescent microspheres were not found (Table T5).

T5. Results from the contamination tracer test, p. 27.

DISCUSSION

Previous studies on microbes associated with ocean floor basalt from the Arctic Ridges and the East Indian Ridge (Thorseth et al., 2001, 2003), based on scanning electron microscopy (SEM) and DNA-based techniques, show high numbers of microbes and several different cell morphologies inhabiting the basalt environments. These microbes were often associated with manganese and iron deposits. Thorseth et al. (2003) investigated alteration of basaltic glass and the endolithic microbes from dredged and drilled (ODP Leg 187) samples from the AAD area by SEM techniques and found a high number of microbes inhabiting the AAD-dredged samples. Microbes were also present in the fracture zones of the Leg 187 samples.

Results from the cultivation approach showed growth in 366 of the 461 basalt enrichment cultures (Table T3). Methane measurements showed the presence of microbes in the basalt capable of producing methane from H_2 and CO_2 (Table T4). This implies that microbial biomass can be produced lithotrophically in the ocean crust basalt without the supply of external energy and carbon sources. The methane produced by these primary producers can then be used by methane-oxidizing bacteria as a source of carbon and energy. Attempts to grow methane-oxidizing bacteria on NMS medium failed, however, and the DNA analysis did not reveal any known methane oxidizers in the enrichment cultures.

Results from basalt enrichment cultures showed iron reduction in four of the IRB cultures (Table T4). The DNA-based analysis showed the presence of microbes closely related to the iron and manganese reducer *Shewanella* (Figs. F2, F3B). The sequences resembling *Shewanella* were mainly retrieved from the iron and manganese microcosms. In addition, DNA analysis showed that the four Fe-TSB cultures contained an unidentified iron reducer. The iron reducers observed in this study grew in media supplied with organic energy and carbon sources. Lithotrophic iron reduction was not observed in these enrichments. Since iron oxidizes spontaneously when oxygen is present, iron oxidation cannot be taken as evidence for microbial activity. The DNA analysis did not yield sequences that could be associated with any known iron-oxidizing bacteria. Thus it was not possible to demonstrate microbial iron oxidation in the basalt samples collected for this study. Manganese, on the other hand, does not oxidize spontaneously in the presence of oxygen, so enrichment mediums for manganese oxidizers (PYGV medium) were included in this work. Observations of manganese oxyhydroxides in basalt samples (Christie, Pedersen, Miller, et al., 2001) indicated that manganese oxidizers could be part of this environment, but attempts to grow manganese oxidizers failed and the large amount of PYGV cultures did not yield DNA sequences belonging to

any known manganese oxidizers. The PYGV medium contains only small amounts of manganese, so direct observation of manganese oxidation in the cultures was not possible.

No evidence for sulfate reduction was found in this study. Sulfide was not detected in cultures aimed at enrichments of SRB (Table T4), and the DNA analysis of basalt samples showed no similarity with any known sulfate reducers (Figs. F2, F3). Sulfate-reducing bacteria could be expected to be found in the sediment samples (Table T4; Figs. F5, F6). A possible explanation for this lack of SRB is that sediments were collected from a depth of 151.9–267.6 mbsf (Table T2). Assuming that the only source of sulfate is seawater and the sulfate was consumed in the upper part of the sediment layer, at this depth the sulfate was most likely depleted. During ODP Leg 182 to the Australian Bight, high concentrations of sulfate and sulfide were found in the sediments (Feary et al., 1999). Almost no sulfides were found in either sediment or basalt cores drilled during Leg 187 (Christie, Pedersen, Miller, et al., 2001). Pore water chemistry was not analyzed.

The PCR-DGGE analysis showed that the DNA band patterns of microbes from basalt differed from microbes from seawater and sediment, with the exception of the dominant band found in both basalt and sediment samples (Fig. F1), resembling *P. flavipulchra* with 99% similarity. If basalt was contaminated with sediment during drilling, it would be expected to be contaminated by all the microbes in the sediment, not by only one bacterium. It is therefore more likely that this bacterium inhabits both basalt and sediment, and its presence in basalt samples was not due to contamination. Both native basalt and basalt enrichment cultures were dominated by gamma *Proteobacteria*, but the DNA analysis showed that few of the microbes found in native samples could be cultured. Comparison of similar (>90%) sequences obtained from native basalt samples and basalt enrichment cultures showed that the sequences cluster within three main groups of *Bacteria* (Fig. F4): the CFB group, the *Bacillus*/*Clostridium* group, and the gamma *Proteobacteria*. Within the CFB group, no sequences showed >90% similarity. Within the *Bacillus*/*Clostridium* cluster, five sequences from enriched basalt samples all showed 97% similarity to the sequence from native basalt, indicating that these microbes belonged to the same genus. Within the gamma *Proteobacteria*, the sequences grouped within five smaller clusters resembling *Pseudomonas stutzeri, Marinobacter* sp., *Thalassolituus oleivorans,* an uncultured gamma *Proteobacterium*, and *P. flavipulchra,* respectively. The similarity between sequences from enriched and native samples in the *P. stutzeri* cluster was 99%–100% and 96%–100% in the *P. flavipulchra* cluster. The sequences from enriched microbes that were 100% similar to the microbes in the native basalt, indicating that these microbes could be identical, would be key organisms for studying the physiology of microbial populations in the basalt. Within the *Marinobacter* cluster, the similarity was 95%–96%, and within the uncultured gamma *Proteobacterium* cluster, 96%–97%, indicating that the sequences belonged to the same genus. Within the *T. oleivorans* cluster, the similarity was 92% between sequences from native and enriched basalt samples, which indicates that these sequences belonged to different genera. In the cases where members of the same species or genera were observed in sequences from both native and enriched basalt samples, physiological studies of these microbes can be performed in order to understand the function they have in the basalt environment.

DGGE separation and 16S rDNA sequencing of PCR products allowed a rapid assessment of community structures in different samples, sites,

and depths (Figs. F1, F2, F3, F4, F5, F6). The 16S rDNA V3 region is highly variable and a good phylogenetic indicator (e.g., Muyzer et al., 1993; Øvreås et al., 1997).

A previous study by Thorseth et al. (2001), investigating microbes in seafloor basalt from the Knipovich Ridge, found bacterial 16S rDNA fragment sequences grouping within the CFB group and the gamma and epsilon *Proteobacteria*. Sequences grouping within the gamma *Proteobacteria* were related to *Pseudoalteromonas, Pseudomonas,* and *Acinetobacter.* These are the same genera found to be abundant in the basalt samples collected in this study. The microbes inhabiting the ocean crust of Arctic Ridges and the AAD could be similar, although probably not identical.

The tracer contamination test showed the presence of fluorescent microspheres on the exterior of all igneous rock samples (Table T5). Microspheres were also found on the surface and inside fractures of some of the thin sections, which could be caused by relocation of microspheres during polishing of the thin sections. This is in accordance with Smith et al. (2000), who did a similar tracer test during ODP Leg 185 and found that microspheres were present in thin sections but not in the interior of rock samples that were crushed after the outer part of the rock was split off. They concluded that the contamination of cores was minimal. In the present study, the DNA profiles (Fig. F1) and sequencing results (Figs. F2, F3, F4, F5, F6) also indicate minimal contamination, as the microbial basalt population differed from that found in seawater and sediment.

In conclusion, the results from this study showed the presence of characteristic microbial populations indigenous to the basalt environment. Our results further suggest that iron reducers and methanogens are key physiological groups present in the ocean crust basalt.

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Figure F1. DGGE analysis of PCR-amplified 16S rDNA gene fragments from uncultured samples of basalt (lanes 1–10), sediment (lane 11), and seawater (lanes 12-13). The positions and numbering of bands discussed in the text are indicated with arrows. The samples included in this figure are taken from Cores 187-1155B-2R (lane 1), 187-1160B-1W (lane 2), 187-1160B-4R (lane 3), 187-1160B-8R (lane 4), 187-1157B-8R (lane 5), 187-1161A-3R (lane 6), 187-1161A-4R (lane 7), 187-1162A-5R (lane 8), 187-1162B-2R (lane 9), 187-1163A-8R (lane 10), 187-1153A-4W (lane 11), and Site 1162 seawater (lane 12), and Site 1164 seawater (lane 13).

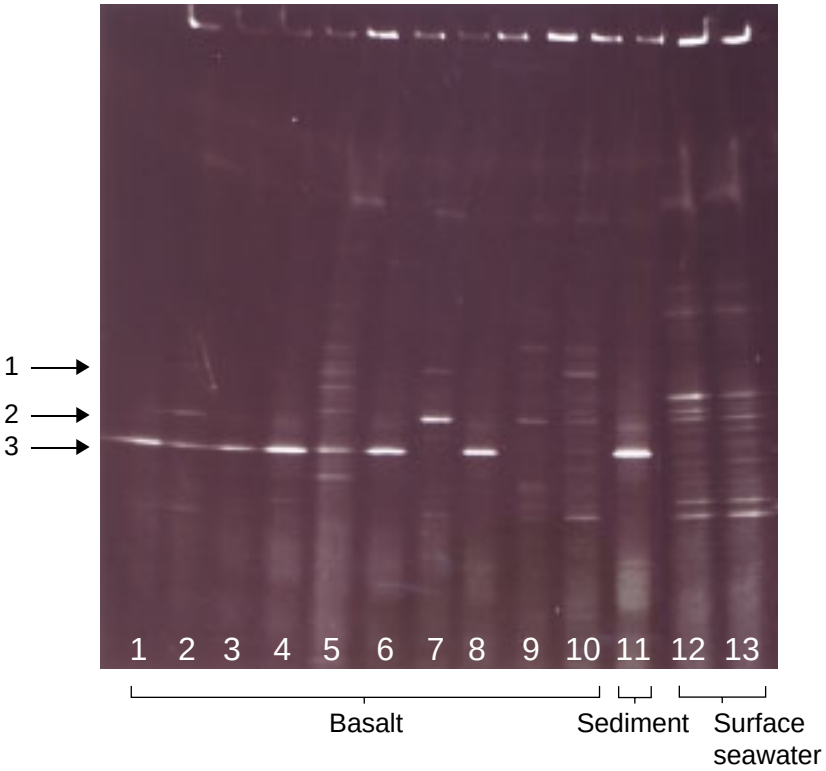

Figure F2. Phylogenetic tree of the partial 16S rDNA sequences from microbes from native basalt samples (in bold, named ODP-Hole-Sequence number) and reference strains (with accession numbers in parenthesis) obtained from GenBank. Reference strains were chosen as the closest known relatives to the sample sequences. Bootstrap values, indicated at the nodes, were obtained from 1000 bootstrap replicates and are reported as percentages. The scale bar corresponds to 0.1 change per nucleotide. CFB = *Cytophaga/Flavobacterium/Bacteroides*.

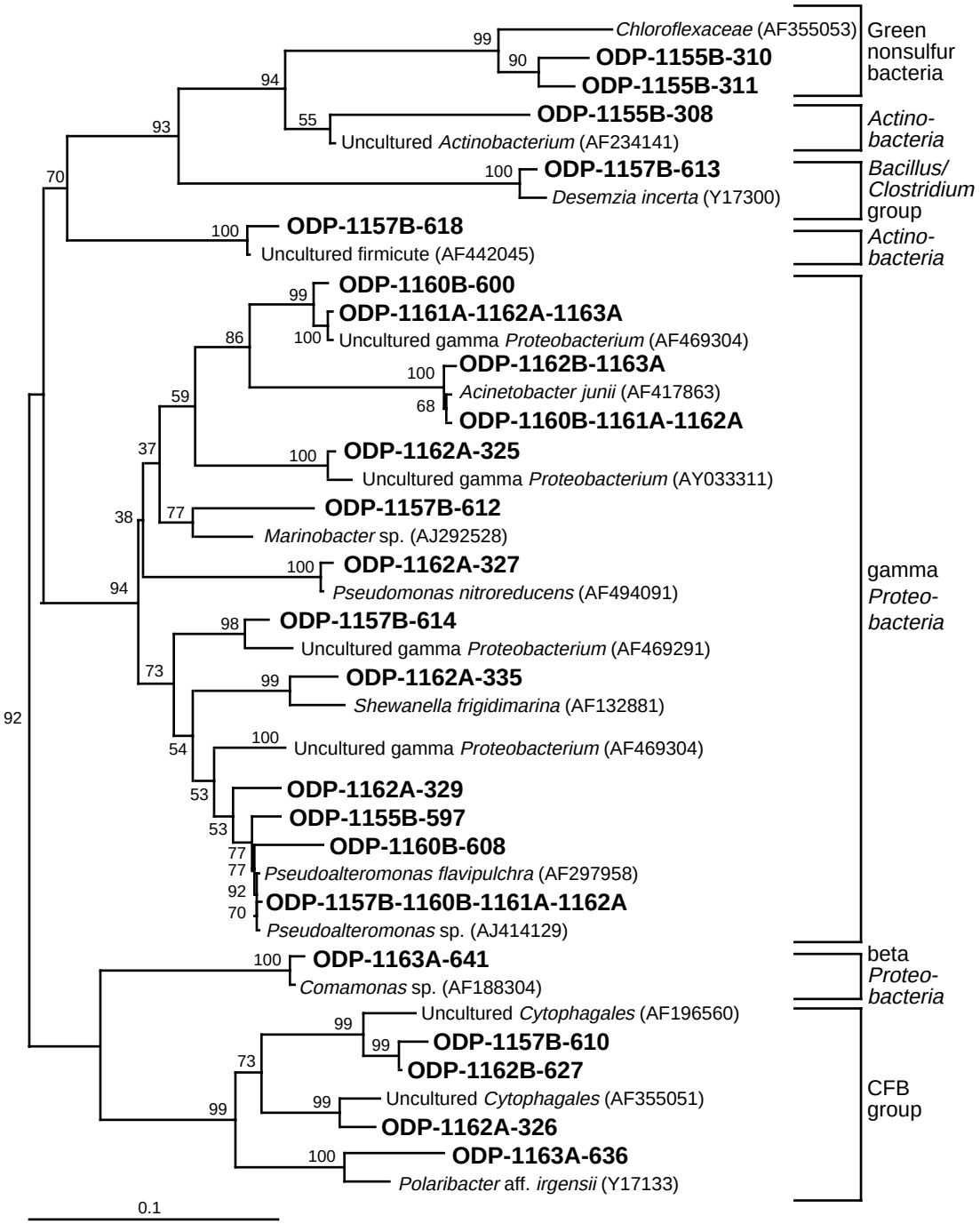

Figure F3. A. Phylogenetic tree of the partial 16S rDNA sequences, except the gamma *Proteobacteria* (see Fig. F3B, p. 16), from microbes from enriched basalt samples (in bold, named Cultured ODP-Hole-Sequence number), and reference strains (accession numbers in parenthesis). Bootstrap values, indicated at the nodes, were obtained from 1000 bootstrap replicates and are reported as percentages. The scale bar corresponds to 0.1 change per nucleotide. CFB = *Cytophaga/Flavobacterium/Bacteroides*. (Continued on next page.)

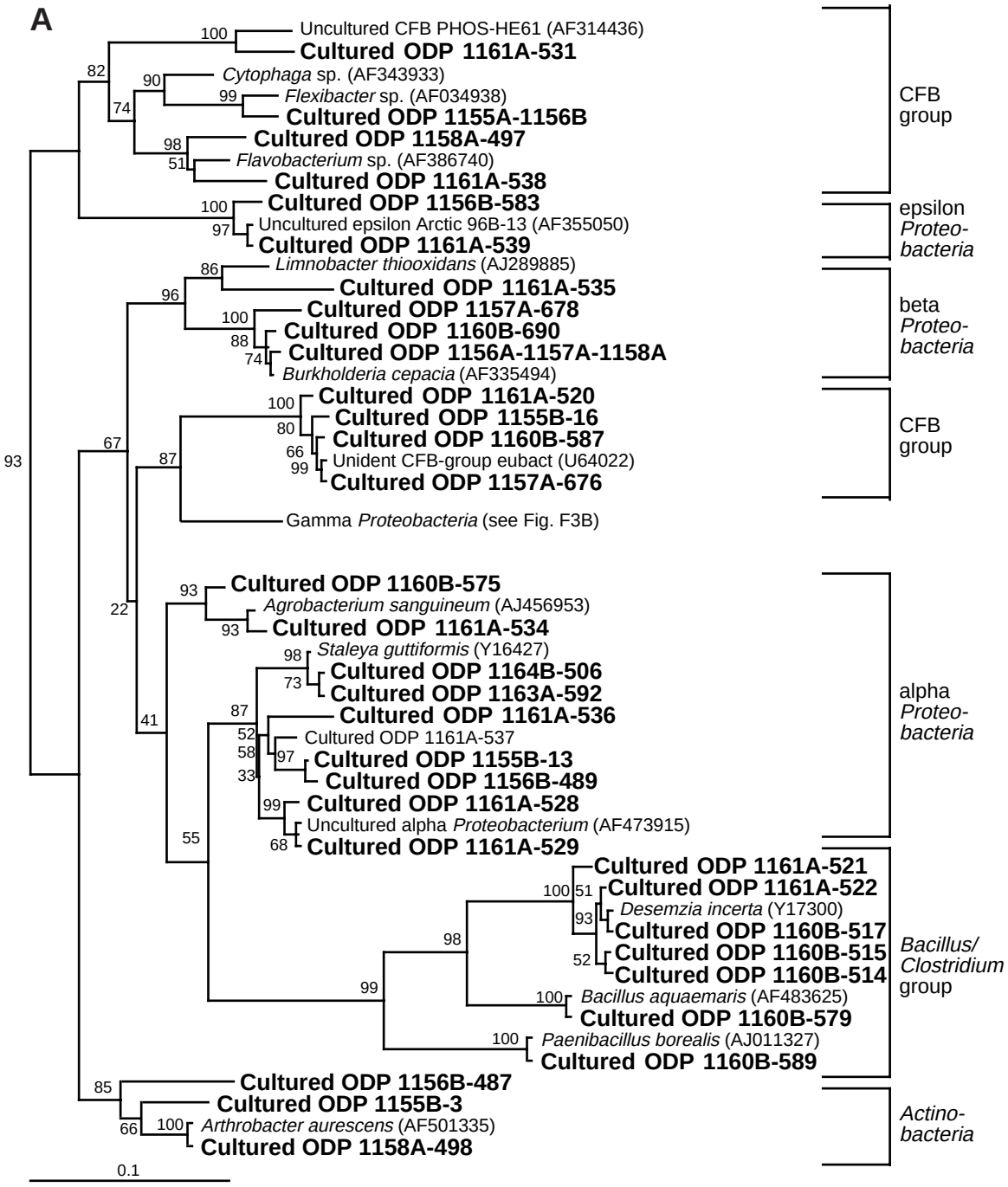

Figure F3 (continued). B. Phylogenetic tree of the partial 16S rDNA sequences grouping within the gamma *Proteobacteria* from microbes from encircled basalt samples and reference strains.

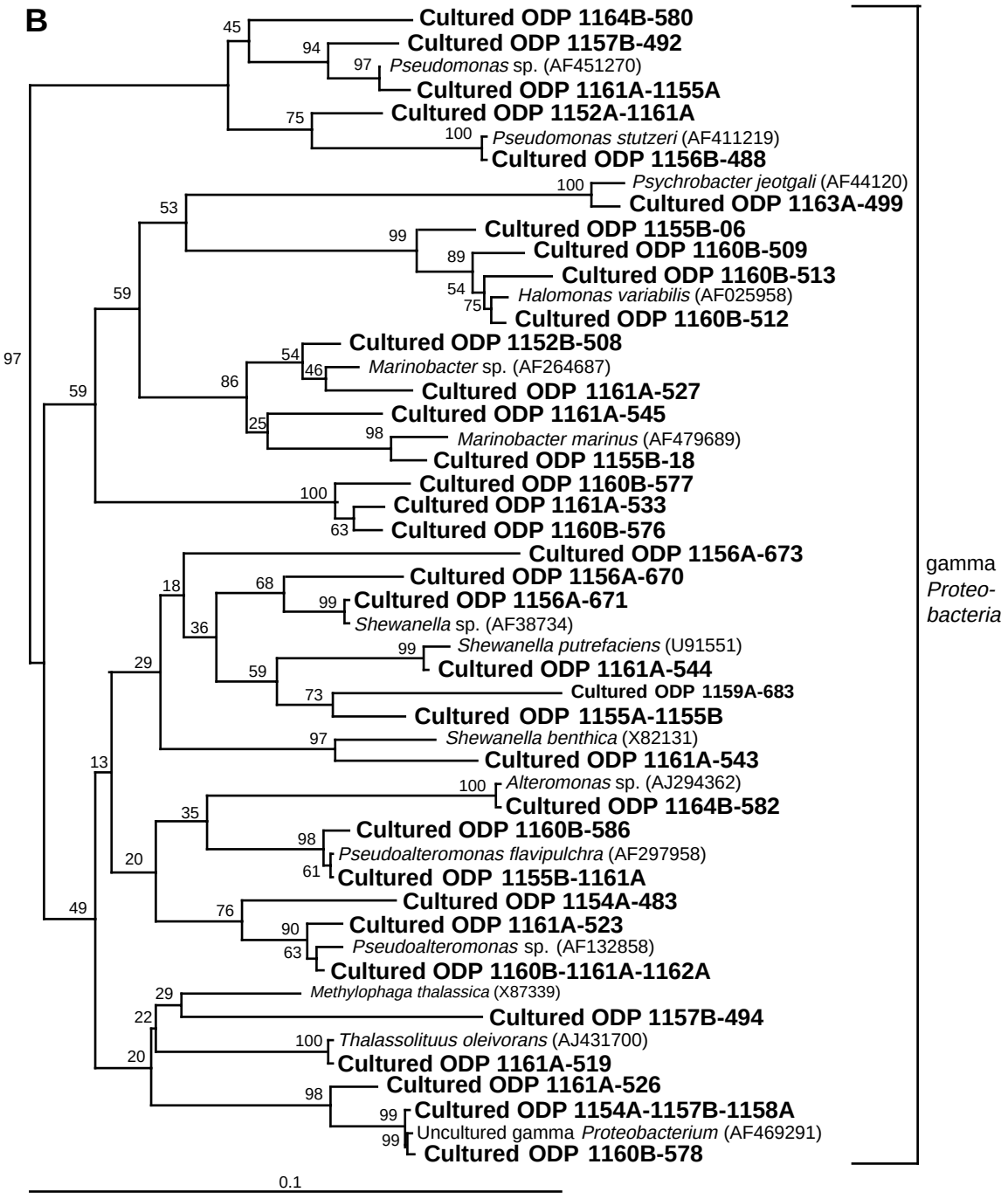

Figure F4. Phylogenetic tree of the partial 16S rDNA sequences from microbes from native basalt samples (in italic bold) compared to sequences from cultured basalt samples (in bold) and reference strains (with accession numbers in parenthesis). Bootstrap values were obtained from 1000 bootstrap replicates and are reported as percentages. The scale bar corresponds to 0.1 change per nucleotide. CFB = *Cytophaga/Flavobacterium/Bacteroides*.

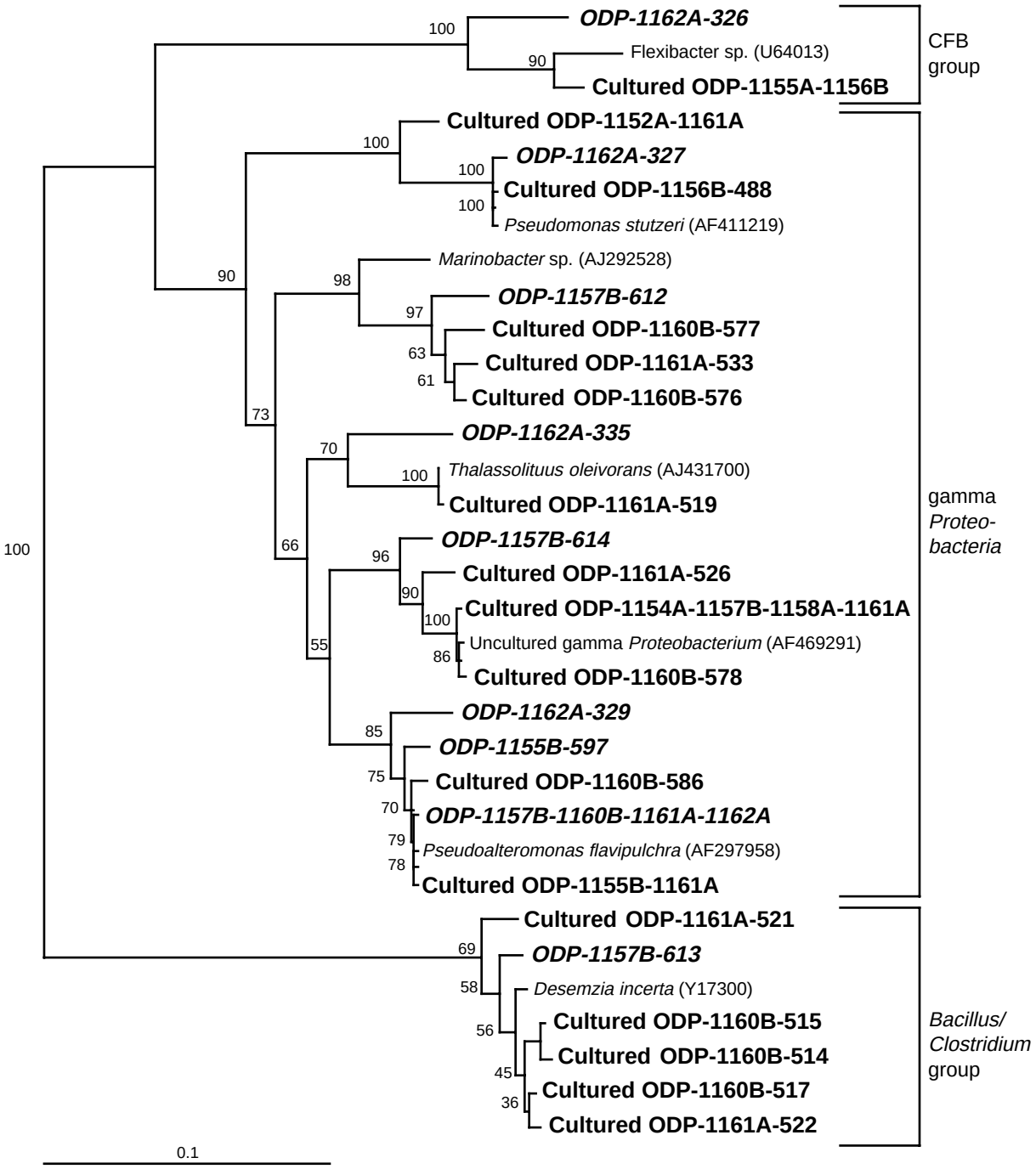

Figure F5. Phylogenetic tree of the partial DNA sequences from microbes from native sediment and seawater samples (in bold, named ODP-Sample type-Hole-Sequence number) and reference strains (accession numbers in parenthesis). Bootstrap values were obtained from 100 bootstrap replicates and are reported as percentages. The scale bar corresponds to 0.1 change per nucleotide.

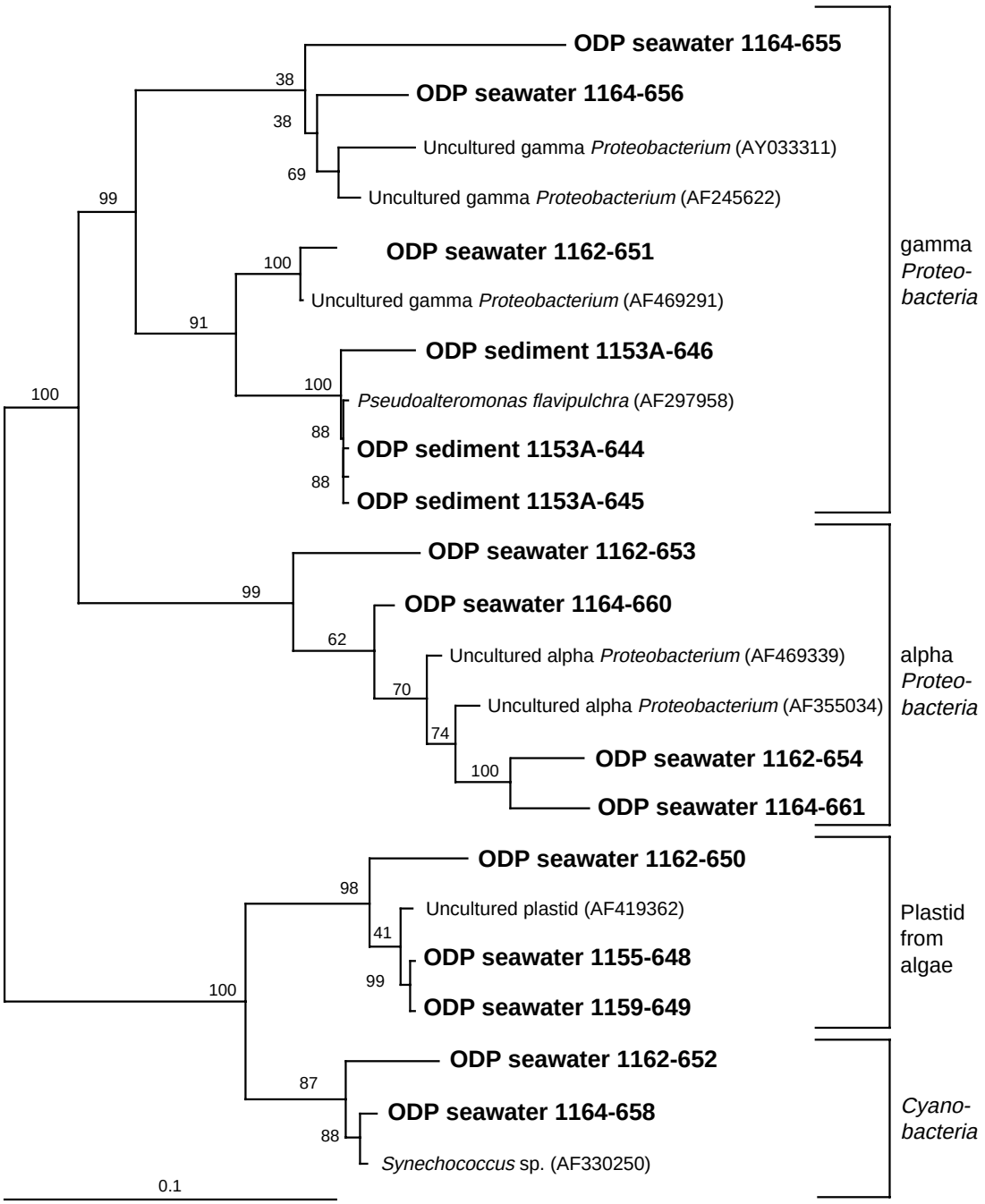

Figure F6. Phylogenetic tree of the partial DNA sequences from microbes from cultured sediment samples (in bold, named Cultured ODP sediment Hole-Sequence number) and reference strains (accession number in parenthesis) Bootstrap values (1000 bootstrap replicates) are reported as percentages. The scale bar corresponds to 0.1 change per nucleotide.

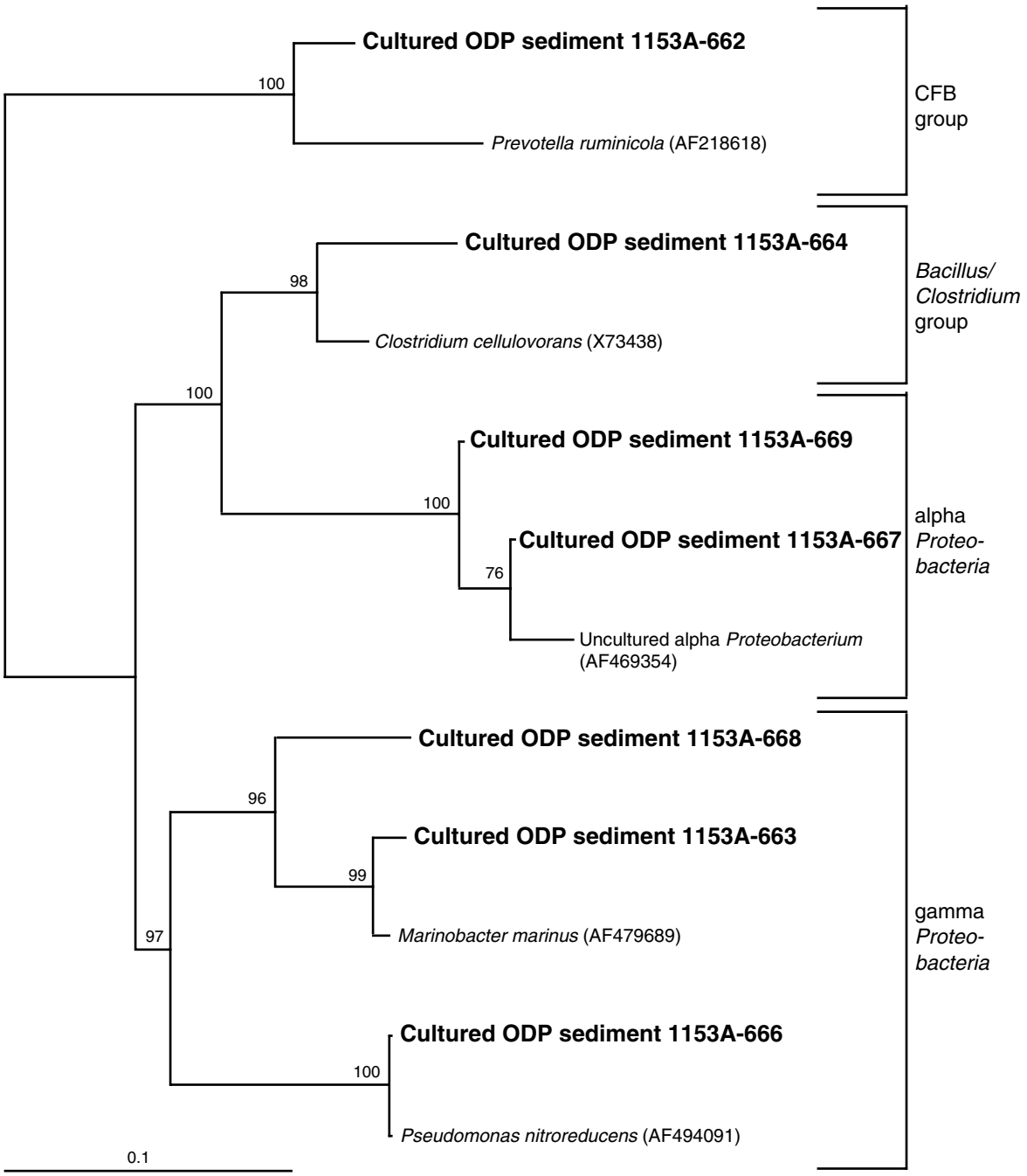

Table T1. Samples of igneous rock collected for microbial studies. (See table notes. Continued on next page.)

Core, section	Piece	Depth (mbsf)	Age (Ma)	Sample type		
				Glass	Crystalline	Breccia
187-1152A-						
1R-1	1A	0–11.2	25	–	+	
187-1152B-						
4R-1	12	35.8–40.8	25	+	+	
187-1153A-						
8R-1	15A	267.6–274.9	28	+	+	
187-1154A-						
3R-1	3A	237.2–241.7	28	–	+	
6R-1	10A	251.3–256.5	28	+	+	
9R-1	3A	265.8–267.6	28	+	+	
187-1155A-						
2R-1	1A	177.3–180.8	24.5	–	+	
187-1155B-						
2R-1	6A	147.9–151.5	24.5	+	+	
3R-1	6A	151.5–155.4	24.5	+	+	
5R-1	14A	160.1–165.1	24.5	+	+	
8R-1	16A	174.7–179.4	24.5	+	+	
187-1156A-						
3R-3	1	124.6–129.6	22	?	+	
187-1156B-						
2R-1	14	181.6–187.4	22	+	+	
5R-1	7	205.9–210.2	22	–	+	
6R-1	4	210.2–215.2	22	+	+	
187-1157A-						
3R-1	20	206–215.4	22.5	+		+
187-1157B-						
2R-1	7	130.6–138.5	22.5	–	+	
4R-2	10	143–147.8	22.5	+	+	
8R-1	6A	161.4–166.4	22.5	+	+	
187-1158A-						
2R-1	8	198.9–203.9	21	–	+	
187-1158C-						
2R-1	10	108–117.4	21	–	+	
187-1159A-						
2R-1	7	145.6–148.6	14	+	+	
3R-1	10A	148.6–152.8	14	+	+	
6R-2	11	161.6–166.7	14	+		+
187-1160B-						
1W-1	3A	0–160.1	21.5	+	+	
4R-1	2	169.5–174.1	21.5	+	+	
6R-1	18	178.9–188	21.5	–	+	
8R-1	0–87 cm*	193.7–197.2	21.5	+	+	
187-1161A-						
3R-1	24	120–129.4	19	+	+	
4R-1	10	129.4–138.8	19	+		+
187-1162A-						
3R-1	3	340.2–345.8	18	?		+
5R-1	19	355.2–364.6	18	?		+
187-1162B-						
2R-1	1	348.4–355	18	+		+
6R-1	25	369.2–374.2	18	+		+
187-1163A-						
2R-1	5	161–165.4	16	+	+	
4R-1	9	171.4–175.9	16	+	+	
8R-1	4	189.9–194.1	16	+		+
10R-1	5	199.1–203.1	16	–	+	

Table T1 (continued).

Core, section	Piece	Depth (mbsf)	Age (Ma)	Sample type		
				Glass	Crystalline	Breccia
187-1164B-						
3R-1	11	155.8–160.8	17	+	+	+
8R-1	7	188.5–197.5	17	+	+	
10R-1	1	206.8–216.1	17	+	+	

Notes: The sample type is indicated with a "+" for crystalline or breccia type of rock and a "+" or "–" for the presence or absence, respectively, of basaltic glass on the sample piece. R = core was sampled using a rotary core barrel, W = sample was taken from a wash core. * = because of a mix-up, the core piece of this sample is uncertain and the whole-core section is reported.

Table T2. Samples of sediment, seawater, and drilling mud collected for controls of microbial studies.

Core, section, interval (cm)	Depth (mbsf)	Sample type		
		Sediment	Seawater	Sepiolite
187-1153	Surface*		+	
187-1153A-	151.9–209.6	+		
4W-2, 92–97				
7W-3, 69–7	243–267.6	+		
187-1155	Surface*		+	
187-1158	Surface*		+	
187-1159	Surface*		+	
187-1162	Surface*		+	
187-1164	Surface*		+	
Drill mud				+
Drill mud			+	+

Notes: The sample type is indicated with a "+" for presence of sediment, seawater, or sepiolite (drilling mud) in the sample. * = seawater was collected from the sea surface.

Table T3. Growth in primary enrichment cultures after ~1 month of incubation at 4°C. (See table notes. Continued on next page.)

	Microbial media																	Microcosms	
Core	Anox	Anox + lac	Anox + succ	Anox + glu	Anox + YE	W_2O + H_2 + CO_2	W_2O + ac	IB	Fe–TSB	Met + H_2 + CO_2	Met + ac	Met + TMA	Ox	Ox + metOH	Ox + YE	NMS + CH_4	PYGV	Fe	Mn
187-1152A-																			
1W	+							+	+				–			+	+		
187-1152B-																			
4R	–	–		+	+	+	+	+	+	+	+		+			+	+		
187-1153A-																			
4W*	+	+		+	+	+	–	+	+		+		–			+	+		
7W*	–	–		–	–	+	+	+	+	+	+		–			+	–		
8R	–	–		–	+	–	+	+	–	+	–		–			+	+		
187-1154A-																			
3R	+	–		+	+	+	+	+	+	+	+		+			+	+		
6R	+		+		–	+		+		+		+	+		+	+	+		
9R	+		–		–	+		–	–	+		+	+	–		+	+		
187-1155A-																			
2R	–	–	–				+	+	+		+	+	+			+	+		+
187-1155B-																			
2R	+	+	+			+	–	+	+	+			+			+	+	+	
3R	+	–		+	+	+	+	+	+		+	+	+			+	+		+
5R	–	–	–				+	+	+	+	+		–			+	+	–	
8R	–	+		–	–	+	+	+	+			+	+			+	+		+
187-1156A-																			
3R	+												+						+
187-1156B-																			
2R	+	+		+	+		+	+	+	–	+		+			+	+	+	+
5R	+	+	+		–		+	+	+	+		+	+			+	+		
6R	–	–						+	+	+						+	+	+	
187-1157A-																			
3R	+	+	+		–	+	+	+	+	+			+			+	+		+
187-1157B-																			
2R	–		–					+	–	–		+	–				+		
4R	+	–	–		–		–	+	+		+		+			+	+	+	
8R	–	–	–		+	–	+	+	–	+			+			+	+		
187-1158A-																			
2R	+	+		+	–		+	+	+	+		+	+			+	+		+
187-1158C-																			
2R	+	+				+	+	+	+	+			+			+	+	+	
187-1159A-																			
2R	+	+					+		+				+						
3R	–	–	–		+		+	+	+	+	+		+			+	+	+	
6R	+	+																	+

Table T3 (continued).

Core	Microbial media																	Microcosms	
	Anox	Anox + lac	Anox + succ	Anox + glu	Anox + YE	W_2O + $H_2 + CO_2$	W_2O + ac	IB	Fe–TSB	Met + $H_2 + CO_2$	Met + ac	Met + TMA	Ox	Ox + metOH	Ox + YE	NMS + CH_4	PYGV	Fe	Mn
187-1160B-																			
1W	+	+		–	–		–	–	+	+		+	+			+	+		+
4R	+	+		–	–	+		+	+	+			–			+	+	+	
6R	–	–		–	+	+	+	+	–	+			–			+	+		
8R																		+	+
187-1161A-																			
3R	+	+	+				–	+	+	+		+	+			+	+		
4R	–	–	+				+	+	+	+		+	+			+	+		+
187-1162A-																			
3R	+	+	+		+	+	+	+	+	+			+			+	+		
5R	+	+	+		+		+	+	+	+	+		+			+	+		
187-1162B-																			
2R	+	+		+	+		–	+	–				+			+	+		
6R	+	+		+	+		+	+	+				–			+	+		
187-1163A-																			
2R	+	+			+		+	+	+				+			+	+	+	
4R	+	+		+	+		+	+	+				+			+	+		
8R	+	+		+	+		+	+	+				+			+	+		
10R	+	+		+	+		+	+	+	+			+			+	+		
187-1164B-																			
3R	+	+		+	+	+	+	+	+	+			+			+	+		
8R	+	+		+	+		+	+	+	+			+			+	+		
10R	+	+		+	+		+	+	+				+						

Notes: Growth was observed by phase-contrast microscopy and is shown as "+" (growth) or "–" (no growth). A blank space means that no enrichment culture was started for this particular sample and medium. Anox = anoxic seawater, Ox = oxic seawater, IB = iron basal culture medium, Met = methanogenic medium 2, lac = lactate, succ = succinate, glu = glucose, YE = yeast extract, ac = actetate, TMA = trimetylamine, and metOH = methanol. * = sediment samples.

Table T4. Results from analysis of the metabolic products H_2S, Fe^{2+}, and CH_4 in enrichment cultures aimed at detecting sulfate-reducing bacteria, iron-reducing bacteria, and methanogenic *Archaea,* respectively. (See table notes. Continued on next page.)

	H_2S production		CH_4 production			Fe(III) reduction	
Core	W_2O + H_2+CO_2	W_2O + acetate	Met + H_2+CO_2	Met + acetate	Met + TMA	IB	Fe-TSB
187-1152A-							
1W						–	–
187-1152B-							
4R	–	–	–	–		–	–
187-1153A-							
4W*	–	–		–		–	–
7W*	–	–	–	–		–	–
8R	–	–	+	–		–	–
187-1154A-							
3R	–		–	–		–	–
6R	–		–		–	–	
9R	–		–		–	–	–
187-1155A-							
2R		–		–	–	–	–
187-1155B-							
2R	–	–	+			–	+
3R	–	–		–	–	–	+
5R		–	–	–		–	–
8R	–	–			–	–	–
187-1156A-							
3R							
187-1156B-							
2R		–	–	–		–	–
5R		–	–		–	–	–
6R			–			–	–
187-1157A-							
3R	–	–	–			–	–
187-1157B-							
2R			+		–	–	–
4R		–		–		–	–
8R	–	–	–			–	–
187-1158A-							
2R		–	+		–	–	–
187-1158C-							
2R	–	–	+			–	–
187-1159A-							
2R		–					–
3R		–	+	–		–	–
6R							
187-1160B-							
1W		–	+		–	–	–
4R	–		+			–	–
6R	–	–	+			–	+
8R							
187-1161A-							
3R		–	+		–	–	+
4R		–	+		–	–	–
187-1162A-							
3R	–	–	–			–	–
5R		–	+	–		–	–
187-1162B-							
2R		–				–	–
6R		–				–	–

Table T4 (continued).

Core	H_2S production		CH_4 production			Fe(III) reduction	
	W_2O + H_2+CO_2	W_2O + acetate	Met + H_2+CO_2	Met + acetate	Met + TMA	IB	Fe-TSB
187-1163A-							
2R		–				–	–
4R		–				–	–
8R		–				–	–
10R		–	–			–	–
187-1164B-							
3R	–	–	–			–	–
8R		–	–			–	–
10R		–				–	–

Notes: Results are shown as "+" or "–". Blank space = no enrichment culture was started for this particular sample and medium. * = enrichments of sediment samples.

Table T5. Results from the contamination tracer test, where 0.5-µm fluorescent microspheres were used to examine possible drilling induced contamination.

Core	Sample type		Observed microspheres		
	Rock	Sediment	Exterior	Inside fractures	On thin section surface
187-1153A-					
3W		+	–	– *	
187-1153A-					
6W		+	–	– *	
8R	+		+	–	–
187-1154A-					
3R	+		+	+	+
6R	+		+	+	+
9R	+		+	–	–
187-1155A-					
2R	+		+	–	–
187-1155B-					
2R	+		+	–	–
9R	+		+	–	–
187-1156A-					
3R	+		+	+	+
187-1156B-					
2R	+		+	+	+
187-1157A-					
2R	+		+	+	+
187-1157B-					
2R	+		+	+	+
8R	+		+	+	+
187-1158A-					
2R	+		+	+	+
187-1158C-					
2R	+		+	+	+
187-1159A-					
2R	+		+	–	–
187-1162A-					
2R	+		+	+	+
3R	+		+	†	†
187-1162B-					
2R	+		+	+	+
11R	+		+	+	+
187-1163A-					
2R	+		+	+	+
187-1164A-					
2R	+		+	†	†
187-1164B-					
2R	+		+	+	+
10R	+		+	+	+

Notes: * = for sediment cores, samples from the center of the cores were examined for microspheres. † = no thin section was made to examine the interior of the rock for microspheres. Table shows presence ("+") and absence ("–") of microspheres on exterior parts, inside fractures, and on thin section surfaces of igneous rock and sediment samples.