Edwards, K.J., Bach, W., Klaus, A., and the Expedition 336 Scientists
Proceedings of the Integrated Ocean Drilling Program, Volume 336

Data report: cultivation of microorganisms from basaltic rock and sediment cores from the North Pond on the western flank of the Mid-Atlantic Ridge, IODP Expedition 336[1]

Hisako Hirayama,[2] Mariko Abe,[2] Junichi Miyazaki,[2] Sanae Sakai,[2] Yuriko Nagano,[3] and Ken Takai[2]

Chapter contents

Abstract 1
Introduction 1
Materials and methods 2
Results 4
Acknowledgments 5
References 5
Figure 8
Tables 9

[1]Hirayama, H., Abe, M., Miyazaki, J., Sakai, S., Nagano, Y., and Takai, K., 2015. Data report: cultivation of microorganisms from basaltic rock and sediment cores from the North Pond on the western flank of the Mid-Atlantic Ridge, IODP Expedition 336. *In* Edwards, K.J., Bach, W., Klaus, A., and the Expedition 336 Scientists, *Proc. IODP,* 336: Tokyo (Integrated Ocean Drilling Program Management International, Inc.). doi:10.2204/iodp.proc.336.204.2015
[2]Department of Subsurface Geobiological Analysis and Research, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka 237-0061, Japan. Correspondence author:
hirayamah@jamstec.go.jp
[3]Department of Marine Biodiversity Research, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka 237-0061, Japan.

Abstract

Cultivation experiments targeting chemolithoautotrophic microorganisms were performed using subseafloor basaltic cores (the deepest sample is from 315 meters below seafloor [mbsf] and overlying sediment cores (the deepest sample is from 91.4 mbsf) from North Pond on the western flank of the Mid-Atlantic Ridge. The cores were recovered by the R/V *JOIDES Resolution* during Integrated Ocean Drilling Program Expedition 336. Different bacteria were grown under different media and temperature conditions. In the enrichment cultures of the basaltic cores under aerobic conditions, frequently detected bacteria at 8°C and 25°C were members of the genera *Ralstonia* (the class Betaproteobacteria) and *Pseudomonas* (Gammaproteobacteria), whereas members of the genera *Paenibacillus* (Bacilli) and *Acidovorax* (Betaproteobacteria) were conspicuous at 37°C. *Bacillus* spp. (Bacilli) were outstanding at 37°C under anaerobic conditions. In the enriched cultures of the sediment cores, bacterial growth was observed at 15°C but not at 37°C, and the bacteria detected at 15°C mostly belonged to gammaproteobacterial genera such as *Pseudomonas*, *Halomonas*, and *Marinobacter*. All of the bacteria detected in this study were enriched only, and subcultivation of the enriched cultures in the respective original media did not succeed. The presence of hydrogenotrophic methanogens was examined by a culture-dependent or a culture-independent analysis in the basalt and sediment cores but was not proven. A fungal isolate was obtained from a single basaltic core and belonged to the genus *Exophiala* of the order Chaetothyriales.

Introduction

Ocean crust of young age is generally rich in reduced substances such as Fe(II) and sulfur (Bach and Edwards, 2003). Hydrogen was found to be produced from the ocean crust by abiotic basalt-water reactions at low temperature (Stevens and Mckinley, 1995, 2000). These reduced substances in the ocean crust can serve as electron donors for endolithic microorganisms. Meanwhile, seawater is oxic and contains oxygen, nitrate, and sulfate that are preferably utilized as electron acceptors by microorganisms. The circulation of seawater through the upper ocean crust can therefore energize potential endolithic communities in which chemolithoauto-

trophs utilizing Fe(II), sulfur, and hydrogen play a role as primary producers. This type of crustal biosphere is most likely to exist on young ridge flanks (Fisher et al., 2003; Lever et al., 2013; McCarthy et al., 2011). The North Pond site located on the western flank of the Mid-Atlantic Ridge is one of ideal study sites to examine the ridge flank crustal biosphere (see the **"Expedition 336 summary"** chapter [Expedition 336 Scientists, 2012a]).

The results of several cultivation studies targeting endolithic or crustal fluid microorganisms have been reported previously. The results from experiments using basaltic core samples collected during Ocean Drilling Program (ODP) Leg 187 at Southeast Indian Ridge (maximum penetration was 374 meters below seafloor [mbsf]) presented the methane production and Fe(III) reduction in enrichment cultures, indicating the existence of active hydrogenotrophic methanogens and Fe(III)-reducing microorganisms within the cores (Lysnes et al., 2004). Cultivation of seafloor basalts collected at low- and high-temperature fluid vent systems along the Mid-Atlantic Ridge detected diverse bacteria belonging to the classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria, and Bacilli, and some of the isolates that were assigned to the genus *Bacillus* were shown to grow on a medium specific for manganese oxidizers (Rathsack et al., 2009). In a study of an Integrated Ocean Drilling Program (IODP) site on the Juan de Fuca Ridge flank, in situ colonization systems deployed in the borehole for 4 years revealed the presence of active and cultivable Fe(II)-oxidizing bacteria belonging to eight genera, including gammaproteobacterial *Alcanivorax*, *Marinobacter*, and *Halomonas* as the predominant genera; however, all cultivated bacteria that utilized low-organic compounds for their growth were not proven to be facultative chemolithoautotrophs in that study (Smith et al., 2011). The existence of methanogens and sulfate reducers in the subseafloor basalt was indicated by a combination of molecular phylogenetic, isotopic, and cultivation analyses using cores collected at the Juan de Fuca Ridge flank during IODP Expedition 301 (Lever et al., 2013). Ocean crust is also inferred to provide habitats for fungi; it has been used to examine fossilized fungi-like filamentous or spherical structures found in thin sections of basaltic cores collected during ODP legs (Ivarsson et al., 2012; Schumann et al., 2004).

Although many microorganisms have been previously enriched or isolated from ocean crust as mentioned above, most of those seem to be heterotrophs. We therefore tried to cultivate chemolithoautotrophic microorganisms from cores of basalt and overlying sediments retrieved by the R/V *JOIDES Resolution* from a North Pond site during IODP Expedition 336.

Materials and methods

Subsampling of cores

Pieces of rock cores collected for microbiology were subsampled for several analyses (see the **"Methods"** chapter [Expedition 336 Scientists, 2012b]), and a portion was used for this study. Each piece of rock (10–20 cm^3 per piece) was immediately transferred into the anaerobic chamber settled in the onboard cold room after the subsampling and then smashed using a tungsten carbide cylinder mortar. The smashed rock was put into a 100 mL glass bottle and sealed tightly with a butyl rubber cap in the anaerobic chamber. The bottles were kept cool before starting shore-based cultivations.

A 10 cm whole-round sediment sample was cut from a sediment core on the catwalk for this study (see the **"Methods"** chapter [Expedition 336 Scientists, 2012b]) and kept at 4°C before further processing. The following process was promptly conducted on the onboard laboratory bench to minimize laboratory contamination and exposure to oxygen. The surface of the whole-round sample was trimmed with a stainless steel spatula, and 10 cm^3 of the sediment was taken from the center with a cut syringe. The syringe sample was put into a 100 mL glass bottle, and the headspace was purged with nitrogen gas. The bottle was sealed tightly with a butyl rubber cap. The bottles were kept at 4°C before starting shore-based cultivations. Another portion of the sediment sample was taken for DNA analysis; a syringe sample (7–10 cm^3) was put in a plastic tube, and frozen at –20°C and kept frozen before the shore-based analysis.

Preparation of growth media

Cultivation media used in this study are summarized in Table **T1.** For hydrogenotrophic methanogens, 20 mL of marine medium salts in artificial seawater (MMJ) medium was prepared in a 70 mL glass vial as previously reported (Takai et al., 2002). For other microorganisms, respective cultivation media were prepared using the basic seawater medium. The basic seawater medium was prepared by adding 0.06 g KH_2PO_4, 0.17 g Na_2HPO_4, and 0.25 g NH_4NO_3 in 1 L of commercial artificial seawater MARINE ART SF-1 (Osaka Yakken), which did not contain any organic compound. A 3 mL portion of each growth medium was prepared in a 15 mL test tube with a butyl rubber cap. Headspace was replaced with each gas composition shown in Table **T1.**

Enrichment cultures

Slurry was prepared using each core sample in an onshore laboratory after the expedition. The smashed rock sample was suspended with 20–40 mL of the anaerobic artificial seawater, which was injected in a glass bottle by a syringe. The sediment sample was suspended with 20 mL of the anaerobic artificial seawater. The total 33 rock cores and 30 sediment cores were subjected to enrichments. A 0.3 mL portion of slurry was inoculated into each medium, and enrichments were performed for 4–8 months at temperatures shown in Tables T2, T3, and T4.

Bacterial and archaeal rRNA gene analyses

Some microbial cells in the enrichment cultures were distinguished from rock or sediment particles by microscopy; however, exact cell counting of the enrichment cultures was difficult because of deceptive cell-like particles in the cultures. Genomic DNA was then extracted from 1 mL of every culture using ISOIL for Beads Beating (NIPPON GENE) according to the manufacturer's instructions. The volume of the final DNA extract was 15 µL. Bacterial and archaeal 16S rRNA genes were amplified from the extracted DNA by polymerase chain reaction (PCR) in each 15 µL volume of reaction mixture containing the following:

- 1xGC buffer I (Takara Bio),
- 0.33 mM each dNTP (deoxynucleotide),
- 0.33 µM each primer,
- 1.25 U (units) of LA Taq polymerase (TaKaRa Bio), and
- 3.7 µL of genomic DNA extract.

Primers were Bac27F and Uni1492R for bacteria and Arc21F and Uni1492R for archaea (DeLong, 1992; Lane, 1991). The amplification was performed using Veriti Thermal Cycler (Applied Biosystems) with the following program:

1. 96°C for 1 min,
2. 37 cycles of 96°C for 25 s,
3. 50°C (archaea) or 53°C (bacteria) for 45 s,
4. 72°C for 90 s, and
5. 72°C for 10 min.

In the PCR amplification, a negative control containing no DNA extract was always examined to exclude contamination. In addition, contamination in the DNA extraction kit was sometimes examined by doing the extraction procedure without a sample and the following PCR amplification.

Aliquots of 2 µL of PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. The amplified 16S rRNA gene fragments shown as clearly visible bands were sequenced by direct sequencing or after cloned into vector pCR2.1 with TA Cloning Kit (Invitrogen). Partial 16S rRNA gene sequences were analyzed by 3130×l Genetic Analyzer (Applied Biosystems). The obtained sequences were compared against the DNA database using BLAST (**blast.st-va.ncbi.nlm.nih.gov/Blast.cgi**).

Analysis of a fungal isolate

A fungal isolate was routinely cultivated on an agar plate prepared with 2 g yeast extract, 2 g peptone, 2 g maltose, and 15 g agar per 1 L of artificial seawater MARINE ART SF-1. DNA was extracted and analyzed as described above for bacterial and archaeal DNA analyses. DNA fragments including 18S, 5.8S, and 28S rRNA coding regions and the internal transcribed spacer (ITS) regions were obtained by PCR. PCR amplification was first conducted using the following three sets of primers (**biology.duke.edu/fungi/mycolab/primers.htm**):

- EU347F and EUK-B (Medlin et al., 1988; Puitika et al., 2007),
- ITS1 and ITS4 (White et al., 1990), and
- LT0R and LR5.

After sequencing the PCR products, blank regions were determined by primer walking to extend the sequences. The obtained sequence was compared against the DNA database using BLAST and analyzed to construct a phylogenetic tree as described previously (Hirayama et al., 2013).

Measurement of methane production

Methane production in rock core enrichment cultures targeting methanogens was examined by injecting 0.1 mL of headspace gas from each vial into a gas chromatograph GC-3200 (GL Science) equipped with a thermal conductivity detector and a SHINCARBON ST 50/80 column (Shinwa Chemical Industries). The detection limit was approximately 10 µM.

Real-time PCR of the methyl coenzyme-M reductase subunit A gene (*mcrA*)

Genomic DNA was extracted from 1 g of frozen sediment using ISOIL for Beads Beating. A 2 µL portion of the total 20 µL DNA extract was used for a real-time PCR experiment to examine the presence of methanogens in the sediment cores by amplification of the methyl coenzyme-*M* reductase subunit *A* gene *mcrA*, which is the key enzyme gene of methanogens. SYBR green dye-based PCR amplification and detection were performed using SYBR Premix Ex Taq (TaKaRa Bio) and a 7500 Real-Time PCR system (Applied Biosystems) as described previously (Nunoura et al., 2008).

Results

In a negative control for PCR experiment, a reaction mixture added with pure water instead of DNA extract sometimes generated a slightly visible PCR band of 16S rRNA gene fragments. The retrieved sequences always indicated 99% identity to that of *Nesterenkonia aethiopica* in the class Actinobacteria. We considered enrichment cultures from which sequences similar to the negative control sequence were detected as growth-negative.

Anaerobic enrichments from rock cores

Cultivations targeting anaerobic hydrogenotrophic methanogens were performed at three different temperatures of 15°C, 37°C, and 55°C, and each headspace gas was examined by gas chromatography analysis; however, methane production was not detected from any of the enrichment cultures. The other targeted anaerobic metabolism was hydrogen oxidation. The medium containing hydrogen as an electron donor and sulfate and nitrate as electron acceptors was used for enrichments. Apparent microbial growth was not detected in any of the enrichment cultures at 15°C, whereas the enrichment cultures at 37°C showed bacterial growth from nine core samples (Table **T2**). The detected bacteria were affiliated to the genera *Bacillus*, *Methylobacterium*, and *Sphingomonas* by direct sequencing analysis of 16S rRNA genes. *Bacillus* spp. have previously been cultivated from subsurface basaltic cores and the inside of seafloor basaltic rocks (Lysnes et al., 2004; Rathsack et al., 2009). Although two *Bacillus* spp., *Bacillus schlegelii* and *Bacillus tusciae,* were previously known to be facultatively chemolithoautotrophic hydrogen-oxidizing bacteria, these species have recently been reclassified in novel genera *Hydrogenibacillus* (Kämpfer et al., 2013) and *Kyrpidia* (Klenk et al., 2011), respectively. To our knowledge, no other *Bacillus* spp. are reported to be capable of hydrogen oxidation.

The archaeal 16S rRNA gene was not amplified from any of the anaerobic cultures.

Aerobic enrichments from rock cores

For aerobic cultivations, reduced sulfur compounds (elemental sulfur and thiosulfate) and hydrogen were used as electron donors. Two enrichment cultures targeting sulfur oxidizers indicated bacterial growth, and *Moraxella* sp. was detected from one of the two examined cultures. Enrichments targeting hydrogen oxidizers were more prolific than those targeting sulfur oxidizers. Bacterial growth was detected from 10, 8, and 13 core samples cultivated at 8°C, 25°C, and 37°C, respectively (Table **T3**).

Members of the genera *Ralstonia* and *Pseudomonas* were most frequently detected in the enrichment cultures at 8°C and 25°C. *Ralstonia* and *Pseudomonas* spp. have previously been detected in cultivation analyses of the subsurface and seafloor basaltic rocks and also in culture-independent microbial community analyses of deep subsurface gabbroic rock cores (Lysnes et al., 2004; Mason et al., 2010; Rathsack et al., 2009). *Ralstonia* spp. are known to be tough microorganisms (Mijnendonckx et al., 2013), and they might be living in various harsh environments, including nutrient-starved endolithic habitats. In the genus *Ralstonia*, only one species, *Ralstonia eutropha*, was previously known to be a facultatively chemolithoautotrophic hydrogen-oxidizing bacterium; however, *R. eutropha* was reclassified in the genus *Cupriavidus* later and now is recognized as a synonym of *Cupriavidus necator* (Vandamme and Coenye, 2004). Currently, it seems that there is no published data about chemolithotrophic hydrogen-oxidizing species of the genera *Ralstonia* and *Pseudomonas*.

A PCR amplification of the culture of the sample from Section 336-U1382A-6R-1A at 8°C generated a dense product band, but direct sequencing of the product did not succeed. The product was then cloned, and 16S rRNA gene sequences similar to those of *Salinibacterium* and *Sphingomonas* spp. were obtained. *Salinibacterium amurskyense* is a marine heterotroph capable of growing at 4°C (Han et al., 2003). The genus *Sphingomonas* is known for the ability to degrade a wide range of recalcitrant environmental pollutants (Yabuuchi and Kosako, 2005). In the enrichment culture of Section 336-U1383C-24R-1A at 25°C, visible growth of black fungi-like cells was observed, and then the culture was analyzed separately as described below.

In the enrichment cultures at 37°C, *Paenibacillus* and *Acidovorax* spp. were frequently detected. A *Paenibacillus* species has previously been cultivated from subsurface basaltic cores (Lysnes et al., 2004). Several *Paenibacillus* spp. have been associated with Fe(III) reduction and have been reported to predominate in the Fe(III)-reducing consortia of subsurface sediments in terrestrial heavy metal–contaminated sites (Ahmed et al., 2012; Petrie et al., 2003). Although the genus *Acidovorax* is generally characterized by chemoorganotrophic growth, *Acidovorax ebreus* has reported as a mixotroph to utilize Fe(II) as the electron donor (Byrne-Bailey et al., 2010). The lithoautotrophic growth by hydrogen oxidation has also been

reported in strains of two species of the genus *Acidovorax*, *Acidovorax facilis* and *Acidovorax delafieldii* (Willems et al., 1990).

The archaeal 16S rRNA gene was not amplified from any of the aerobic cultures.

Detection of the *mcrA* gene of methanogens from sediment cores

To select sediment cores suitable for the cultivation of methanogens, amplification of the *mcrA* genes in DNA from sediment cores was attempted using real-time PCR. The *mcrA* gene was, however, not detected in any of the sediment cores (Table **T2**). Therefore, cultivation of methanogens was not performed on the sediment cores.

Enrichments from sediment cores

Sediment core samples were used for inoculation of aerobic enrichments in the medium for sulfur oxidizers at temperatures of 15°C and 37°C. Bacterial growth was observed in seven core samples at 15°C, whereas there was no clear indication of microbial growth in the enrichment cultures at 37°C (Table **T4**). The bacteria detected in the cultures at 15°C were members of the genera *Pseudomonas*, *Halomonas*, *Marinobacter*, and *Paracoccus*. Members of these genera are known to be typical inhabitants of subsurface sediments (Parkes et al., 2014). It has been reported that strains belonging to these genera are obligately heterotrophic sulfur oxidizers, which means they can utilize sulfur compounds as electron donors and organic compounds as carbon sources, or facultatively autotrophic sulfur oxidizers (Petri et al., 2001; Sorokin, 2003; Van Spanning, 2005). In thiosulfate-oxidizing heterotrophic *Pseudomonas stutzeri*, the presence of thiosulfate dehydrogenase participating in thiosulfate oxidation and the exhibition of it's enzymatic activity were proven by using an expressed recombinant protein (Denkmann et al., 2012).

The archaeal 16S rRNA gene was not amplified from any of the aerobic cultures.

A fungal isolate from the rock core

Fungal growth was observed in the enriched culture from Core 336-U1383C-24R-1A rock at 25°C as described above. The isolated fungal strain was designated NPf1. A sequence of ~4.4 kbp including 18S, 5.8S, and 28S rRNA coding regions and the internal transcribed spacer regions (ITS1 and ITS2) was obtained from the isolate (GenBank/EMBL/DDBJ accession number LC017736). The isolate NPf1 was affiliated to the genus *Exophiala* of the order Chaetothyriales by similarity analysis (Fig. **F1**). It has been reported that many fungal isolates from terrestrial rocks are grouped into the order Chaetothyriales (Sterflinger et al., 1997; Ruibal et al., 2008).

We succeeded in retrieving partial 16S rRNA gene sequences by the direct sequencing of PCR products from most of the growth-positive enrichment cultures, suggesting that the detected microorganisms were certainly grown in the enrichment cultures. However, we did not achieve the subcultivation of the detected bacteria in the respective original media in any case. Because the enrichment cultures contained rock particles as inocula transferred from the slurry, rock minerals might be needed for their growth.

Acknowledgments

This research used samples provided by the Integrated Ocean Drilling Program (IODP). We thank the captain, crew, and technicians of the R/V *JOIDES Resolution*, Co-Chief scientists Katrina J. Edwards and Wolfgang Bach, and the IODP Expedition 336 shipboard scientific party, especially the members of the microbiology team. We also thank Kentaro Nakamura for helpful discussion and Takuro Nunoura and Miho Hirai for their assistance in the *mcrA* detection procedure.

References

Ahmed, B., Cao, B., McLean, J.S., Ica, T., Dohnalkova, A., Istanbullu, O., Paksoy, A., Fredrickson, J.K., and Beyenal, H., 2012. Fe(III) reduction and U(VI) immobilization by *Paenibacillus* sp. strain 300A, isolated from Hanford 300A subsurface sediments. *Appl. Environ. Microbiol.,* 78(22):8001–8009. **doi:10.1128/AEM.01844-12**

Bach, W., and Edwards, K.J., 2003. Iron and sulfide oxidation within the basaltic ocean crust: implications for chemolithoautotrophic microbial biomass production. *Geochim. Cosmochim. Acta,* 67(20):3871–3887. **doi:10.1016/S0016-7037(03)00304-1**

Byrne-Bailey, K.G., Weber, K.A., Chair, A.H., Bose, S., Knox, T., Spanbauer, T.L., Chertkov, O., and Coates, J.D., 2010. Completed genome sequence of the anaerobic iron-oxidizing bacterium *Acidovorax ebreus* strain TPSY. *J. Bacteriol.,* 192(5):1475–1476. **doi:10.1128/JB.01449-09**

DeLong, E.F., 1992. Archaea in coastal marine environments. *Proc. Natl. Acad. Sci. U. S. A.,* 89(12):5685–5689. **doi:10.1073/pnas.89.12.5685**

Denkmann, K., Grein, F., Zigann, R., Siemen, A., Bergmann, J., van Helmont, S., Nicolai, A., Pereira, I.A.C., and Dahl, C., 2012. Thiosulfate dehydrogenase: a widespread unusual acidophilic c-type cytochrome. *Environ. Microbiol.,* 14(10):2673–2688. **doi:10.1111/j.1462-2920.2012.02820.x**

Expedition 336 Scientists, 2012a. Expedition 336 summary. *In* Edwards, K.J., Bach, W., Klaus, A., and the

Expedition 336 Scientists, *Proc. IODP,* 336: Tokyo (Integrated Ocean Drilling Program Management International, Inc.). **doi:10.2204/iodp.proc.336.101.2012**

Expedition 336 Scientists, 2012b. Methods. *In* Edwards, K.J., Bach, W., Klaus, A., and the Expedition 336 Scientists, *Proc. IODP,* 336: Tokyo (Integrated Ocean Drilling Program Management International, Inc.). **doi:10.2204/iodp.proc.336.102.2012**

Fisher, A.T., Davis, E.E., Hutnak, M., Spiess, V., Zühlsdorff, L., Cherkaoui, A., Christiansen, L., Edwards, K., Macdonald, R., Villinger, H., Mottl, M.J., Wheat, C.G., and Becker, K., 2003. Hydrothermal recharge and discharge across 50 km guided by seamounts on a young ridge flank. *Nature,* 421(6923):618–621. **doi:10.1038/nature01352**

Han, S.K., Nedashkovskaya, O.I., Mikhailov, V.V., Kim, S.B., and Bae, K.S., 2003. *Salinibacterium amurskyense* gen. nov., sp. nov., a novel genus of the family Microbacteriaceae from the marine environment. *Int. J. Syst. Evol. Microbiol.,* 53(6):2061–2066. **doi:10.1099/ijs.0.02627-0**

Hirayama, H., Fuse, H., Abe, M., Miyazaki, M., Nakamura, T., Nunoura, T., Furushima, Y., Yamamoto, H., and Takai, K., 2013. *Methylomarinum vadi* gen. nov., sp. nov., a methanotroph isolated from two distinct marine environments. *Int. J. Syst. Evol. Microbiol.,* 63(3):1073–1082. **doi:10.1099/ijs.0.040568-0**

Ivarsson, M., Bengtson, S., Belivanova, V., Stampanoni, M., Marone, F., and Tehler, A., 2012. Fossilized fungi in subseafloor Eocene basalts. *Geology,* 40(2):163–166. **doi:10.1130/G32590.1**

Kämpfer, P., Glaeser, S.P., and Busse, H.-J., 2013. Transfer of *Bacillus schlegelii* to a novel genus and proposal of *Hydrogenibacillus schlegelii* gen. nov., comb. nov. *Int. J. Syst. Evol. Microbiol.,* 63(5):1723-1727. **doi:10.1099/ijs.0.045146-0**

Klenk, H.-P., Lapidus, A., Chertkov, O., Copeland, A., Del Rio, T.G., Nolan, M., Lucas, S., Chen, F., Tice, H., Cheng, J.-F., Han, C., Bruce, D., Goodwin, L., Pitluck, S., Pati, A., Ivanova, N., Mavromatis, K., Daum, C., Chen, A., Palaniappan, K., Chang, Y., Land, M., Hauser, L., Jeffries, C.D., Detter, J.C., Rohde, M., Abt, B., Pukall, R., Göker, M., Bristow, J., Markowitz, V., Hugenholtz, P., and Eisen, J.A., 2011. Complete genome sequence of the thermophilic, hydrogen-oxidizing *Bacillus tusciae* type strain (T2) and reclassification in the new genus, *Kyrpidia* gen. nov. as *Kyrpidia tusciae* comb. nov. and emendation of the family Alicyclobacillaceae da Costa and Rainey, 2010. *Stand. Genomic. Sci.,* 5(1):121–134. **doi:10.4056/sigs.2144922**

Lane, D.J., 1991. 16S/23S rRNA sequencing. *In* Stackebrandt, E., and Goodfellow, M. (Eds.), *Nucleic Acid Techniques in Bacterial Systematics:* New York (Wiley), 115–148.

Lever, M.A., Rouxel, O., Alt, J.C., Shimizu, N., Ono, S., Coggon, R.M., Shanks, W.C., III, Laphan, L., Elvert, M., Prieto-Mollar, X., Hinrichs, K.-U., Inagaki, F., and Teske, A., 2013. Evidence for microbial carbon and sulfur cycling in deeply buried ridge flank basalt. *Science,* 339(6125):1305–1308. **doi:10.1126/science.1229240**

Lysnes, K., Torsvik, T., Thorseth, I.H., and Pedersen, R.B., 2004. Microbial populations in ocean floor basalt: results from ODP Leg 187. *In* Pedersen, R.B., Christie, D.M., Miller, D.J. (Eds.), *Proc. ODP, Sci. Results,* 187: College Station, TX (Ocean Drilling Program), 1–27. **doi:10.2973/odp.proc.sr.187.203.2004**

Mason, O.U., Nakagawa, T., Rosner, M., Van Nostrand, J.D., Zhou, J., Maruyama, A., Fisk, M.R., and Giovannoni, S.J., 2010. First investigation of the microbiology of the deepest layer of ocean crust. *PLoS One,* 5(11):e15399. **doi:10.1371/journal.pone.0015399**

McCarthy, M.D., Beaupré, S.R., Walker, B.D., Voparil, I., Guilderson, T.P., and Druffel, E.R.M., 2011. Chemosynthetic origin of ^{14}C-depleted dissolved organic matter in a ridge-flank hydrothermal system. *Nat. Geosci.,* 4(1):32–36. **doi:10.1038/ngeo1015**

Medlin, L., Elwood, H.J., Stickel, S., and Sogin, M.L., 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene,* 71(2):491–499. **doi:10.1016/0378-1119(88)90066-2**

Mijnendonckx, K., Provoost, A., Ott, C.M., Venkateswaran, K., Mahillon, J., Leys, N., and Van Houdt, R., 2013. Characterization of the survival ability of *Cupriavidus metallidurans* and *Ralstonia pickettii* from space-related environments. *Microb. Ecol.,* 65(2):347–360. **doi:10.1007/s00248-012-0139-2**

Nunoura, T., Oida, H., Miyazaki, J., Miyashita, A., Imachi, H., and Takai, K., 2008. Quantification of *mcrA* by fluorescent PCR in methanogenic and methanotrophic microbial communities. *FEMS Microbiol. Ecol.,* 64(2):240–247. **doi:10.1111/j.1574-6941.2008.00451.x**

Parkes, R.J., Cragg, B., Roussel, E., Webster, G., Weightman, A., and Sass, H., 2014. A review of prokaryotic populations and processes in sub-seafloor sediments, including biosphere:geosphere interactions. *Mar. Geol.,* 352:409–425. **doi:10.1016/j.margeo.2014.02.009**

Petri, R., Podgorsek, L., and Imhoff, J.F., 2001. Phylogeny and distribution of the *sox*B gene among thiosulfate-oxidizing bacteria. *FEMS Microbiol. Lett.,* 197(2):171–178. **doi:10.1016/S0378-1097(01)00111-2**

Petrie, L., North, N.N., Dollhopf, S.L., Balkwill, D.L., and Kostka, J.E., 2003. Enumeration and characterization of iron(III)-reducing microbial communities from acidic subsurface sediments contaminated with uranium(VI). *Appl. Environ. Microbiol.,* 69(12):7467–7479. **doi:10.1128/AEM.69.12.7467-7479.2003**

Puitika, T., Kasahara, Y., Miyoshi, N., Sato, Y., and Shimano, S., 2007. A taxon-specific oligonucleotide primer set for PCR-based detection of soil ciliate. *Microbes Environ.,* 22(1):78–81. **doi:10.1264/jsme2.22.78**

Rathsack, K., Stackebrandt, E., Reitner, J., and Schumann, G., 2009. Microorganisms isolated from deep sea low-temperature influenced oceanic crust basalts and sediment samples collected along the Mid-Atlantic Ridge. *Geomicrobiol. J.,* 26(4):264–274. **doi:10.1080/01490450902892456**

Ruibal, C., Platas, G., and Bills, G.F., 2008. High diversity and morphological convergence among melanised

fungi from rock formations in the Central Mountain System of Spain. *Persoonia*, 21:93–110. **doi:10.3767/003158508X371379**

Schumann, G., Manz, W., Reitner, J., and Lustrino, M., 2004. Ancient fungal life in north Pacific Eocene oceanic crust. *Geomicrobiol. J.,* 21(4):241–246. **doi:10.1080/01490450490438748**

Smith, A., Popa, R., Fisk, M., Nielsen, M., Wheat, C.G., Jannasch, H.W., Fisher, A.T., Becker, K., Sievert, S.M., and Flores, G., 2011. In situ enrichment of ocean crust microbes on igneous minerals and glasses using an osmotic flow-through device. *Geochem., Geophys., Geosyst.,* 12(6):Q06007. **doi:10.1029/2010GC003424**

Sorokin, D.Y., 2003. Oxidation of inorganic sulfur compounds by obligately organotrophic bacteria. *Microbiology,* 72(6):641–653. **doi:10.1023/B:MICI.0000008363.24128.e5**

Sterflinger, K., De Baere, R., de Hoog, G.S., De Wachter, R., Krumbein, W.E., and Haase, G., 1997. *Coniosporium perforans* and *C. apollinis*, two new rock-inhabiting fungi isolated from marble in the Sanctuary of Delos (Cyclades, Greece). *Antonie Van Leeuwenhoek,* 72(4):349–363. **doi:10.1023/A:1000570429688**

Stevens, T.O., and McKinley, J.P., 1995. Lithoautotrophic microbial ecosystems in deep basalt aquifers. *Science,* 270(5235):450–454. **doi:10.1126/science.270.5235.450**

Stevens, T.O., and McKinley, J.P., 2000. Abiotic controls on H_2 production from basalt-water reactions and implications for aquifer biogeochemistry. *Environ. Sci. Technol.,* 34(5):826–831. **doi:10.1021/es990583g**

Takai, K., Inoue, A., and Horikoshi, K., 2002. *Methanothermococcus okinawensis* sp. nov., a thermophilic, methane-producing archaeon isolated from a Western Pacific deep-sea hydrothermal vent system. *Int. J. Syst. Evol. Microbiol.*, 52(4):1089–1095. **doi:10.1099/ijs.0.02106-0**

Van Spanning, R.J.M., Stouthamer, A.H., Baker, S.C., and van Verseveld, H.W., 2005. Class I. Alphaproteobacteria*,* Order III. Rhodobacterales*,* Family 1. Rhodobacteraceae*,* Genus XII. *Paracoccus*. *In* Garrity, G.M., Brenner, D.J., Krieg, N.R., and Staley, J.T. (Eds.), *Bergey's Manual of Systematic Bacteriology* (Vol. 2): *The Proteobacteria* (Part C): New York (Springer), 197–204.

Vandamme, P., and Coenye, T., 2004. Taxonomy of the genus *Cupriavidus*: a tale of lost and found. *Int. J. Syst. Evol. Microbiol.,* 54(6):2285–2289. **doi:10.1099/ijs.0.63247-0**

White, T.J., Bruns, T., Lee, S., and Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J. (Eds.), *PCR Protocols: A Guide to Methods and Applications*: London (Academic Press), 315–322. **doi:10.1016/B978-0-12-372180-8.50042-1**

Willems, A., Falsen, E., Pot, B., Jantzen, E., Hoste, B., Vandamme, P., Gillis, M., Kersters, K., and De Ley, J., 1990. *Acidovorax*, a new genus for *Pseudomonas facilis*, *Pseudomonas delafieldii*, E. Falsen (EF) group 13, EF group 16, and several clinical isolates, with the species *Acidovorax facilis* comb. nov., *Acidovorax delafieldii* comb. nov., and *Acidovorax temperans* sp. nov. *Int. J. Syst. Bacteriol.,* 40(4):384–398. **doi:10.1099/00207713-40-4-384**

Yabuuchi, E., and Kosako, Y., 2005. Class I. Alphaproteobacteria*,* Order IV. Sphingomonadales, Family I. Sphingomonadaceae, Genus I. *Sphingomonas*. *In* Garrity, G.M., Brenner, D.J., Krieg, N.R., and Staley, J.T. (Eds.), *Bergey's Manual of Systematic Bacteriology* (Vol. 2): *The Proteobacteria* (Part C): New York (Springer), 234–258.

Initial receipt: 5 September 2014
Acceptance: 25 February 2015
Publication: 28 April 2015
MS 336-204

Figure F1. Neighbor-joining phylogenetic tree based on partial 28S rRNA gene sequences (464 bp) showing the position of the isolate NPf1 among representative species of the order Chaetothyriales. Bootstrap analyses were performed (100 replicates), and only bootstrap values >50% are shown. Solid dots = spp. which are reported to be isolated from terrestrial rocks (Ruibal et al., 2008).

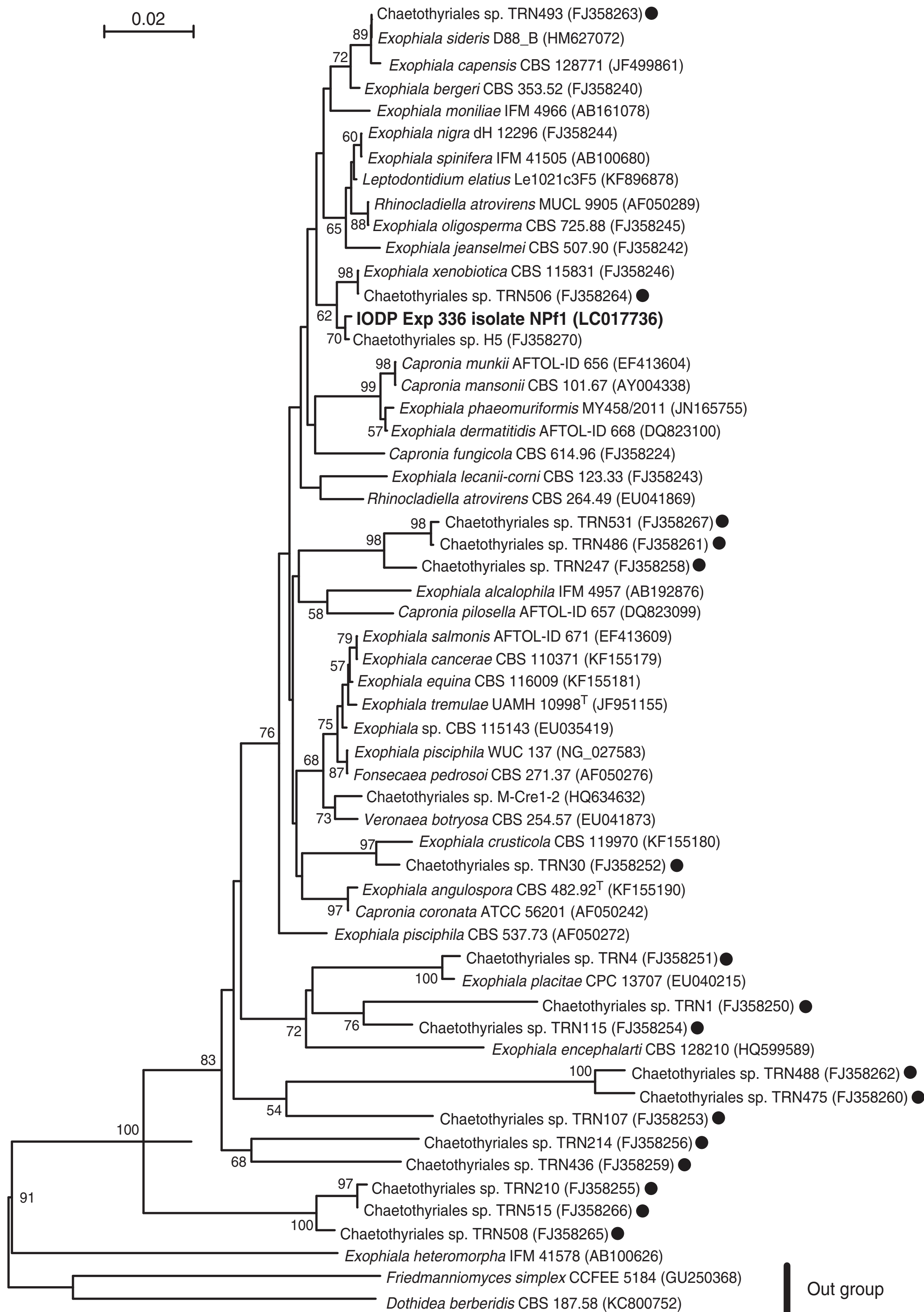

Table T1. Cultivation media used in this study.

Target microorganisms	Medium	Headspace gas	Reference
Methanogens	MMJ medium	$H_2:CO_2$ (80:20, 300 kPa)	Takai et al., 2002
Anaerobic hydrogen oxidizers	DSMZ medium 383 using the basic seawater medium with resazurin (1 mg/L) instead of Solution A	$H_2:N_2:CO_2$ (70:20:10, 200 kPa)	www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium383.pdf
Aerobic hydrogen oxidizers	Basic seawater medium supplemented with the following per 1 L: 0.5 g $NaHCO_3$, 2.5 mg $NiCl_2 \cdot 6H_2O$, 2.5 mg $Na_2WO_4 \cdot 2H_2O$, 0.1 mg $Na_2SeO_3 \cdot 5H_2O$, 1 mL trace element solution SL-10 (DSMZ medium 320), and 1 mL vitamin complex solution (DSMZ medium 141)	$H_2:N_2:O_2:CO_2$ (49:47:3:1, 200 kPa)	www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium320.pdf www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium141.pdf
Aerobic sulfur oxidizers	The medium described above for aerobic hydrogen oxidizers with 1 mM sodium thiosulfate and ~0.1 g elemental sulfur per tube	$N_2:O_2$ (95:5, 100 kPa)	

Basic seawater medium = 0.06 g KH_2PO_4, 0.17 g Na_2HPO_4, and 0.25 g NH_4NO_3 per L of artificial seawater MARINE ART SF-1. MMJ = marine medium salts in artificial seawater.

Table T2. Detection of anaerobic microorganisms in the enrichment cultures from the basaltic rock cores.

		Target metabolism:	Methanogen			Hydrogen oxidizer (sulfate reducer and nitrate reducer)		
		Energy source:	Hydrogen			Hydrogen		
		Temperature:	15°C	37°C	55°C	15°C	37°C	
		Examination by:	GC	GC	GC	PCR	PCR	16S rRNA gene sequences*
Core	Depth (mbsf)	Section, piece						
336-U1382A-								
3R	113.6–118.3	1A	–	–	–	–	–	
		2B	–	–	–	–	–	
		3A	–	–	–	–	+	*Bacillus niacini* 98% (Bacilli)
		4A	–	–	–	–	–	
		4B	–	–	–	–	+	*Methylobacterium fujisawaense* 99% (AP)
4R	123.0–124.8	2E	–	–	–	–	–	
5R	132.5–134.6	1B	–	–	–	–	+	*Bacillus niacini* 98% (Bacilli)
		2E	–	–	–	–	–	
6R	142.4–143.8	1A	–	–	–	–	+	*Sphingomonas ursincola* 99% (AP)
		1C	–	–	–	–	–	
7R	153.1–153.9	2B	–	–	–	–	–	
8R	161.3–164.0	1C	–	–	–	–	–	
		4D	–	–	–	–	–	
		4E	–	–	–	–	–	
9R	171.5–173.8	1B	–	–	–	–	+	Not identified
336-U1383C-								
2R	69.9–72.2	1C	–	–	–	–	+	*Bacillus niabensis* 99% (Bacilli)
		2D	–	–	–	–	–	
4R	86.6–88.1	2D	–	–	–	–	–	
5R	96.4–97.2	1A	–	–	–	–	+	*Bacillus niabensis* 99% (Bacilli)
8R	124.7–126.0	1B	–	–	–	–	–	
11R	153.8–154.5	1C	–	–	–	–	–	
17R	202.1–203.1	1A	–	–	–	–	–	
19R	212.1–212.6	1A	–	–	–	–	+	*Bacillus niacini* 99% (Bacilli)
		1C	–	–	–	–	+	*Bacillus niabensis* 99% (Bacilli)
20R	219.4–220.7	1A	–	–	–	–	–	
23R	248.1–248.3	1A	–	–	–	–	–	
24R	256.6–257.1	1A	–	–	–	–	–	
29R	299.9–300.0	1A	–	–	–	–	–	
30R	304.0–306.3	1A	–	–	–	–	–	
		2B	–	–	–	–	–	
		3C	–	–	–	–	–	
31R	312.2–314.8	1B	–	–	–	–	–	
336-U1384A-								
12X	95.2	1C	–	–	–	–	–	

* = closest microorganisms indicated by Blast search. GC = gas chromatography, PCR = polymerase chain reaction. Phylogenetic groups are shown in parentheses. AP = Alphaproteobacteria.

Table T3. Detection of aerobic microorganisms in the enrichment cultures from the basaltic rock cores.

		Target metabolism:	Hydrogen oxidizer						Sulfur oxidizer		
		Energy source:	Hydrogen						Elemental sulfur and thiosulfate		
		Temperature:	8°C		25°C		37°C		15°C	37°C	
		Examination by:	PCR	16S rRNA gene sequences	PCR	16S rRNA gene sequences	PCR	16S rRNA gene sequences	PCR	PCR	16S rRNA gene seq.
Core	Depth (mbsf)	Section, piece									
336-U1382A-											
3R	113.6–118.3	1A	–		–		–		–	–	
		2B	–		+	*Ralstonia pickettii* 99%–100% (BP)	–		–	–	
		3A	–		–		+	*Paenibacillus darwinianus* 97% (Bacilli)	–	–	
		4A	–		–		–		–	–	
		4B	–		–		–		–	–	
4R	123.0–124.8	2E	–		–		–		–	–	
5R	132.5–134.6	1B	+	*Pseudomonas fluorescens* 98% (GP)	–		–		–	–	
		2E	–		–		–		–	–	
6R	142.4–143.8	1A	+	*Salinibacterium amurskyense* 100% (Act) *Sphingomonas changbaiensis* 96% (AP) *Sphingomonas indica* 97% (AP)	+	Not identified	–		–	–	
		1C	+	*Ralstonia pickettii* 99%–100% (BP)	–		+	*Paenibacillus thermoaerophilus* 92% (Bacilli)	–	–	
7R	153.1–153.9	2B	+	Not identified	–		–		–	+	*Moraxella osloensis* 98% (GP)
8R	161.3–164.0	1C	–		+	*Pseudomonas tolaasii* 97% (GP)	–		–	–	
		4D	–		–		–		–	–	
		4E	–		–		–		–	–	
9R	171.5–173.8	1B	+	*Pseudomonas fluorescens* 98% (GP)	+	*Ralstonia pickettii* 99%–100% (BP)	–		–	–	
336-U1383C-											
2R	69.9–72.2	1C	–		–		+	*Paenibacillus thermoaerophilus* 92% (Bacilli)	–	–	
		2D	+	*Ralstonia pickettii* 99%–100% (BP)	–		+	*Acidovorax ebreus* 99% (BP)	–	+	Not identified
4R	86.6–88.1	2D	+	*Pseudomonas fluorescens* 98% (GP)	–		–		–	–	
5R	96.4–97.2	1A	–		–		+	*Acidovorax ebreus* 99% (BP)	–	–	
8R	124.7–126.0	1B	+	*Ralstonia pickettii* 99%–100% (BP)	–		+	Not identified	–	–	
11R	153.8–154.5	1C	–		–		+	Not identified	–	–	
17R	202.1–203.1	1A	+	*Sphingomonas paucimobilis* 99% (AP)	–		+	*Phenylobacterium koreense* 94% (AP)	–	–	
19R	212.1–212.6	1A	–		–		+	*Acidovorax ebreus* 99% (BP)	–	–	
		1C	–		–		+	*Paenibacillus darwinianus* 97% (Bacilli)	–	–	
20R	219.4–220.7	1A	–		–		–		–	–	
23R	248.1–248.3	1A	–		–		–		–	–	
24R	256.6–257.1	1A	–			*Exophiala* sp. (Fungi)	+	*Paenibacillus darwinianus* 97% (Bacilli)	–	–	
29R	299.9–300.0	1A	+	*Ralstonia pickettii* 99%–100% (BP)	+	Not identified	+	*Paenibacillus thermoaerophilus* 92% (Bacilli)	–	–	
30R	304.0–306.3	1A	–		+	*Pseudomonas fluorescens* 99% (GP)	–		–	–	
		2B	–		+	*Ralstonia pickettii* 99%–100% (BP)	–		–	–	
		3C	–		–		+	*Acidovorax ebreus* 99% (BP)	–	–	
31R	312.2–314.8	1B	–		–		–		–	–	
336-U1384A-											
12X	95.2	1C	–		–		–		–	–	

PCR = polymerase chain reaction. 16S rRNA gene sequences = closest microorganisms indicated by Blast search. Phylogenetic groups are shown in parentheses. AP = Alphaproteobacteria, BP = Betaproteobacteria, GP = Gammaproteobacteria, Act = Actinobacteria. Not identified = direct sequencing did not succeed.

Table T4. Detection of microorganisms in the sediment cores: real-time PCR for methanogens and the cultivation of sulfur oxidizers.

		Target metabolism:	Methanogen (anaerobic)	Sulfur oxidizer (aerobic)		
		Energy source:	—	Elemental sulfur and thiosulfate		
		Incubation temperature:	—	15°C		37°C
		Examination by:	Q-PCR	PCR	16S rRNA gene sequences	PCR
Core	Section	Depth (mbsf)				
336-U1382B-						
1H	4	3.90–4.00	–	–		–
2H	6	12.87–12.97	–	–		–
3H	5	21.90–22.00	–	–		–
4H	4	28.65–28.75	–	–		–
6H	7	50.28–50.38	–	–		–
7H	6	60.61–60.71	–	+	*Paracoccus marcusii* 100% (AP)	–
8H	6	69.95–70.05	–	–		–
10H	5	86.91–87.01	–	–		–
336-U1383D-						
1H	3	2.54–2.64	–	–		–
2H	5	11.25–11.35	–	–		–
3H	6	22.27–22.37	–	–		–
4H	5	30.08–30.18	–	–		–
5H	5	39.80–39.90	–	+	*Pseudomonas xanthomarina* 99% (GP)	–
336-U1383E-						
1H	2	2.0–2.1	–	+	*Halomonas axialensis* 99% (GP)	–
2H	6	10.58–10.68	–	–		–
3H	6	20.08–20.18	–	+	*Halomonas axialensis* 98% (GP)	–
4H	5	27.06–27.16	–	+	*Pseudomonas xanthomarina* 99% (GP)	–
5H	4	36.25–36.35	–	–		–
6H	5	45.7–47.0*	–	+	*Marinobacter adhaerens* 98% (GP)	–
336-U1384A-						
1H	2	1.97–2.07	–	+	*Marinobacter adhaerens* 99% (GP)	–
2H	6	12.04–12.14	–	–		–
3H	5	19.78–19.88	–	–		–
4H	6	30.79–30.89	–	–		–
5H	6	40.04–40.14	–	–		–
6H	4	47.90–48.00	–	–		–
7H	4	57.08–57.18	–	–		–
8H	5	67.47–67.57	–	–		–
9H	5	76.91–77.01	–	–		–
10H	5	86.5–86.6	–	–		–
11H	2	91.3–91.4	–	–		–

* = depth at which the master core for microbiology was collected. Q-PCR = real-time PCR experiment. PCR = polymerase chain reaction. 16S rRNA gene sequences = closest microorganisms indicated by Blast search. Phylogenetic groups are shown in parentheses. AP = Alphaproteobacteria, GP = Gammaproteobacteria.