Distribution of Microorganisms in the Subsurface of the Manus Basin Hydrothermal Vent Field in Papua New Guinea

Hiroyuki Kimura,¹ Ryuji Asada,² Andrew Masta,³ and Takeshi Naganuma^{1*}

School of Biosphere Sciences, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima 739-8528,¹ and Department of Earth Sciences, Kanazawa University, Kakuma, Kanazawa, 920-1192,² Japan, and School of Medicine and Health Sciences, University of Papua New Guinea, Boroko, NCD 111, Papua New Guinea³

Received 2 July 2002/Accepted 8 October 2002

The distribution of microorganisms in the subsurfaces of hydrothermal vents was investigated by using subvent rock core samples. Microbial cells and ATP were detected from cores taken at depths of less than 99.4 and 44.8 m below the seafloor (mbsf), respectively. Cores from various depths were incubated anaerobically with a heterotrophic medium. Growth at 60 and 90°C was ascribed to a *Geobacillus* sp. in the 448.6- to 99.4-mbsf cores and a *Deinococcus* sp. in the 64.8- to 128.9-mbsf cores, respectively, based on the 16S ribosomal DNA analysis.

Since the discovery of deep-sea hydrothermal vents, a number of thermophiles and hyperthermophiles have been isolated from chimneys, sediments, and ambient water of the hydrothermal vent fields (reviewed by Reysenbach et al. [21]). In addition, non-culture-dependent 16S ribosomal DNA (rDNA) analysis has been applied to a variety of vent samples (see, e.g., references 11, 14, 15, 23, 29, and 30). However, most of the previous studies were limited to the surfaces of hydrothermal vent systems, while interest in the subsurface habitats of hydrothermal vents (subvents) has been increasing.

Only a few subvent microbiological studies have been conducted with sediment, sedimentary rock layers, and igneous rocks from relatively shallow depths (less than 52 m below the seafloor [mbsf]) (4, 5, 22, 26, 27). Here, we report the first evidence for the occurrence of a deep-sea subvent biosphere (maximum depth, 128.9 mbsf), by using igneous rock core samples from a back-arc basin hydrothermal vent field.

Sample collection and contamination test. Igneous rock core samples (0 to 386.7 mbsf) were collected from sites 1188 and 1189 during the leg 193 cruise of the Ocean Drilling Program (ODP), targeting hydrothermal vent fields at the PACMANUS site (water depth, 1,640 to 1,690 m), in Manus Basin, Papua New Guinea (Table 1). The in situ temperature was measured in holes 1188F and 1189B with an ultrahigh-temperature multisensor memory thermometer (Geophysical Research Corporation, Tulsa, Okla.). The postdrilling temperatures (0 and 5 days after drilling termination) at the bottom of holes 1189B and 1188F were 68°C (115 mbsf) and 312°C (386.7 mbsf), respectively (Table 1); the in situ temperature in hole 1189B was still influenced by the introduction of drilling fluid (surface seawater used in the ODP).

Contamination of the rock cores by introduced drilling fluid and entrained ambient water is a general and major concern in subsurface microbiology. Prevention of core contamination is

hardly realistic and has rarely been done. Instead, it is practically important to check the degree of contamination in recovered core samples. A contamination test using perfluorocarbons (PFC) as a chemical tracer has been taken as the standard protocol in the ODP (24, 25) and was done in this study for the cores of completely altered volcanic rocks that were collected from 86.9 to 96.6 mbsf in hole 1188A. A core surface fragment (5.23 g) yielded a gas chromatography signal corresponding to 2.73×10^{-10} g of PFC, while no PFC were detected from the interior of the core (Table 2). These results showed that contamination by drilling fluid was occasionally limited to the core surfaces, and thus the interiors of certain cores were regarded as contamination-free. There is a trade-off between contamination tests and enrichment cultures, and single samples were not used for both purposes simultaneously. However, the rocks used for enrichment cultures were chosen from the rocks closely similar to the tested rocks whose interiors were proved to be noncontaminated.

Cell counts and ATP concentrations. Total cell counts in the core interiors were determined by direct counting of DAPI (4'-diamidino-2-phenylindole)-stained cells with an epifluorescence microscope (4). We counted the cells in 70 to 200 microscopic fields twice for each sample. The detection limit by this protocol was 10^4 cells cm^{-3} .

Total cell counts decreased with the increase in depth below the seafloor (Fig. 1). Maximum cell densities of 4.4×10^7 and 1.3×10^7 cells cm^{-3} were observed in the shallow zones of <59.6 mbsf (hole 1188A) and 9.7 mbsf (hole 1189A), respec-

TABLE 1. Depths for core recovery and the bottom temperatures in the boreholes

Site	Hole	Casing depth (mbsf)	Core recovery depth (mbsf)	Bottom temp (°C)
1188	1188A		0–211.6	No data
	1188F	0–218.0	218.0–386.7	
1189	1189A		0–125.8	No data
	1189B	0–31.0	31.0–206.0	

* Corresponding author. Mailing address: School of Biosphere Sciences, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima 739-8528, Japan. Phone: 81-824-24-7986. Fax: 81-824-22-7059. E-mail: takn@hiroshima-u.ac.jp.

TABLE 2. Gas chromatographic detection of PFC tracers in rock fragment from core surfaces and interiors^a

Sample	Sample wt (g)	Total PFC wt (g) (10 ⁻¹⁰) ^b
Cotton swab		7.84
Core surface	5.23	2.73
Core interior	5.81	ND
Core interior	6.13	ND
Laboratory air		ND

^a The cotton swab that wiped the inside of the core liner and the laboratory atmosphere were used as positive and negative controls for PFC determination, respectively.

^b ND, not detected.

tively, while total cell counts were below the detection limit in the deeper zones of >68.9 mbsf (hole 1188A), >77.7 mbsf (hole 1189A), and >79.1 mbsf (hole 1189B). Other stains, such as acridine orange and SYBR Green I (Molecular Probes Inc., Eugene, Oreg.) (20), were also used; however, DAPI staining resulted in the lowest background fluorescence and thus was thought to provide more reliable cell count estimates.

To estimate live-cell counts in subvent rocks, ATP concentrations were measured by the luciferin-luciferase bioluminescence assay with an ATP photometer (TOA Electric Ltd., Tokyo, Japan). ATP was detected only from the shallow zones

of <48.8 mbsf (hole 1188A) and <39.1 mbsf (hole 1189A). Maximum ATP concentrations of 65.5 and 18.2 pg of ATP cm⁻³ were found in the uppermost samples of holes 1188A and 1189A, respectively (Fig. 1). ATP concentrations were converted to live-cell counts by using the ratios of ATP to carbon (28 to 510 pg of ATP) (12) and carbon to cell (39 fg of carbon cell⁻¹) (31); the conversion factor was thus 0.08 to 1.4 fg of ATP per cell or 0.7 × 10³ to 13 × 10³ cells per 1 pg of ATP (7). The estimated maximum live-cell counts were (0.5 to 8.6) × 10⁵ and (0.1 to 2.4) × 10⁵ cells cm⁻³ for holes 1188A and 1189A, respectively, which corresponded to 0.5 to 5.6% of the total cell counts.

Enrichment cultures. The interiors of the cores from different depths were anaerobically added to the anaerobic enrichment medium for *Thermococcus*-like heterotrophic sulfur reducers (1). This medium is successfully used for the enrichment of hyperthermophiles from hydrothermal-vent sediment and sedimentary cores (26). Therefore, this medium was chosen in anticipation of increased probabilities for any microbial growth by heterotrophs and facultative autotrophs.

In the anaerobic cultures at 60°C, microbial growth was found only with the cores from 59.8, 69.1, and 87.9 mbsf (hole 1188A), 48.6 and 68.4 mbsf (hole 1189A), and 79.1 and 99.4 mbsf (hole 1189B). Epifluorescence microscopy showed the

FIG. 1. Depth profiles of total cell counts (circles) and ATP concentrations (triangles) in rock core samples recovered from holes 1188A (filled symbols) and 1188F (open symbols) (A) and holes 1189A (filled symbols) and 1189B (open symbols) (B).

FIG. 2. Epifluorescence photomicrographs of DAPI-stained cells cultured at 60°C from the rock from 99.4 mbsf in hole 1189B (A) and cultured at 90°C from the rock from 118.1 mbsf in hole 1189B (B). Scale bars, 40 μm .

presence of DAPI-stained rods, some of which were dividing (Fig. 2A). In contrast, no microbial growth was found with the rocks from shallower and deeper zones. In the anaerobic cultures at 90°C, rods and cocci were found only with the cores

from 69.1, 87.9, and 106.8 mbsf (hole 1188A), 68.4 and 106.9 mbsf (hole 1189A), and 99.4, 118.1, and 128.9 mbsf (hole 1189B) (Fig. 2B). Again, no microbial growth was found with the rocks from shallower and deeper zones.

FIG. 3. Phylogenetic positions based on 1,489 nucleotides of 16S rDNA sequences of the monospecific bacterial populations grown in anaerobic 60 and 90°C cultures. The tree topography and evolutionary distances were determined by the neighbor-joining method. Values at the nodes indicate the cluster probabilities (percentages) after 1,000 bootstrap trials.

The rock samples that yielded microbial growth at 60 and 90°C were from the depths where both direct cell counts and ATP concentrations were below detection limits. Therefore, this enrichment culture complemented conventional quantitative approaches, such as direct cell counting and ATP measurement, and demonstrated the presence of viable microorganisms that otherwise would have been overlooked.

16S rDNA analysis. Bulk DNA was extracted from four enriched samples, i.e., two 60°C cultures (hole 1189A at 48.6 mbsf and hole 1189B at 99.4 mbsf) and two 90°C cultures (hole 1189A at 68.4 mbsf and hole 1189B at 118.1 mbsf), for the 16S rDNA analysis. A total of four PCR clone libraries consisting of 32 to 40 clones for the amplified 16S rDNA (ca. 1,500 bp) were constructed (8). The clones of a library were >98% identical in the 400 nucleotides of the 5' ends of 16S rDNA sequences and were grouped into a single operational taxonomic unit (OTU). A total of four OTUs were thus formed. Randomly selected clones from the OTUs were sequenced over 1.4 kb for the 16S rDNA-based phylogenetic analysis.

The OTUs from the 60°C cultures were closely related to *Geobacillus thermoleovorans* (98 to 99% nucleotide homology), isolated from a hot oil field (18), and to *Geobacillus vulcani* (97 to 98% nucleotide homology), isolated from a shallow marine hydrothermal vent (2) (Fig. 3). Major strains of the genus *Geobacillus* were collected from geothermal areas, such as the

oil field subsurface (16, 17, 19) and hydrothermal vents (2). Most *Geobacillus* species are known to grow in a thermophilic temperature range from 45 to >70°C, and some species are known to proliferate anaerobically (3, 28).

On the other hand, OTUs from the 90°C cultures were most closely related to *Deinococcus geothermalis*, with 95 to 96% nucleotide homology (Fig. 3). An isolate of *D. geothermalis* from a hot spring has the optimum and maximum growth temperatures of 48 to 50 and 55°C, respectively (9). The 16S rDNA sequences of the genus *Deinococcus* have been recovered from 1,500- to 2,000-m deep hot springs in Iceland, where temperatures range from 76 to 91.4°C. The Icelandic 16S rDNA sequences show 99% homology to *D. geothermalis* sequences (13). *Deinococcus* species were previously described as strictly aerobic; however, certain *Deinococcus* strains grow anaerobically at 65°C (13). *Deinococcus radiodurans* is known for anaerobic reduction of Fe(III), Cr(VI), U(VI), and Tc(VII) (10), challenging the view of *Deinococcus* as aerobic. Therefore, detection of the *Deinococcus* 16S rDNA in anaerobic 90°C cultures in this study does not necessarily overthrow the opposing view. Unfortunately, both *Geobacillus* and *Deinococcus* strains were lost during subculturing. It is known that certain extremophilic species require unique conditions or agents that occur in extreme environments, and it is often difficult to establish persistent culture collections (26).

Archaeal 16S rDNA was not detected in the 60 and 90°C cultures by PCR despite repeated trials using common archaeal primer sets (6). This simply means that archaeal species were not recovered from the rock core samples and does not deny the possibility of the existence of archaeal populations, particularly hyperthermophiles, in the subvent habitat. No attempt was made to extract DNA directly from the core interiors, because a limited DNA yield from less than a few grams of rocks was expected. Further enrichment cultures with a wide range of media and conditions will allow the recovery of archaeal and other bacterial species.

We are grateful to the JOIDES *Resolution* crew and scientists on the cruise of leg 193 and the curator of the ODP for obtaining and sharing samples and data of this leg.

This work was partly supported by the Special Coordination Fund "Archaeal Park Project" of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan; a grant-in-aid for scientific research from the MEXT (no. 11204205 to H. Yasuda, Kochi University, Kochi, Japan), and the Collaborative Research Fund program "Strategy for Life under Extreme Conditions" of the Graduate University for Advanced Studies, Hayama, Japan.

REFERENCES

- Baross, J. A. 1995. Isolation, growth, and maintenance of hyperthermophiles, p. 15–23. In F. T. Robb and A. R. Place (ed.), *Archaea: a laboratory manual. Thermophiles*. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
- Caccamo, D., C. Gugliandolo, E. Stackebrandt, and T. L. Maugeri. 2000. *Bacillus vulcani* sp. nov., a novel thermophilic species isolated from a shallow marine hydrothermal vent. *Int. J. Syst. Evol. Microbiol.* **50**:2009–2012.
- Claus, D., and R. C. W. Berkeley. 1986. Genus *Bacillus* Cohn 1872, 174^{AL}, p. 1105–1139. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 2. Williams & Wilkins, Baltimore, Md.
- Cragg, B. A., and R. J. Parkes. 1994. Bacterial profiles in hydrothermally active deep sediment layers from Middle Valley (NE Pacific) sites 857 and 858. *Proc. Ocean Drill. Prog. Sci. Results* **139**:509–516.
- Cragg, B. A., M. Summit, and R. J. Parkes. 2000. Bacterial profiles in a sulfide mound (site 1035) and an area of active fluid venting (site 1036) in hot hydrothermal sediments from Middle Valley (northwest Pacific). *Proc. Ocean Drill. Prog. Sci. Results* **169**:1–18. [Online.] http://www.odp.tamu.edu/publications/169_SR/VOLUME/CHAPTERS/SR169_02.PDF.
- DeLong, E. F. 1992. Archaea in coastal marine environments. *Proc. Natl. Acad. Sci. USA* **89**:5685–5689.
- Egeberg, P. K. 2000. Adenosine 5'-triphosphate (ATP) as a proxy for bacteria numbers in deep-sea sediments and correlation with geochemical parameters (site 994). *Proc. Ocean Drill. Prog. Sci. Results* **164**:393–398.
- Elsaied, H., H. Kimura, and T. Naganuma. 2002. Molecular characterization and endosymbiotic localization of the gene encoding D-ribulose 1,5-bisphosphate carboxylase-oxygenase (RuBisCO) form II in the deep-sea vestimentiferan trophosome. *Microbiology* **148**:1947–1957.
- Ferreira, A. C., M. F. Nobre, F. A. Rainey, M. T. Silva, R. Wait, J. Burghardt, A. P. Chung, and M. S. da Costa. 1997. *Deinococcus geothermalis* sp. nov. and *Deinococcus murrayi* sp. nov., two extremely radiation-resistant and slightly thermophilic species from hot springs. *Int. J. Syst. Bacteriol.* **47**:939–947.
- Fredrickson, J. K., H. M. Kostander, S. W. Li, A. E. Plymale, and M. J. Daly. 2000. Reduction of Fe(III), Cr(VI), U(VI), and Tc(VII) by *Deinococcus radiodurans* R1. *Appl. Environ. Microbiol.* **66**:2006–2011.
- Jeanthon, C. 2000. Molecular ecology of hydrothermal vent microbial communities Antonie Leeuwenhoek **77**:117–133.
- Karl, D. M. 1980. Cellular nucleotide measurements and applications in microbial ecology. *Microbiol. Rev.* **44**:739–796.
- Marteinson, V. T., S. Hauksdóttir, C. F. V. Hobel, H. Kristmannsdóttir, G. O. Hreggvidsson, and J. K. Kristjánsson. 2001. Phylogenetic diversity analysis of subterranean hot springs in Iceland. *Appl. Environ. Microbiol.* **67**:4242–4248.
- Moyer, C. L., F. C. Dobbs, and D. M. Karl. 1995. Phylogenetic diversity of the bacterial community from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. *Appl. Environ. Microbiol.* **61**:1555–1562.
- Moyer, C. L., J. M. Tiedje, F. C. Dobbs, and D. M. Karl. 1998. Diversity of deep-sea hydrothermal vent Archaea from Loihi Seamount, Hawaii. *Deep-Sea Res. Part II* **45**:303–317.
- Nazina, T. N., A. E. Ivanova, and A. V. Blagov. 1992. Microbiological characteristics of oil fields of Mangyshlak peninsula. *Microbiology* **61**:216–221.
- Nazina, T. N., A. E. Ivanova, I. A. Borzenkov, S. S. Belyaev, and M. V. Ivanov. 1995. Occurrence and geochemical activity of microorganisms in high-temperature water-flooded oil fields of Kazakhstan and Western Siberia. *Geomicrobiol. J.* **13**:181–192.
- Nazina, T. N., T. P. Tourova, A. B. Poltarau, E. V. Novikova, A. A. Grigoryan, A. E. Ivanova, A. M. Lysenko, V. V. Petrunyaka, G. A. Osipov, S. S. Belyaev, and M. V. Ivanov. 2001. Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov., and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. kaustophilus*, *G. thermoglucosidasius*, and *G. thermodenitrificans*. *Int. J. Syst. Evol. Microbiol.* **50**:1331–1337.
- Nazina, T. N., T. P. Tourova, A. B. Poltarau, E. V. Novikova, A. E. Ivanova, A. A. Grigoryan, A. M. Lysenko, and S. S. Belyaev. 2000. Physiological and phylogenetic diversity of thermophilic spore-forming hydrocarbon-oxidizing bacteria from oil fields. *Microbiology* **69**:96–102.
- Noble, R. T., and J. A. Fuhrman. 1998. Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. *Aquat. Microb. Ecol.* **14**:113–118.
- Reysenbach, A.-L., D. Götz, and D. Yernool. 2002. Microbial diversity of marine and terrestrial thermal springs, p. 345–421. In J. T. Staley and A.-L. Reysenbach (ed.), *Biodiversity of microbial life. Foundation of Earth's biosphere*. Wiley-Liss, Inc., New York, N.Y.
- Reysenbach, A.-L., N. G. Holm, K. Hershberger, D. Prieur, and C. Jeanthon. 1998. In search of a subsurface biosphere at a slow-spreading ridge. *Proc. Ocean Drill. Prog. Sci. Results* **158**:355–360.
- Reysenbach, A.-L., K. Longnecker, and J. Kirshtein. 2000. Novel bacterial and archaeal lineages from an in situ growth chamber deployed at a Mid-Atlantic Ridge hydrothermal vent. *Appl. Environ. Microbiol.* **66**:3798–3806.
- Smith, D. C., A. J. Spivack, M. R. Fisk, S. A. Haveman, H. Staudigel, and the Leg 185 Shipboard Scientific Party. 2000. Methods for quantifying potential microbial contamination during deep ocean coring. *Ocean Drill. Prog. Tech. Note* **28**. [Online.] <http://www-odp.tamu.edu/publications/tnotes/tn28/INDEX.HTM>.
- Smith, D. C., A. J. Spivack, M. R. Fisk, S. A. Haveman, H. Staudigel, and the Ocean Drilling Program Leg 185 Shipboard Scientific Party. 2000. Tracer-based estimates of drilling-induced microbial contamination of deep-sea crust. *Geomicrobiol. J.* **17**:207–219.
- Summit, M., and J. A. Baross. 2001. A novel microbial habitat in the mid-ocean ridge seafloor. *Proc. Natl. Acad. Sci. USA* **98**:2158–2163.
- Summit, M., A. Peacock, D. Ringelberg, D. C. White, and J. A. Baross. 2000. Phospholipid fatty acid-derived microbial biomass and community dynamics in hot, hydrothermally influenced sediments from Middle Valley, Juan De Fuca Ridge. *Proc. Ocean Drill. Prog. Sci. Results* **169**:1–19. [Online.] http://www-odp.tamu.edu/publications/169_SR/VOLUME/CHAPTERS/SR169_03.PDF.
- Sunna, A., S. Tokajian, J. Burghardt, F. Rainey, G. Antranikian, and F. Hashwa. 1997. Identification of *Bacillus kaustophilus*, *Bacillus thermocatenulatus*, and *Bacillus* strain HSP as members of *Bacillus thermoleovorans*. *Syst. Appl. Microbiol.* **20**:232–237.
- Takai, K., and K. Horikoshi. 1999. Genetic diversity of archaea in deep-sea hydrothermal vent environments. *Genetics* **152**:1285–1297.
- Takai, K., T. Komatsu, F. Inagaki, and K. Horikoshi. 2001. Distribution of archaea in a black smoker chimney structure. *Appl. Environ. Microbiol.* **67**:3618–3629.
- Vrede, K., M. Haldal, S. Norland, and G. Bratbak. 2002. Elemental composition (C, N, P) and cell volume of exponentially growing and nutrient-limited bacterioplankton. *Appl. Environ. Microbiol.* **68**:2965–2971.