APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2003, p. 644–648 Vol. 69, No. 1
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.1.644–648.2003


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

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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 $2.73 \times 10^{-10}$ 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′6′-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 $4.4 \times 10^7$ and $1.3 \times 10^7$ 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 | 312 |
| 1189 | 1189A | | 0–125.8 | No data |
| | 1189B | 0–31.0 | 31.0–206.0 | 68 |

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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 $0.7 \times 10^3$ to $13 \times 10^3$ cells per 1 pg of ATP (7). The estimated maximum live-cell counts were (0.5 to 8.6) $\times 10^5$ and (0.1 to 2.4) $\times 10^5$ 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

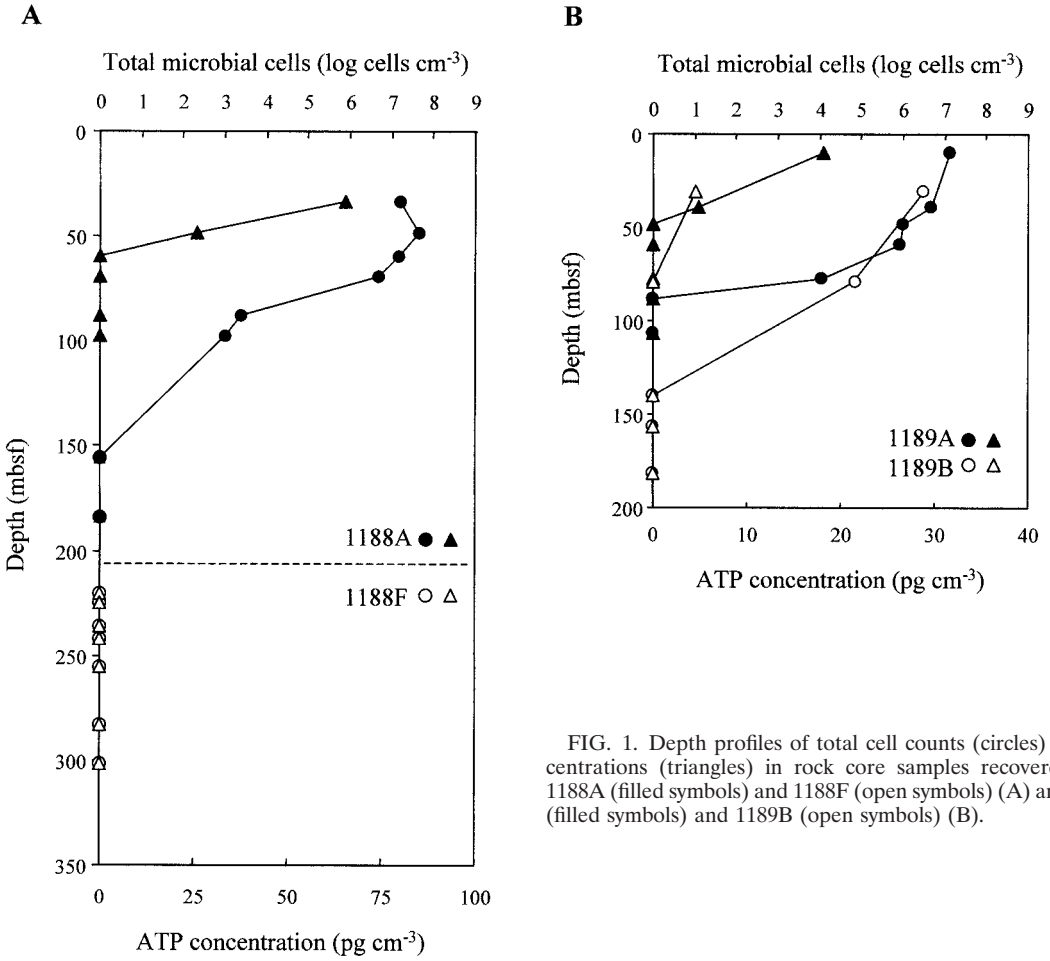


FIG. 1. Depth profiles of total cell counts (circles) and ATP concentrations (triangles) in rock core samples recovered from holes 1188A (filled symbols) and 1188F (open symbols) (A) and holes 1189A (filled symbols) and 1189B (open symbols) (B).

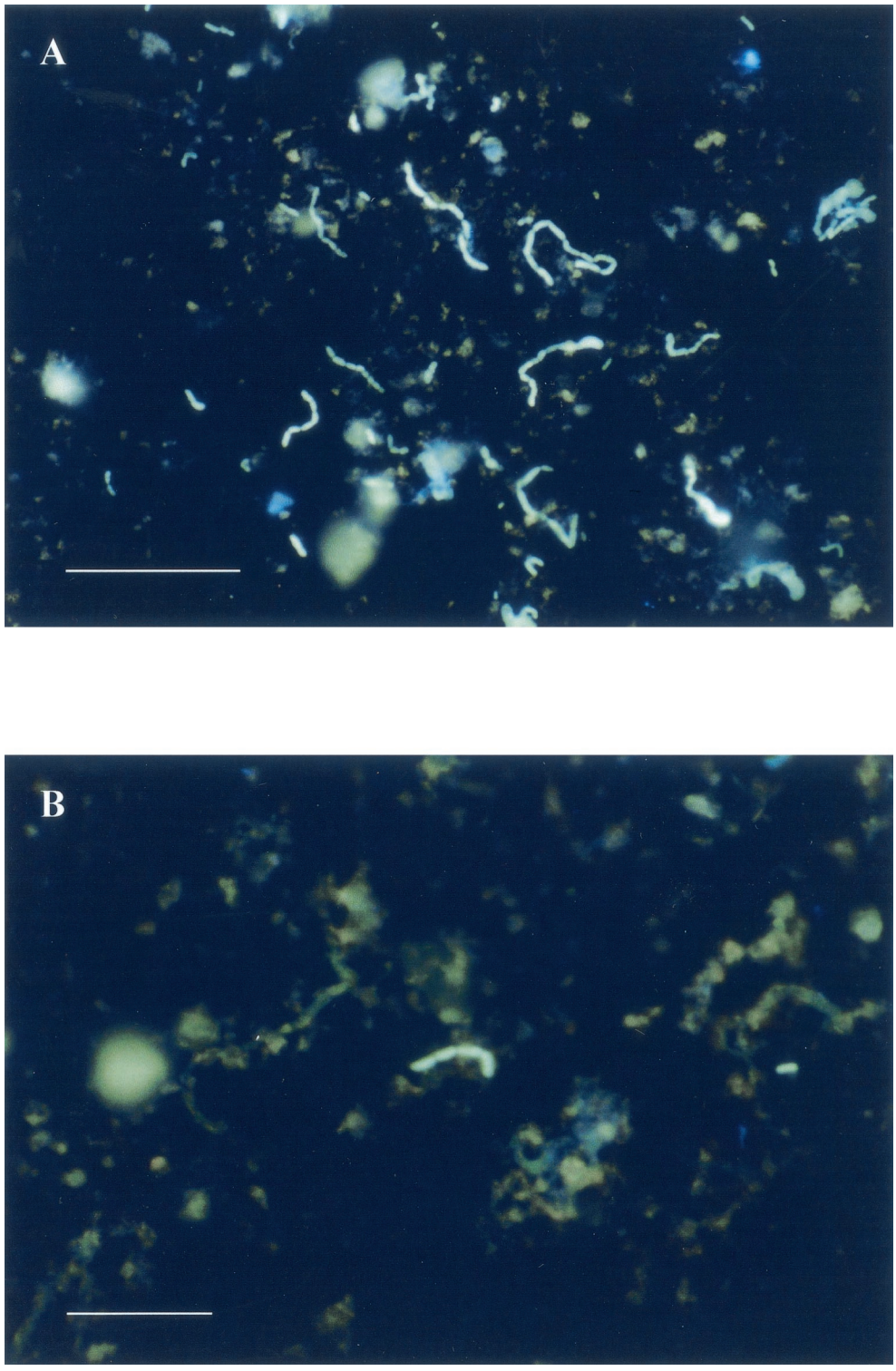


FIG. 2. Epifluorescence photomicrographs of DAPI-stained cells cultured at 60°C from the rock from 99.4 mbsf in hole 1189B (A) and cultured at 90°C from the rock from 118.1 mbsf in hole 1189B (B). Scale bars, 40 μm.

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 (hole1189A), 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.

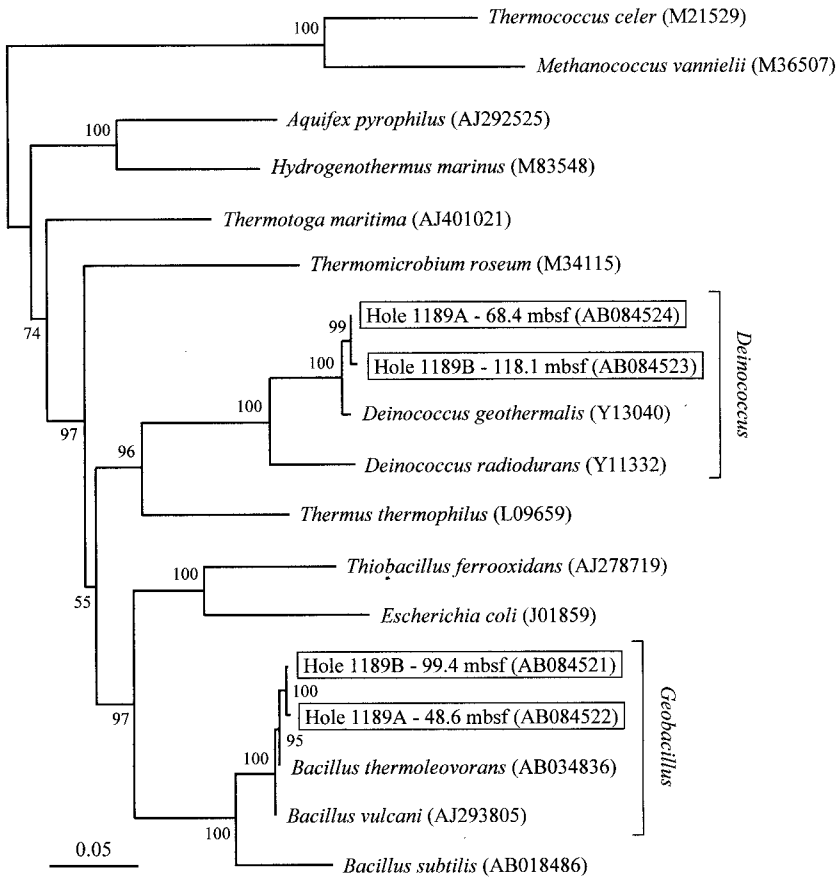


FIG. 3. Phylogenetic positions based on 1,489 nucleotides of 16S rDNA sequences of the monospecific bacterial populations grown in anaerobic 60 and 90°C cultures. The tree topography and evolutionary distances were determined by the neighbor-joining method. Values at the nodes indicate the cluster probabilities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

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