ENZYMES FROM DEEP-SEA MICROORGANISMS

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Summary

In an attempt to characterize the microbial flora on the deep-sea floor, we isolated thousands of microbes from samples collected at various deep-sea (1 050–10 897 m) sites located in the Mariana Trench and off southern Japan. Various types of bacteria, such as alkaliphiles, thermophiles, psychrophiles, and halophiles were recovered on agar plates at atmospheric pressure at a frequency of 0.8×10^2–2.3×10^4/g of dry sea mud. No acidophiles were recovered. Similarly, non extremophilic bacteria were recovered at a frequency of 8.1×10^2–2.3×10^5. These deep-sea isolates were widely distributed and detected at each deep-sea site, and the frequency of isolation of microbes from the deep-sea mud was not directly influenced by the depth of the sampling site. Phylogenetic analysis of deep-sea isolates based on 16S rDNA sequences revealed that a wide range of taxa were represented in the deep-sea environment. Among these deep-sea isolates, we have isolated a *Pseudomonas*-like amylase producer, strain MS300, which displayed a large halo on starch medium. Strain MS300 produced two major and two minor α-maltotetraohydrolases (G4-amylase). The two major G4-amylases share the same molecular weight of 55 000 but had different pI values of 5.0 and 4.7, respectively. The optimum temperature for activity of both major G4-amylases is 40°C,

and the optimum pH is 6.8 for one and 8.9 for the other. MS300 produced more amylase under higher hydrostatic pressure than under atmospheric pressure.

1. Introduction

The major environment of the Earth is the deep-sea environment, at an average depth of 3 800 m, because the ocean occupies 70% of the earth. The deep sea is an extreme environment in terms of land plants and animals, including human beings, as it is characterized by high hydrostatic pressure, low temperature, and lack of light. However, the Earths surface, exposed to strong UV light, desiccation, and atmospheric pressure, would be an extreme environment for organisms living in the deep sea. Microorganisms living in the deep sea have developed specific characteristics that allow them to thrive in that environment. Bacteria have been isolated from deep-sea mud and from benthic organisms, such as amphipods and sea cucumbers in the bathypelagic zone. However, little information is available on the bacterial diversity and the distribution of useful microbes for industrial application in sediments of the deep-sea floor.

On March 2, 1996, the 3 m long unmanned submersible *Kaiko* touched the bottom of the Challenger Deep in the Mariana Trench and successfully scooped out a mud sample, the first obtained at a depth of 10 897 m. We isolated thousands of microbes from this deep-sea mud sample and found that the microbial flora contained was composed of actinomycetes, yeasts, and a range of bacteria including various extremophilic genera.

To explore in greater detail the microbial diversity in various shallower deep-sea environments, we attempted to isolate and characterize a number of bacteria from deep-sea mud collected by the manned submersibles *Shinkai 2000* and *Shinkai 6500*. We recorded considerable bacterial diversity and the occurrence of extremophilic bacteria at several deep-sea sites located off the southern part of Japan. It is well known that some microbes produce different types of enzyme depending on the growth environment. Thus, it is expected that the deep sea will be a good source for new types of enzyme producers, because the deep-sea ecosystem differs from that on land. We therefore attempted to explore unique enzyme-producing bacteria from deep-sea isolates and focused on new types of amylase producers, as the first step in industrial application of deep-sea microbes.

2. Collection of Deep-Sea Mud

Samples of deep-sea sediment were collected from the Iheya Ridge, Nankai Islands (1 050 m depth, 27°47.18'N, 126°54.15'E), using the manned submersible *Shinkai 2000* and from the Izu-Ogasawara Trench (2 759 m, 30°07.05'N, 139°58.42'E; and 3 400 m depth, 29°04.2'N, 140°43.3'E) using the manned submersible *Shinkai 6500*, employing cylinder mud samplers (Figure 1). The 3 m long submersible *Kaiko* touched the bottom of the Challenger Deep and successfully scooped out a mud sample, the first in the world obtained at the great depth of 10,897 m, as shown in Figure 2B, C, and D. A sterile 50 ml-Falcon tube filled with a very fine mud of grayish-brownish particles was inserted tightly in a tube holder and brought to the sea surface without being contaminated by upper-ocean bacteria (Figure 2A and D).

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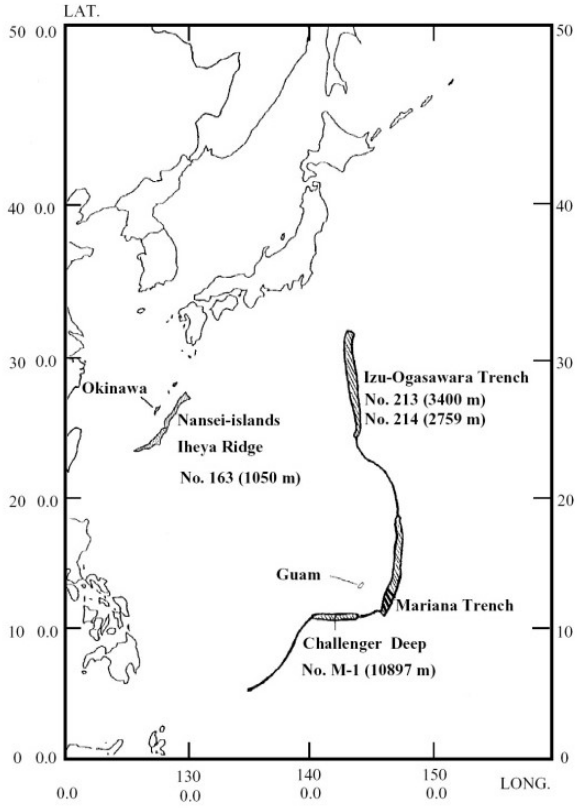

Figure. 1. Deep-sea sites for collection of mud samples.

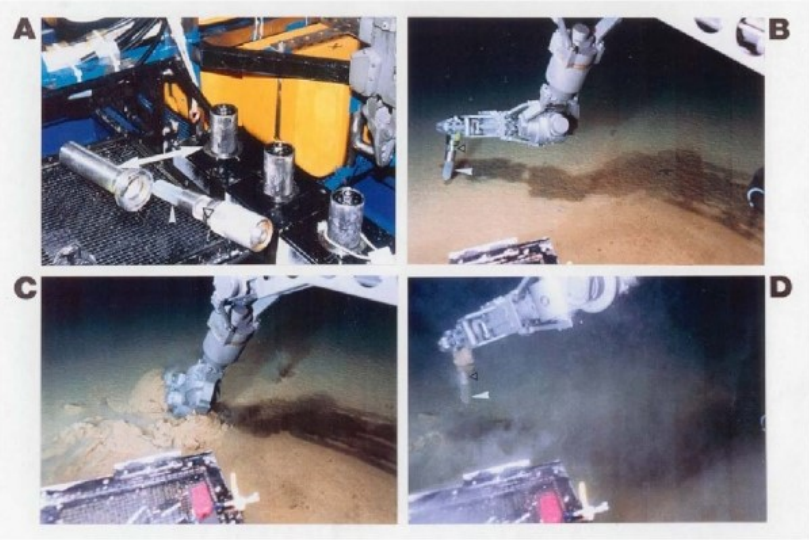

Figure 2. A, B, C, and D. Collection of a mud sample from a depth of 10,897 m in the Challenger Deep.

3. Isolation of Microorganisms From Deep-Sea Mud

The ChallengerDeep mud sample was diluted two-fold with Marine broth 2216, and 100 to 200 μl (5 to 10 mg as dry weight) of the suspension was spread on Marine agar or

half-strength nutrient agar plates used as a basal medium. In addition, modified Marine agar plates supplemented with 1% skimmed milk or 1% potato starch, which had varied pH values (pH 3, 7, or 10) and concentrations of sodium chloride (0%, 2%, or 15%), were used for isolation. The alkaline and acidic media contained 1% sodium carbonate and 50 mM citric acid, respectively. The alkaline or acidic stock solution concentrated 10-fold was autoclaved separately and then added to the plain or modified Marine agar medium. The agar plates were incubated at 4° to 75°C at atmospheric pressure (0.1 MPa) or at 100 MPa for 1 to 4 weeks. Cultivation techniques, at high pressure, were as described previously by authors from our laboratory.

3.1. Bacteria From The Mariana Trench

Various non-extremophilic bacteria were isolated from the mud sample with a frequency of 2.2×10^4 to 2.3×10^5 colonies per gram of dry sea mud under the conditions described in Table 1. This is the first report of the isolation of viable bacteria from the bottom of the Challenger Deep and is significant to our understanding of the nature of microbial flora in the deepest-sea mud. Previously, by means of microscopic observations, ZoBell confirmed the existence of viable bacterial cells in mud collected at a depth of 10 400 m from the Philippine Trench, but none of those bacteria were cultivated. We have obtained thousands of isolates from the Challenger Deep. Some were found to be producers of enzymes, such as proteases and amylases (Figure 3A and B). Although barophilic, halophilic, and acidophilic bacteria have not yet been detected in mud samples from the Challenger Deep, various other types of extremophilic bacteria were isolated at 0.1 MPa (Table 1). Among the extremophiles found were numerous alkaliphiles and thermophiles that could be expected to thrive in an extreme environment different from the *in situ* conditions of high hydrostatic pressure and low temperature in the Challenger Deep. The population of psychrophilic isolates tended to be smaller than those of alkaliphiles and thermophiles. In addition, we successfully isolated filamentous fungi and actinomycetes at 0.1 MPa at almost the same frequency (2.0×10^2 per gram of dry sea mud) as that of psychrophilic bacteria.

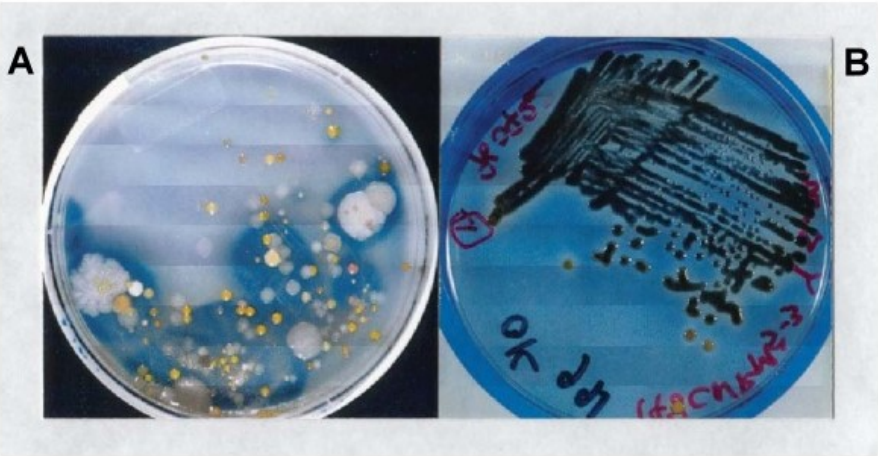

Figure 3. A and B. Deep-sea enzyme-producing isolates grown on modified Marine agar plates.

Category	Isolation conditions	Origin (Depth)	No.	Bacterial recovered (colonies g^{-1} dry sea mud)
Alkaliphile	**pH 9.7 ± 0.3** 25°C, 0.1 MPa	163 (1050m) 214 (2759m) 213 (3400m) MI (10897m)		3.0-6.1 x 10^2 0.2-2.3 x 10^4 0.9 x 10^2 0.4-1.2 x 10^3
Thermophile	**55°C** pH 7.3 ± 0.2, 0.1 MPa	163 (1050m) 214 (2759m) 213 (3400m) MI (10897m)		0.8-2.3 x 10^2 1.1-7.8 x 10^2 1.0-6.0 x 10^2 0.6-3.5 x 10^3
Psychrophile	**4°C** pH 7.3 ± 0.2, 0.1 Mpa	163 (1050m) 214 (2759m) 213 (3400m) MI (10897m)		0.8-5.3 x 10^2 1.4-7.8 x 10^2 1.0 x 10^2 2.0 x 10^2
Halophile	**15%-NaCl, 25°C** pH 7.3 ± 0.2, 0.1 Mpa	163 (1050m) 214 (2759m) 213 (3400m) MI (10897m)		4.6 x 10^2 3.6 x 10^3 0.9 x 10^2 ————
Acidophile	**pH 3.7 ± 0.2** 25°C, 0.1 MPa	163 (1050m) 214 (2759m) 213 (3400m) MI (10897m)		———— ———— ———— ————
Non-extremophile	25°C, 0.1 Mpa pH 7.3 ± 0.2	163 (1050m) 214 (2759m) 213 (3400m) MI (10897m)		0.5-6.6 x 10^3 0.2-1.1 x 10^5 8.1-9.4 x 10^2 0.2-2.3 x 10^5

———— : no growth obtained

Table 1. Isolation of extremophilic bacteria from several deep-sea sites.

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Biographical Sketch

Hideto Takami received his Bachelors degree in agricultural chemistry and education from Tokyo University of Agriculture in 1984, and his M.S. degree in 1986 from Yamanashi University. He then worked for one and a half year at Kurita Water Industry Co. Ltd. and for three and a half years at RIKEN Institute as a visiting research associate. He received a Ph.D. degree in 1993 from Tokyo Institute of Technology, Department of Bioengineering. He next went to Michigan State University at East Lansing, where he carried out research as a postdoctoral fellow (1993-1995) on the distribution of 2,4-D degrading bacteria in the pristine soil and 2,4-D metabolic pathways. In May 1995, he joined the department of Frontier Research System for Extremophiles at Japan Marine Science and Technology Center (JAMSTEC), where he is currently senior scientist and group leader of Microbial Genome Research Group. In 1997, he and his colleagues published the first paper on the microbial flora of the deepest mud of Mariana Trench, Challenger Deep. In 1998, he initiated the whole genome sequencing project of alkaliphilic *Bacillus halodurans* which produces various kinds of enzymes. His group completed the sequencing project of *B. halodurans* genome in 2000. His research interests include the adaptation mechanisms of extremophilic bacteria and the evolutionary process of the genomes of microorganisms. He lives with his wife, Yumiko, in Yokohama city.

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