

# Microbial flora in deep-sea sites located near the south part of Japan.

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We obtained a hundred isolates of bacteria from deep-sea mud samples collected at various depths (1,050-10,897 m). Various types of bacteria such as alkaliphiles, thermophiles, psychrophiles, and halophiles were recovered on agar plates at a frequency of  $0.8 \times 10^2$  -  $2.3 \times 10^4$  per g of dry sea mud. Acidophiles were not recovered. It was found that these extremophilic bacteria were widely distributed, being detected at each deep-sea site, and the frequency of isolation of such extremophiles from the deep-sea mud was not directly influenced by the depth of the sampling sites. Growth patterns under high hydrostatic pressure were determined for the deep-sea isolates obtained in this study. No extremophilic strains isolated in this study showed growth at 60 MPa although a few of the other isolates grew slightly at this hydrostatic pressure.

**Key words :** Extremophiles, Halophiles, Psychrophiles, Alkaliphiles, Thermophiles, Deep-sea

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## 1. Introduction

The most concentrated and widespread occurrences of organisms are generally in so-called "moderate" environments with approximately neutral pH, temperatures around 20–37°C, pressures near 0.1 MPa, adequate concentrations of nutrients and moderate salinity. In contrast, the deep-sea is an extreme environment with especially high hydrostatic pressure and low temperature. Microorganisms living there presumably have developed particular characteristics that allow them to thrive in such an environment. Bacteria have been isolated from deep-sea mud and from benthic organisms such as amphipods and sea cucumbers in the bathypelagic zone<sup>1,2</sup>. However, little information is available on bacterial diversity in sediments of the deep-sea floor because most marine biologists have focused on barophilic and psychrophilic inhabitants of the deep-sea environment<sup>3,4</sup>.

On 2 March, 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 and found that the microbial flora there was composed of actinomycetes, yeasts, and a range of bacterial lives including various extremophilic bacteria<sup>5</sup>. In

studies aimed at further exploring the microbial diversity in various deep-sea environments, we attempted to isolate and characterize a number of bacteria from deep-sea mud collected by means of the manned submersibles Shinkai 2000 and 6500<sup>6</sup>. In this paper, we record considerable bacterial diversity and the occurrence of extremophilic bacteria at several deep-sea sites located near the south part of Japan.

## 2. Materials and methods

### 2.1 Collection of deep-sea mud

Samples of deep-sea sediment were collected from the Nankai-Islands Iheya Ridge (1,050 m depth; 27°47.18'N, 126°54.15'E) by means of 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) by means of the manned submersible Shinkai 6500, using cylinder mud samplers (Fig. 1).

### 2.2 Isolation of bacteria from deep-sea mud

The mud samples were 2-fold diluted with sterile Marine Broth 2216 (Difco Lab.)<sup>7</sup>, and 100–200 µl (5–10 mg as dry weight) of the suspension was spread on Marine agar plates used as a basal medium.

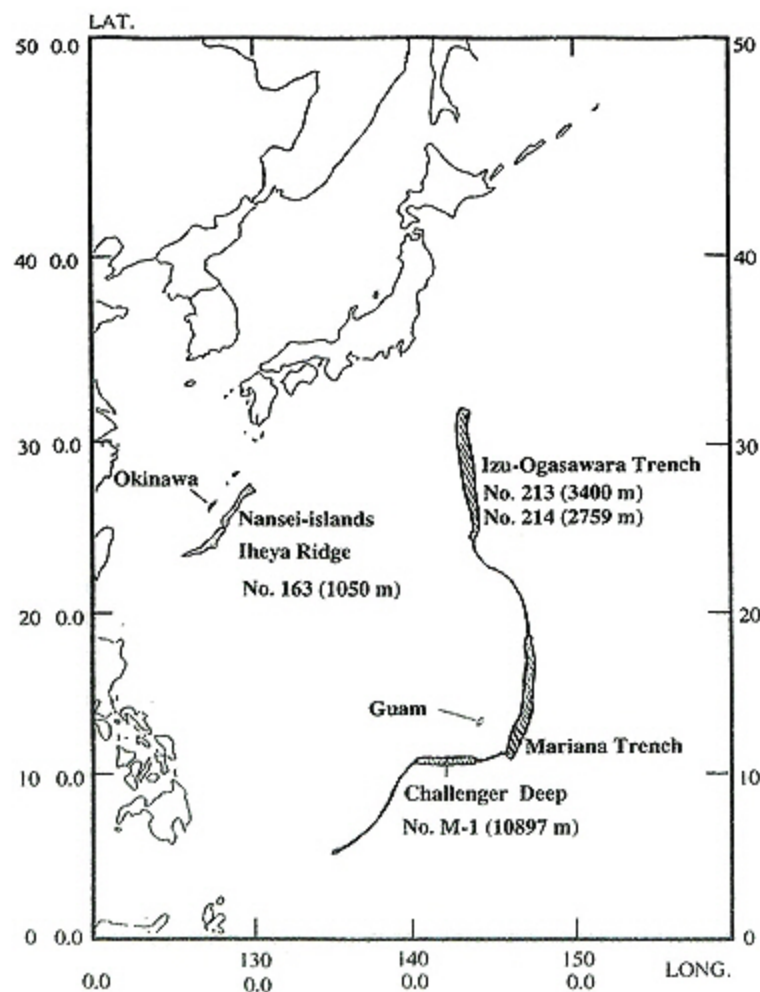


Fig. 1 Deep-sea sites for collection of mud samples. Sediment sample No. 163 was collected from the Nankai-islands Iheya Ridge by means of the manned submersible Shinkai 2000. Sediment samples No. 213 and No. 214 were obtained from the Izu-Ogasawara Trench (30°07.05'N, 139°58.42'E) and (29°04.2'N, 140°43.3'E), respectively, by means of the manned submersible Shinkai 6500. Deep-sea mud (M1) from the Challenger Deep region of the Mariana Trench was collected as described previously<sup>5</sup>.

Modified Marine agar plates supplemented with 1% skim milk or 1% potato starch, with different pH (3, 7, or 9.7) and NaCl concentrations (0, 2, or 15%), were used for isolation. The alkaline and acidic media contained 1% sodium carbonate and 50 mM citric acid, respectively. The sodium carbonate and citric acid stock solutions, 10-fold concentrated, were autoclaved separately and then added to the plain or modified Marine agar medium. The agar plates were incubated at 4 to 55°C at atmospheric pressure (0.1 MPa) for 1 to 4 weeks.

### 2.3 Cultivation of deep-sea isolates under high pressure

A total of twenty-five isolates including examples of extremophiles were selected at random representing several different deep-sea sites (Fig. 1). A Marine agar plate was inoculated with each isolate and overlaid with low melting agarose (1% v/v) dissolved in the same medium. Each agar plate

was slipped into a plastic bag which contains the air corresponding to the headspace of a regular petri dish and sealed with a heat sealer. The air in the plastic bag is pressed into the agar during incubation in the pressure vessel. The bacteria were cultivated at 4°C under pH and salt concentration conditions appropriate for each isolate (Table 2) at 30, 60 or 100 MPa for 4 weeks. The techniques employed for cultivation at high pressure were as described previously<sup>9</sup>.

## 3. Results and discussion

### 3.1 Isolation of extremophilic bacteria from deep-sea mud

Halophiles, psychrophiles, thermophiles and alkaliphiles were isolated at 0.1 MPa from the deep-sea mud collected at the different sites (Fig. 1), although acidophilic bacteria were not detected in the mud samples in this study or the previous study<sup>9</sup>. There was no substantial difference in the frequency of isola-

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

| Category         | Isolation conditions                    | Origin No. (Depth) | Bacterial recovered (colonies g <sup>-1</sup> dry sea mud) |
|------------------|-----------------------------------------|--------------------|------------------------------------------------------------|
| Alkaliphile      | pH 9.7 ± 0.3<br>25°C, 0.1 MPa           | 163 (1050m)        | 3.0 - 6.1 × 10 <sup>2</sup>                                |
|                  |                                         | 214 (2759m)        | 0.2 - 2.3 × 10 <sup>4</sup>                                |
|                  |                                         | 213(3400m)         | 0.9 × 10 <sup>2</sup>                                      |
|                  |                                         | M1(10897m)*        | 0.4 - 1.2 × 10 <sup>3</sup>                                |
| Thermophile      | 55 °C<br>pH 7.3 ± 0.2, 0.1 MPa          | 163 (1050m)        | 0.8 - 2.3 × 10 <sup>2</sup>                                |
|                  |                                         | 214 (2759m)        | 1.1 - 7.8 × 10 <sup>2</sup>                                |
|                  |                                         | 213(3400m)         | 1.0 - 6.0 × 10 <sup>2</sup>                                |
|                  |                                         | M1(10897m)*        | 0.6 - 3.5 × 10 <sup>3</sup>                                |
| Psychrophile     | 4°C<br>pH 7.3 ± 0.2, 0.1 MPa            | 163 (1050m)        | 0.8 - 5.3 × 10 <sup>2</sup>                                |
|                  |                                         | 214 (2759m)        | 1.4 - 7.8 × 10 <sup>2</sup>                                |
|                  |                                         | 213(3400m)         | 1.0 × 10 <sup>2</sup>                                      |
|                  |                                         | M1(10897m)*        | 2.0 × 10 <sup>2</sup>                                      |
| Halophile        | 15%-NaCl, 25°C<br>pH 7.3 ± 0.2, 0.1 MPa | 163 (1050m)        | 4.6 × 10 <sup>2</sup>                                      |
|                  |                                         | 214 (2759m)        | 3.6 × 10 <sup>3</sup>                                      |
|                  |                                         | 213(3400m)         | 0.9 × 10 <sup>2</sup>                                      |
|                  |                                         | M1(10897m)*        | —                                                          |
| Acidophile       | pH 3.7 ± 0.2<br>25°C, 0.1 MPa           | 163 (1050m)        | —                                                          |
|                  |                                         | 214 (2759m)        | —                                                          |
|                  |                                         | 213(3400m)         | —                                                          |
|                  |                                         | M1(10897m)*        | —                                                          |
| Non-extremophile | 25°C, 0.1 MPa<br>pH 7.3 ± 0.2           | 163 (1050m)        | 0.5 - 6.6 × 10 <sup>3</sup>                                |
|                  |                                         | 214 (2759m)        | 0.2 - 1.1 × 10 <sup>5</sup>                                |
|                  |                                         | 213(3400m)         | 8.1 - 9.4 × 10 <sup>2</sup>                                |
|                  |                                         | M1(10897m)*        | 0.2 - 2.3 × 10 <sup>5</sup>                                |

— : no growth obtained, \*Takami et al.(1997)

tion of thermophilic and psychrophilic bacteria among the three deep-sea mud samples examined in this study (Table 1). The population of alkaliphilic isolates from deep-sea site No. 214 ( $0.2 - 2.3 \times 10^4$ ) was larger than those from the other deep-sea sites, and the frequency of isolation of alkaliphiles did not vary depending on the depth of the sampling site. On the other hand, halophilic bacteria were isolated from mud samples collected at depths of 1,050 m (No. 163), 2,759 m (No. 214) and 3,400 m (No. 213) under the conditions described in Table 1 whereas halophiles were not detected in our previous study<sup>9</sup>. Some were found to be alkaliphilic halophiles growing at pH 9.3 (Table 2). The halophilic strain HTE831 (Table 2) grew on Marine agar plates containing 2% (0.34 M) to 23.4% (4 M) NaCl and strain HTC018 (Table 2) grew on Marine agar plates containing 15% (2.6 M) NaCl but not those containing 2% NaCl.

Other halophiles and alkaliphilic halophiles isolated grew well on Marine agar plates containing 2% to 15% NaCl (data not shown).

Various non-extremophilic bacteria also were isolated at 0.1 MPa from the mud samples at a frequency of  $0.5 - 6.6 \times 10^3$  (No. 163) to  $0.2 - 1.1 \times 10^5$  (No. 214) colonies per gram of dry sea mud under the conditions described in Table 1. The frequency of isolation of non-extremophilic bacteria did not change depending on the depth of habitat. Some of them were found to be producers of enzymes such as proteases and amylases (Table 3). In this study, it was found that the frequency of isolation of extremophilic and non-extremophilic bacteria at 0.1 MPa was not directly related to the depth of sampling sites. We presume that the frequency of isolation of bacteria from the deep-sea sediment at 0.1 MPa may be influenced by other factors, such

Table 2 Growth patterns of deep-sea isolates under high hydrostatic pressure

| Strain No.(Origin No.)        | pH  | Growth under |     |    |           |
|-------------------------------|-----|--------------|-----|----|-----------|
|                               |     | 0.1          | 30  | 60 | 100 (MPa) |
| <b>Alkaliphile</b>            |     |              |     |    |           |
| HTB110 ( 214)                 | 9.5 | ++           | -   | -  | -         |
| HTB113 ( 214)                 | 9.5 | +++          | +   | -  | -         |
| HTB119 ( 214)                 | 9.5 | +++          | +   | -  | -         |
| HTB127 ( 214)                 | 9.5 | ++           | -   | -  | -         |
| HTB132 ( 214)                 | 9.5 | +++          | -   | -  | -         |
| <b>Halophile</b>              |     |              |     |    |           |
| HTE830 (163)                  | 7.4 | ++           | -   | -  | -         |
| HTB068 (214)                  | 7.4 | +++          | +++ | -  | -         |
| HTB069 (214)                  | 7.4 | +++          | +++ | -  | -         |
| <b>Alkaliphilic halophile</b> |     |              |     |    |           |
| HTB 122 (214)                 | 9.3 | +++          | ++  | -  | -         |
| HTB 123 (214)                 | 9.3 | ++           | -   | -  | -         |
| HTB 124 (214)                 | 9.3 | ++           | -   | -  | -         |
| <b>Neutrophile</b>            |     |              |     |    |           |
| HTE777 (163)                  | 7.6 | ++           | -   | -  | -         |
| HTE807 (163)                  | 7.6 | +            | -   | -  | -         |
| HTE811 (163)                  | 7.6 | +            | -   | -  | -         |
| HTE846 (163)                  | 7.6 | +++          | ++  | -  | -         |
| HTE856 (163)                  | 7.6 | +            | +   | +  | +         |
| HTB006 (214)                  | 7.6 | +++          | ++  | +  | -         |
| HTB021 (214)                  | 7.6 | +++          | ++  | +  | +         |
| HTB035 (214)                  | 7.6 | +++          | -   | -  | -         |
| HTB080 (214)                  | 7.6 | +++          | +++ | -  | -         |
| HTB095 (214)                  | 7.6 | +++          | +++ | +  | +         |
| HTC004 (213)                  | 7.6 | +            | -   | -  | -         |
| HTC010 (213)                  | 7.6 | +++++        | +++ | -  | -         |
| HTC011 (213)                  | 7.6 | +++          | ++  | -  | -         |
| HTC028 (213)                  | 7.6 | ++           | -   | -  | -         |

All isolates were incubated at 4°C under high hydrostatic pressure.

Symbols : -, no growth ; +, slight growth ; ++, growth ; +++, good growth ; +++++, very good growth.

as the size and kind of sediment particles, nutrient conditions in the sediment, and the speed of the bottom current. All isolates have been stored in the gas phase area of a nitrogen freezer (-165 to -171°C) for further investigation.

### 3.2 Growth patterns of deep-sea isolates under high pressure

Twenty-five isolates described in Table 2 were cultivated at 4°C (the in situ temperature of the deep-sea floor) under high hydrostatic pressure to examine the growth patterns of the isolates obtained from different depths. As shown in Table 2, the alkaliphilic bacteria did not grow at pressures higher than 30 MPa during a four week period of cultivation, although two strains (HTB113 and HTB119) grew slightly at 30 MPa. Two moderately halophilic isolates, HTB068 and HTB069, grew well at 30 MPa but another one, HTE830, showed no growth at the same hydrostatic pressure. Two of three alkaliphilic halobacteria did not grow at pressures greater than 30 MPa, the same as observed with alkaliphiles, whereas strain HTB122 grew at 30 MPa (Table 2). Eight of fourteen neutrophilic strains grew at 30 MPa and, of these, only three strains grew slightly at 100 MPa. Alkaliphiles were more sensitive to inhibition by pressure at 30 MPa than the neutrophiles although two of them (HTB113 and HTB119) grew slightly, the same as observed in our previous study on isolates from deep-sea mud of the Mariana Trench<sup>9</sup>. It was found that some Mariana isolates grew slowly at 100 MPa, which is similar to the in situ pressure conditions in the Mariana Trench and they presumably had become adapted to such hydrostatic pressure over a long period of time. In the present study, most of the isolates from the deep-sea mud collected at depths of 1,050 m, 2,759 m and 3,400 m were sensitive to hydrostatic pressures higher than 30 MPa. Thus, many of bacteria living in the deep-sea sediment may be well adapted to the in situ hydrostatic pressure of their deep-sea environment but some may not be indigenous.

### 4. Conclusion

We isolated many bacteria from deep-sea sediments and characterized their properties. It was found that deep-sea isolates were composed of various species based on the analysis of 16S rDNA sequences (data not shown). Some of isolates might be novel species. These findings demonstrate that various types of extremophilic bacteria are widely distributed in the deep-sea mud at depths of 1050 -10897 m near the south part of Japan (Fig. 1).

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