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Biodiversity in deep-sea sites located near the south part of Japan

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Abstract We obtained 100 isolates of bacteria from deep-sea mud samples collected at various depths (1050–10 897 m). Various types of bacteria such as alkaliphiles, thermophiles, psychrophiles, and halophiles were recovered on agar plates at a frequency of 0.8×10^2 to 2.3×10^4/g of dry sea mud. No acidophiles were recovered. 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. Phylogenetic analysis of deep-sea isolates based on 16S rDNA sequences revealed that a wide range of taxa were represented in the deep-sea environments. 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 · 16S ribosomal RNA · Phylogenetic tree

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 (Yayanos 1979, 1981). 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 (Jannasch and Taylor 1984; Yayanos 1995).

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 and found that the microbial flora was composed of actinomycetes, yeasts, and a range of bacterial types including various extremophilic bacteria (Takami et al. 1997). 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* (Takagawa et al. 1989). In this article, we record considerable bacterial diversity and the occurrence of extremophilic bacteria at several deep-sea sites located near the south part of Japan.

Materials and methods

Collection of deep-sea mud

Samples of deep-sea sediment were collected from the Nankai Islands Iheya Ridge (1050-m depth: 27°47.18′ N, 126°54.15′ E) by means of the manned submersible *Shinkai 2000* and from the Izu-Ogasawara Trench (2759-m depth: 30°07.05′ N, 139°58.42′ E; and 3400-m depth: 29°04.2′ N, 140°43.3′ E) by means of the manned submersible *Shinkai 6500*, using cylindrical mud samplers (Fig. 1).

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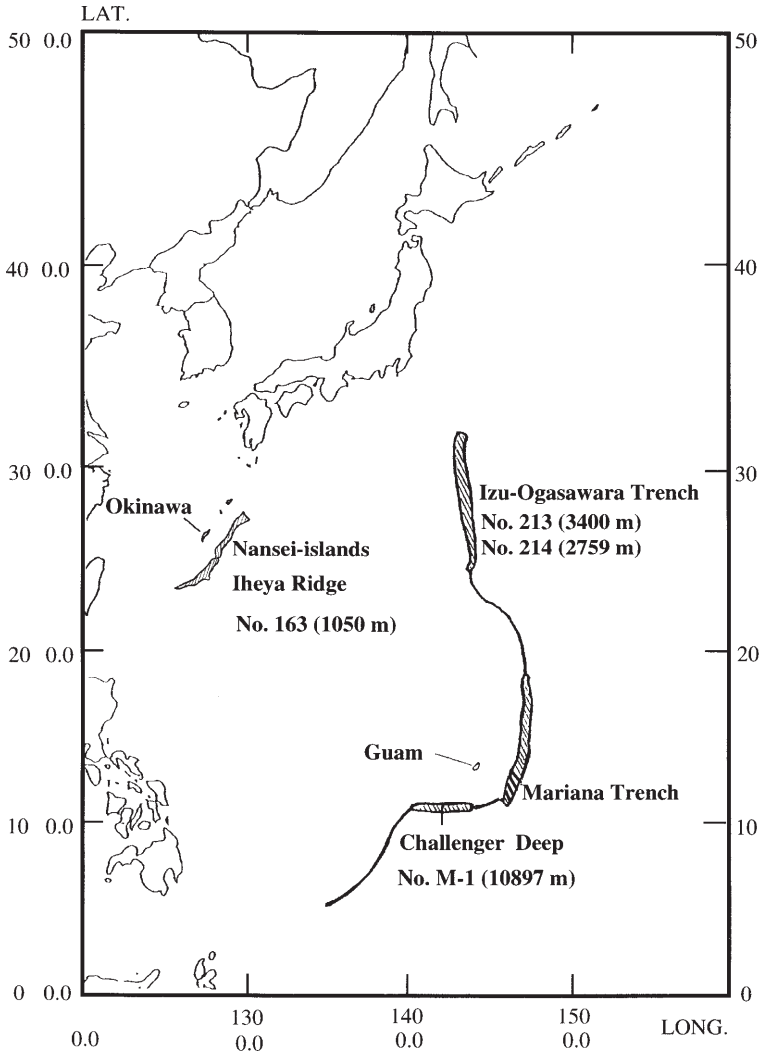

Fig. 1. Deep-sea sites for collection of mud samples. Sediment sample no. 163 was collected from the Nankai Islands, Iheya Ridge, by means of the manned submersible *Shinkai 2000*. Sediment samples no. 213 and no. 214 were obtained from the Izu-Ogasawara Trench (30°07.05′ N, 139°58.42′ E and 29°04.2′N, 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 (Takami et al. 1997)

Isolation of bacteria from deep-sea mud

The mud samples were diluted twofold with sterile marine broth 2216 (Difco, Detroit, MI, USA) (ZoBell 1941), and 100–200 µl (5–10 mg as dry weight) of the suspension was spread on marine agar plates used as a basal medium.

Modified marine agar plates supplemented with 1% skim milk or 1% potato starch, at different pHs (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°–55°C at atmospheric pressure (0.1 MPa) for 1–4 weeks.

Cultivation of deep-sea isolates under high pressure

A total of 25 isolates including examples of extremophiles and representing several different deep-sea sites were selected at random (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 inserted into a plastic bag that contained the amount of air corresponding to the headspace of a petri dish and was 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 1) at 30, 60, or 100 MPa for 4 weeks. The techniques employed for cultivation at high pressure were as described previously (Abe and Horikoshi 1995).

16S rDNA sequencing and analysis

Twenty-four strains were selected on the basis of cell and colony morphology from among the deep-sea isolates categorized as alkaliphiles, halophiles, alkaliphilic halophiles, and neutrophiles for 16S rDNA analysis. Each strain was cultivated on an agar plate under appropriate growth conditions as shown in Table 2. One loop of cell pellet was suspended in 50 µl of 25 mM Tris-HCl, 50 mM glucose, 10 mM EDTA, and lysozyme (1 mg ml^{-1}). The cell suspension was

Table 1. Growth patterns of deep-sea isolates under high hydrostatic pressure

Strain no. (Origin no.)	pH	Growth under			
		0.1 MPa	30 MPa	60 MPa	100 MPa
Alkaliphile					
HTB110 (214)	9.5	++	−	−	−
HTB113 (214)	9.5	+++	+	−	−
HTB119 (214)	9.5	+++	+	−	−
HTB127 (214)	9.5	++	−	−	−
HTB132 (214)	9.5	+++	−	−	−
Halophile					
HTE830 (163)	7.4	++	−	−	−
HTB068 (214)	7.4	+++	+++	−	−
HTB069 (214)	7.4	+++	+++	−	−
Alkaliphilic halophile					
HTB 122 (214)	9.3	+++	++	−	−
HTB 123 (214)	9.3	++	−	−	−
HTB 124 (214)	9.3	++	−	−	−
Neutrophile					
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

Table 2. Deep-sea isolates selected for 16S rDNA analysis

Strain no. (Origin no.)	Growth conditions		Remarks
	Medium[a]	pH	
Alkaliphile			
HTB110 (214)	3M	9.6	
HTB111 (214)	3M	9.6	
HTB113 (214)	3M	9.6	
HTB138 (214)	MC	9.6	
HTB139 (214)	MC	9.6	
HTB143 (214)	MS	9.6	
HTB147 (214)	MS	9.6	
Halophile			
HTE831 (163)	15M	7.4	
HTB148 (214)	15M	7.4	
HTB069 (214)	15M	7.4	
HTC018 (213)	15M	7.4	
Alkaliphilic halophile			
HTB122 (214)	15M	9.3	
HTB123 (214)	15M	9.3	
Neutrophile			
HTE856 (163)	MS	7.6	Amylase producer
HTB082 (214)	3M	7.6	
HTB010 (214)	3M	7.6	
HTB015 (214)	3M	7.6	
HTB019 (214)	3M	7.6	
HTB021 (214)	MC	7.6	Protease producer
HTB028 (214)	MC	7.6	
HTB091 (214)	MS	7.6	Amylase producer
HTB095 (214)	MS	7.6	Amylase producer
HTB096 (214)	MS	7.6	
HTC042 (213)	MS	7.6	

[a] Medium: 3M, marine broth containing 3% NaCl; 15M, marine broth containing 15% NaCl; MC, marine broth containing 1% skim milk; MS, marine broth containing 1% starch. All isolates were incubated at 25°C under atmospheric pressure

incubated at 37°C for 20 min, and 5 µl of 10% SDS solution was added to the cell suspension to extract chromosomal DNA. The DNA was purified by phenol–chloroform treatment and then used as template DNA for polymerase chain reaction (PCR) amplification of 16S rDNA. The following procaryote-specific primers were used for gene amplification and sequencing: EU10F (5′-AGAGTTTGATCCTGGCTCAG-3′), EU1500R (5′-GGTTACCTTGTTACGACTT-3′), EU500F (5′-GTGCCAGCAGCCGCGG-3′), EU500R (5′-GTATTACCGCGGCTGCTG-3′), EU1100F (5′-AAGTCCCGCAACGAGCGCA-3′), and EU1100R (5′-TTGCGCTCGTTGCGGGACT-3′). The amplified fragments were purified with Suprec-02 (Takara Shuzo, Kyoto, Japan) and sequenced using a LI-COR DNA sequencer (model 400L; LI-COR, Lincoln, NE, USA).

16S rDNA sequences were aligned using the Clustal multiple-alignment program (Clustal W) (Thompson et al. 1994). Sites involving gaps were excluded from all analyses. A phylogenetic tree was inferred by the neighbor-joining method (Saitou and Nei 1987), with DNADIST and NEIGHBOR programs in the PHYLIP package, version 3.57 (Felsenstein 1985, 1995). The sequences determined in this study were aligned with sequences previously deposited in GenBank. The nucleotide sequence data reported in this paper have been submitted to DDBJ, EMBL, and GenBank nucleotide sequence databases under the following accession numbers: AB010843 for HTE856, AB0010842 for HTB082, AB0010858 for HTB010, AB010864 for HTB015, AB010860 for HTB019, AB010854 for HTB028, AB010869 for HTB091, AB010853 for HTB095, AB010862 for HTB096, AB010848 for HTC042, AB0010851 for HTB110, AB010870 for HTB111, AB010861 for HTB113, AB010847 for HTB138, AB010859 for HTB021, AB010852 for HTB139, AB010845 for HTB143, AB010850 for HTB147, AB010863 for HTE831, AB010868 for HTB148, AB010846 for HTB069, AB010865 for HTC018, AB010867 for HTB122, and AB010866 for HTB123.

Results and discussion

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 (see Fig. 1), although acidophilic bacteria were not detected in the mud samples in this study or the previous study by Takami et al. (1997). There was no

Table 3. Isolation of extremophilic bacteria from several deep-sea sites

Category	Isolation conditions	Origin no. (depth)	Bacteria recovered (colonies g^{-1} dry sea mud)
Alkaliphile	pH 9.7 ± 0.3	163 (1050 m)	$3.0–6.1 \times 10^2$
	25°C, 0.1 MPa	214 (2759 m)	$0.2–2.3 \times 10^4$
		213 (3400 m)	0.9×10^2
		M1 (10897 m)[a]	$0.4–1.2 \times 10^3$
Thermophile	55°C	163 (1050 m)	$0.8–2.3 \times 10^2$
	pH 7.3 ± 0.2, 0.1 MPa	214 (2759 m)	$1.1–7.8 \times 10^2$
		213 (3400 m)	$1.0–6.0 \times 10^2$
		M1 (10897 m)[a]	$0.6–3.5 \times 10^3$
Psychrophile	4°C	163 (1050 m)	$0.8–5.3 \times 10^2$
	pH 7.3 ± 0.2, 0.1 MPa	214 (2759 m)	$1.4–7.8 \times 10^2$
		213 (3400 m)	1.0×10^2
		M1 (10897 m)[a]	2.0×10^2
Halophile	15% NaCl, 25°C	163 (1050 m)	4.6×10^2
	pH 7.3 ± 0.2, 0.1 MPa	214 (2759 m)	3.6×10^3
		213 (3400 m)	0.9×10^2
		M1 (10897 m)[a]	–
Acidophile	pH 3.7 ± 0.2	163 (1050 m)	–
	25°C, 0.1 MPa	214 (2759 m)	–
		213 (3400 m)	–
		M1 (10897 m)[a]	–
Nonextremophile	25°, 0.1 MPa	163 (1050 m)	$0.5–6.6 \times 10^3$
	pH 7.3 ± 0.2	214 (2759 m)	$0.2–1.1 \times 10^5$
		213 (3400 m)	$8.1–9.4 \times 10^2$
		M1 (10897 m)[a]	$0.2–2.3 \times 10^5$

–, no growth obtained
[a] From Takami et al. (1997)

substantial difference in the frequency of isolation of thermophilic and psychrophilic bacteria among the three deep-sea mud samples examined in this study (Table 3). 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 1050 m (no. 163), 2759 m (no. 214), and 3400 m (no. 213) under the conditions described in Table 3, whereas halophiles were not detected in our previous study (Takami et al. 1997). Some were found to be alkaliphilic halophiles growing at pH 9.3 (see Table 1). The halophilic strain HTE831 (Table 1) grew on marine agar plates containing 2% (0.34 M) to 23.4% (4 M) NaCl, and strain HTC018 (Table 1) 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%–15% NaCl (data not shown).

Various nonextremophilic 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 3. The frequency of isolation of nonextremophilic bacteria did not vary depending on the depth of habitat. Some of these were found to be producers of enzymes such as proteases and amylases (see Table 2). In this study, it was found that the frequency of isolation of extremophilic and nonextremophilic 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 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.

Growth patterns of deep-sea isolates under high pressure

Twenty-five isolates described in Table 1 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 1, the alkaliphilic bacteria did not grow at pressures higher than 30 MPa during a 4-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 stram, HTE830, showed no growth at the same hydrostatic pressure. Two of three alkaliphilic halobacteria did not grow at pressures greater than 30 MPa, as was observed with alkaliphiles, whereas strain HTB122 grew at 30 MPa (Table 1). Eight of 14 neutrophilic strains grew at 30 MPa and, of these, only 3 strains grew slightly at 100 MPa. Alkaliphiles were more sensitive to inhibition by pressure at 30 MPa than the neutrophiles, although 2 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 (Takami et al. 1997). 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 these strains 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 1050,

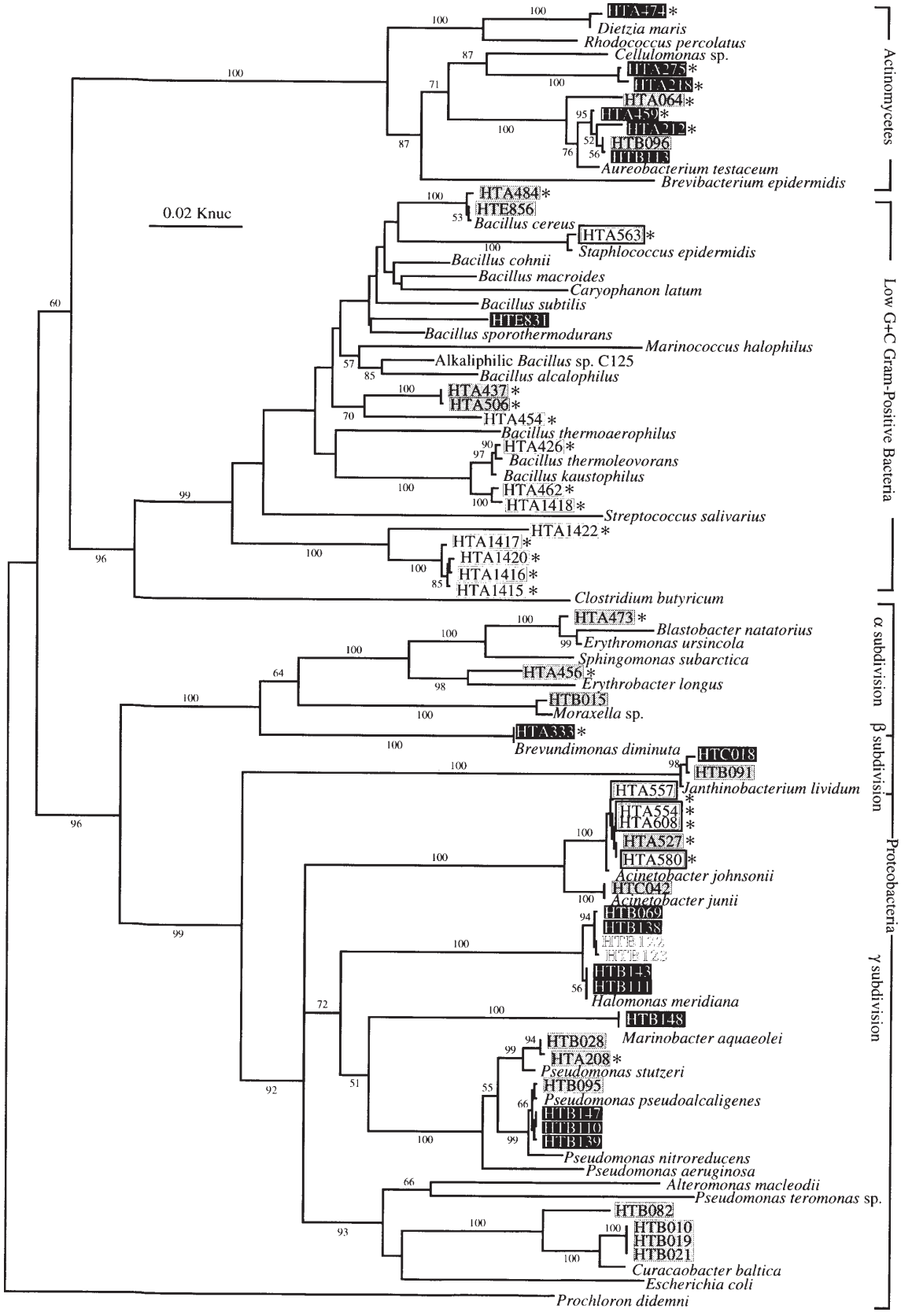

Fig. 2. Unrooted phylogenetic tree shows the relationship of bacteria isolated from the deep-sea to reference organisms. The numbers indicate the percentages of bootstrap samples, derived from 10 000 samples that supported the internal branches. Bootstrap probability values less than 50% were omitted from this figure. *Black box*, alkaliphile; *heavily shaded box*, halophile; *lightly shaded box*, neutrophile; outlined box, thermophile; *double-outline box*, psychrophile; *stram abbreviation in outline characters*, alkaliphilic halophile. *, Isolates from the Mariana Trench (Takami et al. 1997)

2759, and 3400 m were sensitive to hydrostatic pressures higher than 30 MPa. Thus, many 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.

16S rDNA sequencing and analysis

For the deep-sea isolates shown in Table 2, total 16S rDNA sequences were determined. These sequences were aligned and compared with the 16S rDNA sequences of 40 reference strains and 28 Mariana isolates (Takami et al. 1997) categorized as actinomycetes, low G + C gram-positive bacteria, and proteobacteria of the α, β, and γ subdivisions (Olsen et al. 1994). Evolutionary distances among the deep-sea isolates in this study, the Mariana isolates, and the reference strains were computed, and a phylogenetic tree was constructed using the NJ algorithm (Fig. 2).

The 16S rDNA sequences of alkaliphilic strain HTB113 and neutrophilic strain HTB096, both categorized as actinomycetes, were 99.6% similar to each other and showed 99.1% and 98.8% similarity, respectively, to that of Mariana isolate HTA212. The 16S rDNA sequence of neutrophilic strain HTE856 showed 99% and 98.4% similarity to those of *Bacillus cereus* and Mariana isolate HTA484, respectively. Halophilic isolate HTE831, which grew on medium containing 0.34 M to 4 M NaCl, was positioned close to *Bacillus subtilis* in the phylogenetic tree (Fig. 2). The halophilic isolate HTC018 and the neutrophilic isolate HTB091 were categorized as members of the β subdivision of proteobacteria, which were not found among the Mariana isolates (Takami et al. 1997), based on 16S rDNA sequence analysis. The 16S rDNA sequences of HTC018 and HTB091 were 99.4% similar to each other and showed 99.3% and 99.4% similarity, respectively, to that of *Janthiobacterium lividum*, which is categorized as a member of the proteobacteria β subdivision (O'Sullivan et al. 1990).

Two halophilic strains (HTB122 and HTB123), alkaliphilic halophilic strain HTB069, and alkaliphilic strain HTB138, which were categorized as members of the proteobacteria γ subdivision constituted two subclasses, and these were quite distantly related to the Mariana isolates. On the other hand, these isolates were comparatively related to another subclass constituted of two alkaliphilic strains (HTB111 and HTB143) and *Halomonas meridiana* (Dobson et al. 1993). The 16S rDNA sequence of strain HTB148 was 99.9% similar to that of *Marinobacter aquaeolei*, which is phylogenetically distant from any other isolates. Three alkaliphilic isolates (HTB110, HTB139, and HTB147) were found to be closely related to *Pseudomonas pseudoalcaligenes* (Moore et al. 1996). Finally, three neutrophilic isolates, HTB010, HTB019, and HTB021, which constituted a subclass based on the results of 16S rDNA sequence analysis, were distantly related to the Mariana isolates but were related to *Curacaobacter baltica*.

We have thus obtained many isolates that differ in taxonomic affiliation, including some which belong to the proteobacteria β subdivision and some that belong to new subclasses which were not found among the Mariana isolates (Fig. 2). These findings demonstrate that various types of extremophilic bacteria are widely distributed in the deep-sea mud at depths of 1050–10 897 m near the south part of Japan (Fig. 1).

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References

Abe F, Horikoshi K (1995) Hydrostatic pressure promotes the acidification of vacuoles in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 130:307–312

Dobson SJ, McMeekin TA, Franzmann, PD (1993) Phylogenetic relationships between some members of the genera *Deleya*, *Halomonas*, and *Halovibrio*. Int J Syst Bacteriol 43:665–673

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Felsenstein J (1995) PHYLIP: phylogenetic inference package, version 3.57c. University of Washington, Seattle

Jannasch HW, Taylor CD (1984) Deep-sea microbiology. Annu Rev Microbiol 38:487–514

Moore ERB, Mau M, Arnscheidt A, Bottger EC, Hutson RA, Collins MD, van de Peer Y, de Wachter R, Timmis KN (1996) The determination and comparison of the 16S rRNA gene sequences of species of the genus *Pseudomonas* (sensu stricto) and estimation of the natural intragenetic relationships. Syst Appl Microbiol 19:478–492

Olsen GJ, Woese CR, Overbeek R (1994) Winds of (evolutionary) change: breathing new life into microbiology. J Bacteriol 176:1–6

O'Sullivan J, McCullough J, Johnson JH, Bonner DP, Clark JC, Dean L, Trejo WH (1990) Janthinocins A, B and C, novel peptide lactone antibiotics produced by *Janthiobacterium lividum*. I. Taxonomy, fermentation, isolation, physicochemical and biological characterization. J Antibiot (Tokyo) 43:913–919

Saitou N, Nei M (1987) A neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 44:406–425

Takagawa S, Takahashi K, Sano, T, Mori Y, Nakanishi T, Kyo M (1989) 6500 m deep manned research submersible *Shinkai 6500* system. Oceans 89 3:741–746

Takami H, Inoue A, Fuji F, Horikoshi K (1997) Microbial flora in the deepest sea mud of the Mariana Trench. FEMS Microbiol Lett 152:279–285

Thompson JD, Higgins DG, Gibson TJ (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Yayanos AA (1979) Isolation of a deep-sea barophilic bacterium and some of its growth characteristics. Science 205:808–810

Yayanos AA (1981) Obligately barophilic bacterium from the Mariana Trench. Proc Natl Acad Sci USA 78:5212–5215

Yayanos AA (1995) Microbiology to 10 500 meters in the deep sea. Annu Rev Microbiol 49:777–805

ZoBell CE (1941) Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. J Mar Res 4:42–75