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# Molecular analyses of the sediment of the 11000-m deep Mariana Trench

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**Abstract** We have obtained sediment samples from the world's deepest sea-bottom, the Mariana Trench challenger point at a depth of 10898m, using the new unmanned submersible *Kaiko*. DNA was extracted from the sediment, and DNA fragments encoding several prokaryotic ribosomal RNA small-subunit sequences and pressure-regulated gene clusters, typically identified in deep-sea adapted bacteria, were amplified by the polymerase chain reaction. From the sequencing results, at least two kinds of bacterial 16S rRNAs closely related to those of the genus *Pseudomonas* and deep-sea adapted marine bacteria, and archaeal 16S rRNAs related to that of a planktonic marine archaeon were identified. The sequences of the amplified pressure-regulated clusters were more similar to those of deep-sea barophilic bacteria than those of barotolerant bacteria. These results suggest that deep-sea adapted barophilic bacteria, planktonic marine archaea, and some of the world's most widespread bacteria (the genus *Pseudomonas*) coexist on the world's deepest sea-bottom.

**Key words** Mariana Trench · Challenger point · Deep-sea · Barophilic bacteria · *Pseudomonas* · Planktonic marine archaea · Pressure-regulated operon

## Introduction

The deep-sea bottom is a very stable environment under extremely high pressure and at low temperature. The study of microorganisms isolated from the deep-sea promises to provide us with new information about the evolution of life. To investigate the environment and the biology of the deep-sea world, several deep-sea manned and unmanned submersibles have been constructed. A newly constructed unmanned submersible, *Kaiko*, which means "trench" in Japanese, operated by the Japan Marine Science and Technology Center (JAMSTEC), is the most capable of all, with the ability to submerge to the world's deepest bottom depth at 11000m (Kyo et al. 1995). In fact, *Kaiko* was successful in reaching the bottom of the Mariana Trench at the challenger point depth of 10911m on March 24th, 1995, in a test dive (Kyo et al. 1995).

In 1960, J. Piccard and D. Walsh dived to the world's deepest point aboard the manned submersible *Trieste*, and they reported seeing several deep-sea animals and fishes there (Piccard and Dietz 1961). Unfortunately, that dive was not for scientific purposes and no film pictures were made or samples collected, so their report could not be confirmed scientifically.

*Pseudomonas bathycetes* was the first bacterial isolate obtained from a sediment sample collected from the Mariana Trench (Morita 1976). This bacterium is not an organism adapted to the deep-sea environment; its generation time is 33 days under such conditions (2°C, 100MPa) (Schwarz and Colwell 1975) and it grows nonbarophilically at atmospheric pressure and at temperatures as high as 25°C (Pope et al. 1975). With respect to other deep-sea organisms, in 1978, Yayanos was successful in collecting an amphipod (*Hirondellea gigas*) from the Mariana Trench at a depth of 10476m using an insulated trap (Yayanos et al. 1981). An obligately barophilic bacterium, strain MT41, was isolated from this deep-sea amphipod, and this bacterium could grow only under a pressure greater than 518 bars, i.e., about 50MPa or more (Yayanos et al. 1981). However, there has been no real information about biodiversity within the Mariana sediment at a depth of almost 11000m.

On February 28, 1996, we set out to obtain a sediment sample from the world's deepest point. This was *Kaiko* dive number 21 during our most recent Mariana Trench cruise, which involved the use of a sterilized sediment sampler at a depth of 10898m. It seems likely that this was the first time sediment samples have been recovered from the world's deepest point without any microbiological contamination from other depths. We were successful in obtaining sedi-

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ment samples in the course of two more sampling dives at the same point on March 2 and 4, 1996. These were *Kaiko* dive numbers 22 and 23. In this paper, we describe a preliminary analysis of the microbial diversity of the Mariana sediment obtained in these dives, and we show that a pressure-regulated operon previously identified in deep-sea-adapted bacteria (Kato et al. 1995b, 1996b, 1997) is present also in bacteria of the Mariana sediment.

## Materials and methods

### Sample collection

Deep-sea sediment samples were collected from the Mariana Trench, challenger point (11°22.10′N. 142°25.85′E), at a depth of 10898m, by means of sterilized mud samplers (Ikemoto and Kyo 1993) using the newly constructed unmanned submersible *Kaiko* (Fig. 1a). At the bottom of the Mariana Trench challenger point, brown colored soft sediment was widespread and no rock was observed. As shown in Fig. 1b, the mud samples were taken by *Kaiko*'s manipulator and put into the sample holder of the sterilized sampler, then the samples were carried to the sea-surface without changing temperature but with a change in pressure. Most of the samples obtained were apportioned into 2-ml sterilized serum tubes (Corning, NY, USA) and placed in a liquid nitrogen tank (−150°C). A part of each sample was pressurized at about 100MPa in a pressure vessel and these vessels were placed in a refrigerator (2°C–4°C) on the support ship M.S. *Yokosuka*.

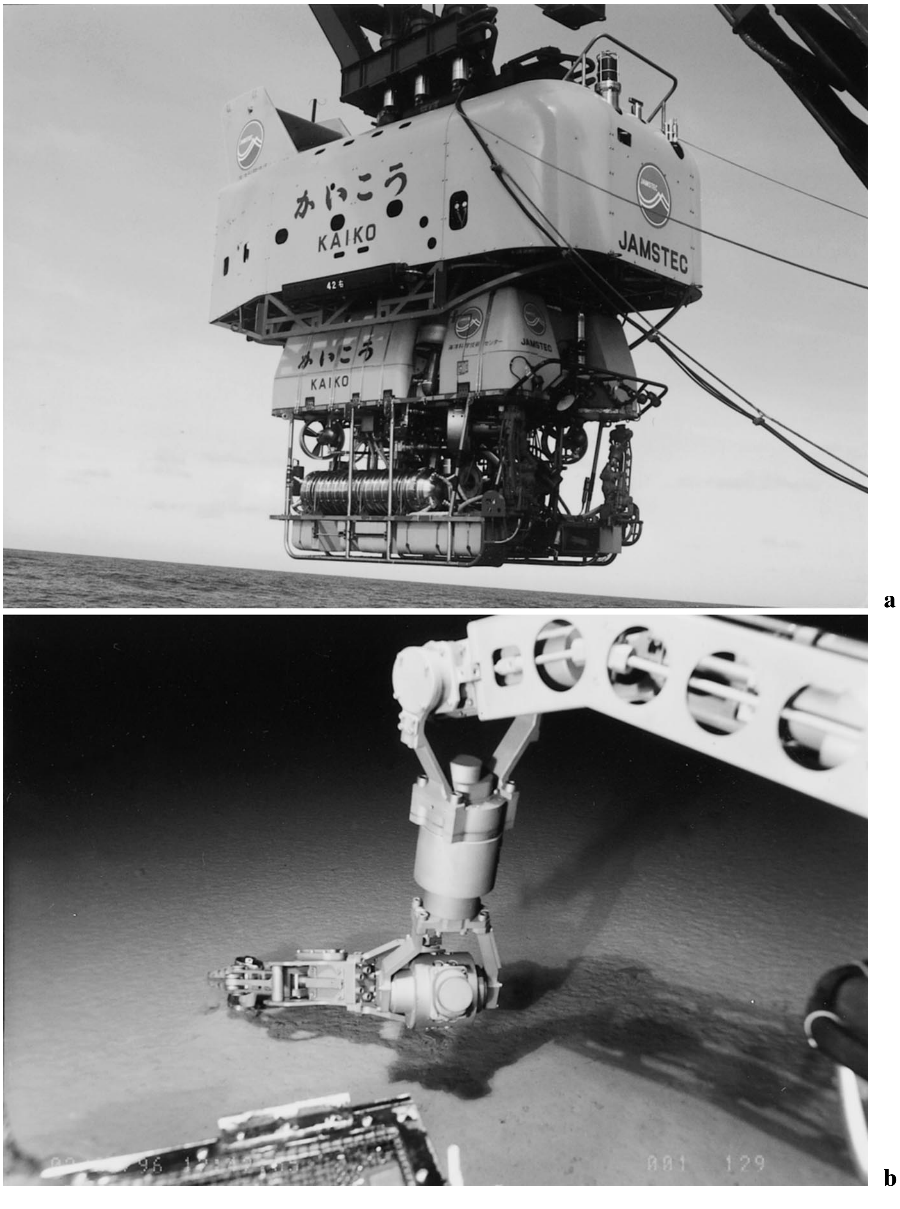

**Fig. 1. a** Unmanned submersible *Kaiko* was launched to investigate the world's deepest sea-bottom, the Mariana Trench challenger point, on February 29, 1996, as dive number 21. **b** We obtained sediment samples with the sterilized mud sampler using *Kaiko*'s manipulator at a depth of around 11 000 m in the Mariana Trench

## DNA extraction

Total DNA was extracted from the sediment samples by a procedure modified from the methods of Herrick et al. (1993). Approximately 20 g of the sediment (wet weight) was suspended in 50 ml of sterilized artificial sea water (30 g NaCl, 0.7 g KCl, 5.3 g $MgSO_4 \cdot 7H_2O$, 10.8 g $MgCl_2 \cdot 6H_2O$, and 1.0 g $CaSO_4 \cdot 2H_2O$ per liter of distilled water), and centrifuged for 5 min at 1000 × *g* and 4°C. The supernatant was pooled in an Erlenmeyer flask and maintained on ice until further centrifugation. That step was repeated three times. The supernatants were mixed and centrifuged for 30 min at 10 000 × *g* and 4°C to collect the microbial cells. The pellets were resuspended in lysozyme solution [150 mM NaCl, 100 mM $Na_2$(EDTA) (pH 8.0), 15 mg of lysozyme per ml] and incubated at 37°C for 1 h with occasional agitation. A sodium dodecyl sulfate (SDS) solution [100 mM NaCl, 500 mM Tris-HCl (pH 8.0), 10% SDS] was added, the reaction mixture was mixed gently by inversion, and incubated at room temperature for 5 min. Three cycles of freezing in liquid nitrogen and thawing in a 65°C water bath were conducted to release nucleic acids. A 7.5 M ammonium acetate solution was added to give a final concentration of 2.5 M, and then acid-washed polyvinylpolypyrrolidone (PVPP) (Sigma, St. Louis, MO, USA) was added to achieve 2% of the total mixture. The mixture was then centrifuged at 6000 × *g* for 10 min. This process resulted in the precipitation of humic materials. Nucleic acids in the supernatant fraction were precipitated by the addition of 2.5 volumes of ice-cold ethanol and 20 μg of glycogen (Sigma) per ml and incubation at −80°C for 2 h or more. The precipitate was collected by centrifugation at 4°C, and washed with 70% ethanol. The dried pellet was resuspended in TE [10 mM Tris-HCl, 1 mM EDTA (pH 7.6)]. The crude DNA extract was subjected to further purification using Gene Clean II (Bio101, La Jolla, CA, USA).

## PCR amplification

Polymerase chain reaction (PCR) amplification was performed with a DNA Thermal Cycler model 9600 (Perkin-Elmer/Cetus, Norwalk, CT, USA) by using 50 μl of the PCR reaction mixture under the conditions recommended by the enzyme manufacturers (Takara Shuzo, Otsu, Japan). A total of 30 cycles of amplification was performed with template DNA denaturation at 95°C for 1.5 min, primer annealing at 55°C for 1.5 min, and primer extension at 72°C for 1.5 min (DeLong 1992). The oligonucleotide primer sequences used were as follows:

| | |
|---|---|
| Eubac27F | AGA GTT TGA TCC TGG CTC AG |
| Eubac1492R | GGT TAC CTT GTT ACG ACT T |
| EukF | AAC CTG GTT GAT CCT GCC AGT |
| EukR | TGA TCC TTC TGC AGG TTC ACC TAC |
| Arch21F | TTC CGG TTG ATC CYG CCG GA |
| Arch958R | YCC GGC GTT GAM TCC AAT T |

where Y is a C or T, M is an A or C. PCR primers "Eubac27F and Eubac1492R for amplification of bacterial 16S rDNA, EukF and EukR for eukaryote 18S rDNA, and Arch21F and Arch958R for archaea 16S rDNA" were designated by DeLong (1992).

| | |
|---|---|
| ORF1.2F | CCT TGG GTT GTT CGT CCT TG (#977-996) |
| ORF1.2R | GCT TCG GTA CGC TCT TCA CG (#1442-1383 complementary) |
| ORF3F | TAG AGA AAC AAC TGA CCT CT (#1893-1912) |
| ORF3R | GCA GCA CCC ATA CCG ACT AA (#2442-2423 complementary) |

The numbers of ORF1.2 and ORF3 primers are those corresponding to the sequence accession number D37767 (Kato et al. 1996b). PCR amplified DNAs were analyzed by electrophoresis on a 1.5% agarose gel.

## Purification and cloning of PCR products

Amplified DNA was purified from the reaction components using a DNA recovery filter SUPREC-02 (Takara Shuzo) and quantities were measured spectrophotometrically. The PCR products were cloned into pT7Blue(R)T-vector, according to the manufacturer's directions (Novagen, Madison, WI, USA). Clones containing the DNA inserts were identified by agarose gel electrophoresis.

## Sequencing of the cloned PCR products

Plasmid DNA of the clones was prepared by the alkaline-SDS general method (Sambrook et al. 1989). DNA was sequenced by the dideoxyribonucleotide chain-termination method using a model 373A DNA Sequencer (Perkin Elmer/Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Sequencing was performed using commercial primers, forward and reverse primers of pUC vectors (Toyobo, Osaka, Japan), and by primer walking using the following oligonucleotide primers, which we discovered from the homology search of several bacterial 16S rDNA sequences, synthesized by means of a model 392 DNA Synthesizer (Perkin Elmer/Applied Biosystems):

For bacterial 16S rDNA

| | |
|---|---|
| 357F | TAC GGG AGG CAG CAG (#343-357) |
| 342R | CTG CTG CCT CCC GTA G (#357-342 complementary) |
| 530F | GTG CCA GCA GCC GCG G (#515-530) |
| 519R | GTA TTA CCG CGG CTG CTG (#536-519 complementary) |
| 803F | GAT TAG ATA CCC TGG TAG (#786-803) |
| 785R | CTA CCA GGG TAT CTA ATC C (#803-785 complementary) |
| 926F | ACT CAA AGG AAT TGA CGG (#909-926) |
| 909R | CCG TCA ATT CAT TTG AGT (#926-909 complementary) |
| 1114F | GCA ACG AGC GCA ACC C (#1099-1114) |

1100R AGG GTT GCG CTC GTT G (#1115-1100 complementary)
1241F TAC ACA CGT GCT ACA ATG (#1224-1241)
1225R CCA TTG TAG CAC GTG TGT (#1242-1225 complementary)

where numbers are those corresponding to the *Escherichia coli* 16S rDNA sequence (accession number JO1695).

For archaeal 16S rRNA

275F GAC TAT TAC AGG TAC GGG CTC TG
677R CCA CAG GTG GTC ATC GAG AGA TC
533F GTG CCA GCM GCC GCG GTA A

The archaeal 275F and 677R primers were developed in this study for Mariana archaea 16S rDNA sequencing by the primer walking procedure. The 533F sequence was one of the common archaeal 16S sequencing primers reported by Robb et al. (1995).

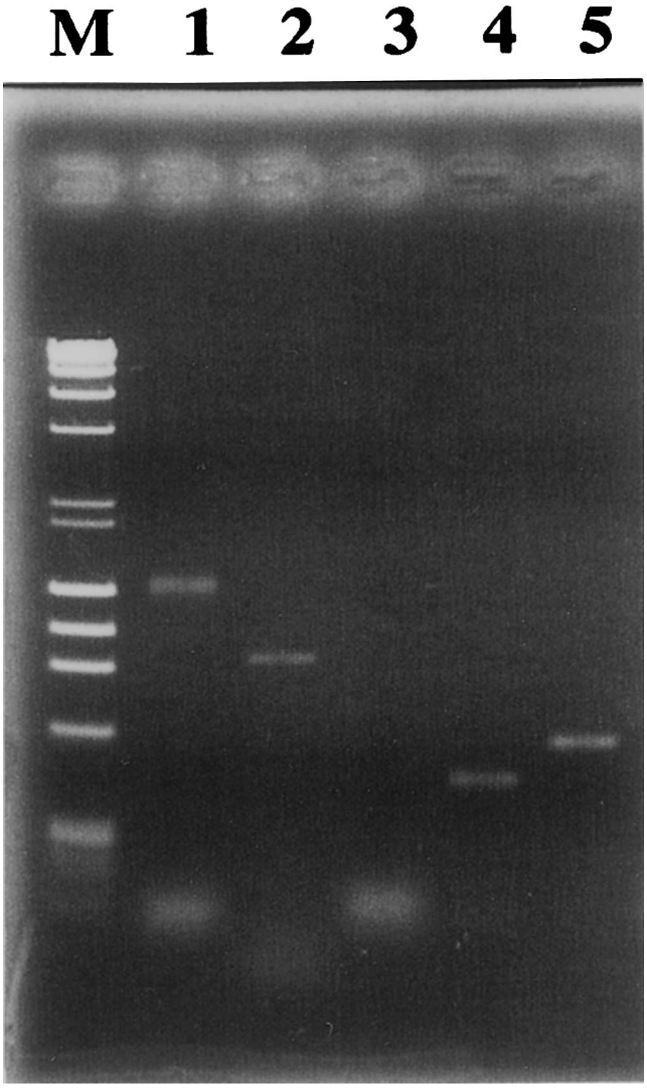

**Fig. 2.** Gel electrophoresis of polymerase chain reaction (PCR) products amplified with several primers. *Lane M*, molecular size markers (*Hin*dIII-digested lambda DNA); *lanes 1 to 5*, amplified fragments of bacterial 16S ribosomal DNAs, archaeal 16S ribosomal DNAs, eukaryotes 18S ribosomal DNAs, pressure-regulated operon (ORF1, 2), and ORF3, respectively, from the DNA purified from the Mariana sediment

### Analysis of DNA sequences and phylogenetic relationships

The DNA sequences determined for the small subunit of rRNA of bacteria and that of archaea, and the pressure-regulated gene clusters, amplified from the Mariana sediment sample, were checked for similarity to sequences in the DNA databases GenBank and EMBL using the GENETYX-MAC/CD program (ver. 34.0, Software, Tokyo, Japan). The percent similarity between each of the amplified pressure-regulated gene clusters was calculated using the GENETYX-MAC program (ver. 8.0, Software). Phylogenetic trees were constructed by the neighbor-joining method using the Clustal V program (Saitou and Nei 1987).

### Nucleotide sequence accession numbers

The amplified rRNA small-subunit DNA sequences and the pressure-regulated gene clusters sequences reported in this paper have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases. The accession numbers of the DNA sequences corresponding to the rRNA small subunit of isolate Mariana bacterium no.1, no.2, no.11, and Mariana archaeon no.1, no.11, no.15 are D87345, D87346, D87347, and D87348, D87349, D87350, respectively. The accession numbers of the pressure-regulated operon DNA sequences, #9, #11, #13, #24, #55, and the ORF3 DNA sequences, #26, #28, #30, #63, #66 are D88194, D88195, D88196, D88197, D11898, and D88199, D88200, D88201, D88202, D88203, respectively.

## Results and discussion

### PCR amplification of DNA from the Mariana Trench

The DNA fragments encoding the rRNA small subunit were amplified by PCR using the primers Eubac27F and Eubac1492R (for bacterial 16S rRNA), Arch21F and Arch958R (for archaeal 16S rRNA), and EukF and EukR (for eukaryote 18S rRNA) from the purified DNA obtained from the Mariana sediment. The pressure-regulated operon (ORF1, 2) and the downstream sequence (ORF3) were also amplified by PCR using the primers ORF1.2F and ORF1.2R, and ORF3F and ORF3R, respectively. The results are shown in Fig. 2.

DNA fragments corresponding to the rRNA small-subunit sequences of bacteria and archaea, and the pressure-regulated gene clusters (ORF1, 2 and ORF3) were clearly amplified, however, no DNA corresponding to the eukaryote rRNA small subunit was amplified. These amplified fragments were cloned and sequenced.

## Phylogenetic relationships of amplified rRNA small-subunit sequences

The amplified bacterial 16S rRNA sequences were determined and compared with sequences in the DNA databases. The results suggested that at least three kinds of sequences from the domain Bacteria were present in DNA isolated from the sediment and those belonged to the Proteobacteria gamma subgroup. The phylogenetic relationships between the amplified sequences and this subgroup of bacteria are shown in Fig. 3. Two of the amplified sequences (Mariana bacterium no. 2 and no. 11) were closely related to the *Pseudomonas* branch, and another one (Mariana bacterium no. 1) belonged to the branch containing *Shewanella* and barophilic bacteria (Kato et al. 1995a, 1996a). The first bacterium isolated from the Mariana Trench was *Pseudomonas bathycetes* (Morita 1976), so it seems reasonable that our results identified members of the genus *Pseudomonas* as microbial residents of the sediment.

The amplified archaeal 16S rRNA sequences were also determined and compared with the DNA databases. The phylogenetic relationships between these amplified sequences and archaea are shown in Fig. 4. The results indicate that at least three kinds of archaeal sequences were present in DNA from the sediment and these belonged to the planktonic archaea group I reported by DeLong (1992). These planktonic archaea were unculturable strains isolated from ocean waters, and group I belongs to one of the major branches of archaea; however, group I shares no close evolutionary relationship with previously cultured archaea (DeLong 1992; Stein et al. 1996). These unculturable archaea are widely distributed in the ocean (DeLong 1992). Recently, the group I archaea were also identified from the midgut of the deep-sea holothurians as a paraphyletic component (McInerney et al. 1995). It is still unknown whether the group I archaea have some physiological function as a symbiont in such deep-sea animals. Our results indicate that planktonic archaea exist at the sea-bottom of the Mariana Trench, and these archaea probably arrived from the middle ocean with planktonic particles which settled there like marine snow.

## Distribution of the pressure-regulated operon (ORF1, 2) and downstream region (ORF3)

As shown in Fig. 2, the pressure-regulated operon, ORF1, 2, and the downstream region, ORF3, found especially in deep-sea-adapted barophilic bacteria (Kato et al. 1995b, 1996b, 1997), were also amplified from the DNA of the Mariana sediment. Several clones which included these

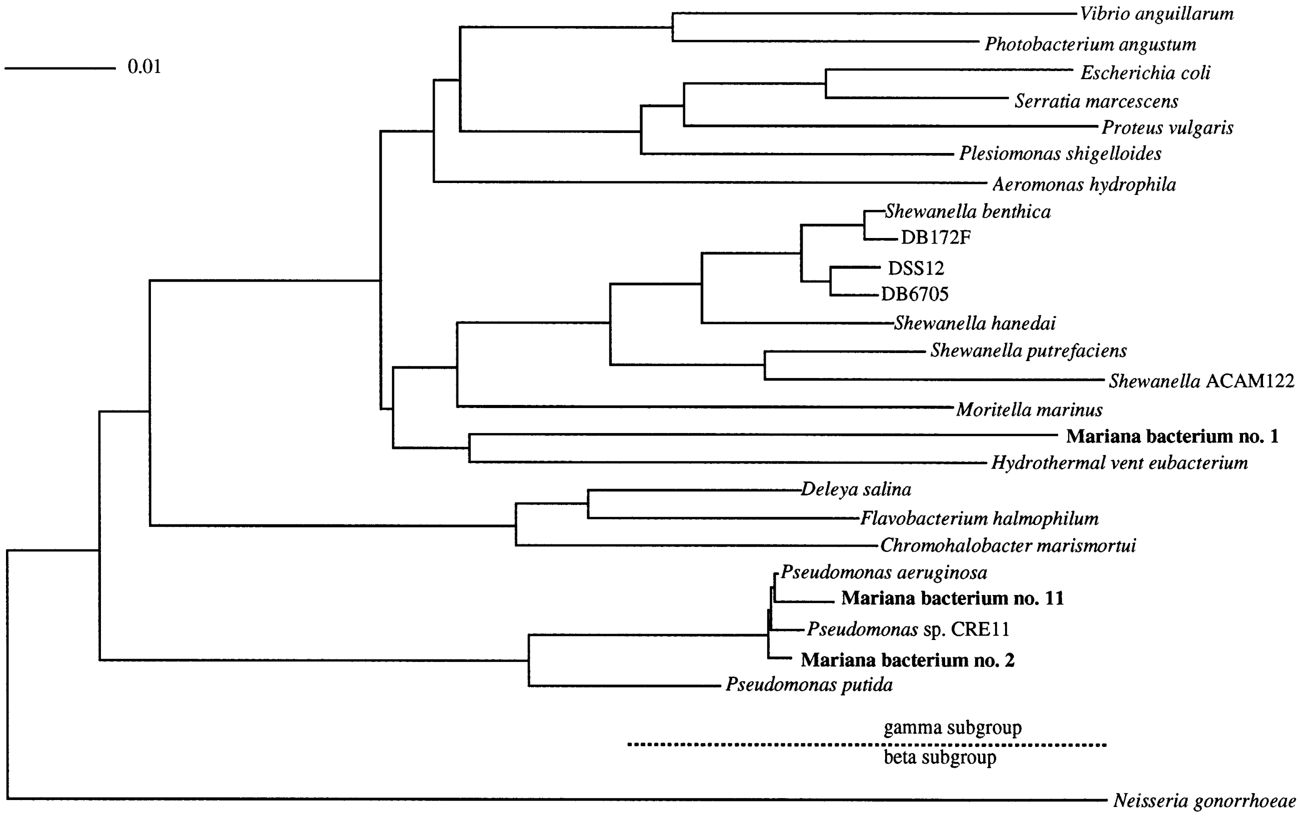

**Fig. 3.** Phylogenetic tree showing the relationship of amplified bacterial 16S rDNA within the Proteobacterium gamma subgroup using the neighbor-joining method. The *scale* represents the average number of nucleotide substitutions per site. The bacterial strains DSS12 (barotolerant) and DB6705 and DB172F (barophilic) were isolated in our laboratory from deep-sea sediment samples collected at a depth of 5100–6500 m (Kato et al. 1995a, 1996a). *Neisseria gonorrhoeae* (Proteobacteria beta subgroup) 16S rDNA sequence was used as an out-group to make the tree

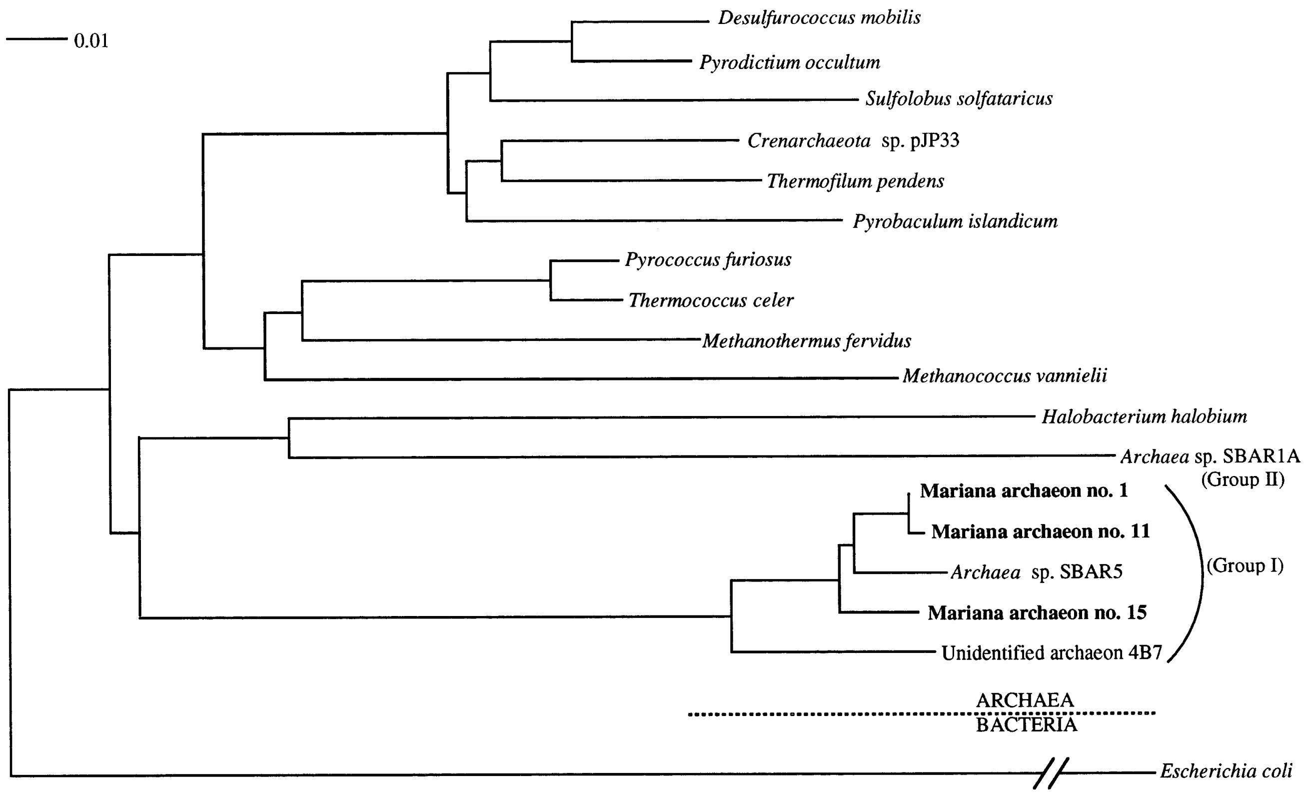

**Fig. 4.** Phylogenetic tree showing the relationship of amplified archaeal 16S rDNA within the Archaeal branch using the neighbor-joining method. The *scale* represents the average number of nucleotide substitutions per site. *Escherichia coli* (bacteria) 16S rDNA sequence was used as an out-group of the tree. *Archaea* spp. designated as clone SBAR5 and clone SBAR1A were reported by DeLong (1992), and unidentified archaeon 4B7 was reported by Stein et al. (1996). *Crenarchaeota* sp. was an unculturable archaeon isolated from a Yellowstone National Park hot spring environment, and designated as clone pJP33 (Barns et al. 1994)

**Table 1.** Similarity of the amplified pressure-regulated operon (ORF1, 2) DNA sequences to those from the Mariana sediment DNA

| | DB6705 | DSS12 | #9 | #11 | #13 | #24 | #55 |
|---|---|---|---|---|---|---|---|
| DB6705 | 100% | 93.4% | 97.9% | 98.6% | 98.3% | 98.3% | 98.2% |
| DSS12 | | 100 | 92.2 | 92.4 | 92.2 | 92.7 | 92.5 |
| #9 | | | 100 | 99.3 | 99.0 | 99.0 | 99.2 |
| #11 | | | | 100 | 99.8 | 99.8 | 99.7 |
| #13 | | | | | 100 | 99.5 | 99.5 |
| #24 | | | | | | 100 | 99.5 |
| #55 | | | | | | | 100 |

amplified ORF1, 2 and ORF3 sequences were obtained and the similarity with other sequences was analyzed, and the results are shown in Tables 1 and 2, respectively. Both of the identified sequences corresponding to ORF1, 2 and ORF3 found in the DNA from the Mariana sediment were more similar to those of the barophilic strain DB6705 than those of the barotolerant strain DSS12 (Kato et al. 1995a). These results suggest that the bacteria of the Mariana sediment which display the pressure-regulated operon are more like barophilic organisms (e.g., the strain DB6705) than barotolerant organisms (e.g., the strain DSS12). In fact, we were able to isolate obligate barophilic strains from the Mariana sediment by maintaining the conditions of 100 MPa and 4°C, and some of these bacteria were found to have the pressure-regulated operon similar to that of the barophilic strains in their chromosomal DNA (Kato et al., paper in preparation).

Recently, we have reported that the downstream region, ORF3, is complementary with the *cydD* gene of *E. coli*, and it functions to stabilize bacterial growth in the high-pressure environment (Kato et al. 1996c). Thus, the pressure-regulated clusters (ORF1, 2 and ORF3) may be components of an important mechanism for high-pressure adaptation. Our results indicate that bacteria adapted to life on the world's deepest ocean floor have these pressure-regulated clusters in their chromosomal DNA.

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**Table 2.** Similarity of the amplified ORF3 DNA sequences to those from the Mariana sediment DNA

| | DB6705 | DSS12 | #26 | #28 | #30 | #63 | #66 |
|---|---|---|---|---|---|---|---|
| DB6705 | 100% | 87.3% | 95.8% | 99.8% | 95.6% | 95.0% | 95.3% |
| DSS12 | | 100 | 88.3 | 87.2 | 88.2 | 87.8 | 88.1 |
| #26 | | | 100 | 95.6 | 99.8 | 99.6 | 99.8 |
| #28 | | | | 100 | 95.4 | 94.8 | 95.1 |
| #30 | | | | | 100 | 99.4 | 99.6 |
| #63 | | | | | | 100 | 99.6 |
| #66 | | | | | | | 100 |

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