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# Isolation and phylogenetic diversity of members of previously uncultivated ε-Proteobacteria in deep-sea hydrothermal fields

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**Abstract**

We report the successful cultivation and partial characterization of novel members of ε-Proteobacteria, which have long been recognized solely as genetic signatures of small subunit ribosomal RNA genes (rDNA) from a variety of habitats occurring in deep-sea hydrothermal fields. A newly designed microhabitat designated 'in situ colonization system' was used for enrichment. Based on phylogenetic analysis of the rDNA of the isolates, most of these represent the first cultivated members harboring previously uncultivated phylotypes classified into the Uncultivated ε-Proteobacteria Groups A, B, F and G, as well as some novel members of Group D. Preliminary characterization of the isolates indicates that all are mesophilic or thermophilic chemolithoautotrophs using $H_2$ or reduced sulfur compounds (elemental sulfur or thiosulfate) as an electron donor and $O_2$, nitrate or elemental sulfur as an electron acceptor. The successful cultivation will enable the subsequent characterization of physiological properties and ecological impacts of a diversity of ε-Proteobacteria in the deep-sea hydrothermal environments.


*Keywords:* Deep-sea hydrothermal vent; ε-Proteobacteria; Thermophile; Sulfur-oxidizing; Hydrogen-oxidizing

## 1. Introduction

During the last decade, a number of molecular phylogenetic surveys for microbial ecosystems in global deep-sea hydrothermal systems have revealed that a great diversity of microorganisms inhabits the niches associated with the formation of steep physical and geochemical gradients [1]. In high temperature habitats adjacent to hot, anoxic hydrothermal fluids, strictly anaerobic to microaerobic, (hyper-)thermophilic, chemolithoautotrophic or organotrophic Archaea and Bacteria dominate the microbial communities, while psychrophilic to mesophilic chemolithoautotrophs bloom in the lower temperature of ambient habitats [1]. Recovery of abundant small subunit ribosomal RNA genes (rDNA) phylogenetically associated with the ε-Proteobacteria delineated the predominant occurrence of chemolithoautotrophs within the ε-Proteobacteria, potentially obtaining energy by oxidation of reduced sulfur compounds and hydrogen with dissolved free oxygen and nitrate [2–6]. However, their physiological properties and ecological impacts have long been unclear because of their resistance to cultivation.

Recently, isolation and characterization of the previously uncultivated ε-Proteobacteria have been reported [7–9]. Both *Caminibacter hydrogeniphilus* and *Nautilia lithotrophica* were identified as novel anaerobic, thermophilic hydrogen-oxidizers using elemental sulfur as a primary electron acceptor [8,9]. The isolates represent the first cultivated ε-Proteobacteria of the previously uncultivated ε-Proteobacteria Group D, one of the phylogenetic groups within the ε-Proteobacteria based on the classification by Corre et al. [6], but they appear to be minor fractions of the existing deep-sea hydrothermal vent ε-Proteobacteria

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Table 1
Summary of the samples for isolation of novel ε-Proteobacteria

| Deep-sea hydrothermal field | Habitat | Location | Depth (m) | Properties | Representative strains |
|---|---|---|---|---|---|
| Okinawa Trough | | | | | |
| Iheya North (OTIN) | 'NBC' chimney (inside part) | 27°47.451′N, 126°53.799′E | 968 | max. 311°C vent water | str. B155-1 |
| | 'NBC' chimney (intermediate part) | 27°47.451′N, 126°53.799′E | 968 | max. 311°C vent water | str. MI55-1 |
| | 'E9' chimney (inside part) | 27°47.431′N, 126°53.735′E | 984 | max. 238°C vent water | str. E9137-1 |
| | 'E9' chimney (surface part) | 27°47.431′N, 126°53.735′E | 984 | max. 238°C vent water | str. E9S37-1 |
| | 'B4' in situ colonization system (ISCS) | deployed in 303°C vent water of 'NBC' chimney for 3 days | 968 | another vent orifice of 'NBC' chimney having max. 303°C vent water | str. B455-1 |
| | 'NBC' chimney (tube-dwelling polychaete's nest) | attached to the surface of the 'NBC' chimney | 968 | active nest of tube-dwelling polychaete (8.9°C) | str. GO25-1 |
| | White microbial mat (surface sediments, 0–7 cm) | 27°47.389′N, 126°53.960′E | 1052 | approx. 8°C sediments with $CO_2$ gas bubbling and elemental sulfur | str. 42BKT |
| | White microbial mat (bottom sediments, 7–15 cm) | 27°47.389′N, 126°53.960′E | 1052 | approx. 22°C sediments with $CO_2$ gas bubbling and elemental sulfur | str. BKB25Ts-Y, str. BKB55-1 |
| Iheya Ridge (OTIR) | Yellow microbial mat | 27°33.019′N, 126°58.263′E | 1392 | approx. 4°C sediments with elemental sulfur | str. 49MY |
| Hatoma Knoll (OTHK)[a] | White microbial mat | 24°51.449′N, 123°50.573′E | 1528 | pH = 6.5, oxidation–reduction potential = −187 mV | str. OK-5, str. OK-10 |
| Indian ocean | | | | | |
| Central Indian Ridge (CIR) | ISCS deployed in 365°C vent water for 7 days | 25°19.215′S, 70°02.393′E | 2450 | under 365°C during the deployment | str. 365-55-1% |
| | ISCS deployed in 250°C vent water for 3 days | 25°19.217′S, 70°02.445′E | 2420 | under approx. 250°C during the deployment | str. 1-37-1% |
| | ISCS deployed adjacent to 250°C vent water for 3 days | 25°19.217′S, 70°02.445′E | 2420 | under approx. 38°C during the deployment | str. 2-37-10% |

[a]The sample was recovered by a grab sampler of RV *Kairei* and other samples were retrieved using the manned submersibles *Shinkai 2000* or *Shinkai 6500*.

community [7]. Despite these results, and the presence of some deep-sea hydrothermal ε-Proteobacteria, the phylogenetic tree within the ε-Proteobacteria is crowded with environmental rDNA clones. The organisms harboring these sequences have remained uncultivated and uncharacterized [6]. In this study, we report the cultivation and partial characterization of novel isolates of ε-Proteobacteria representing nearly all of the previously uncultivated subgroups inhabiting the deep-sea hydrothermal environments.

## 2. Materials and methods

### 2.1. Sample collection and description

From deep-sea hydrothermal vent systems in the Okinawa Trough (Iheya North, Iheya Ridge and Hatoma Knoll), the Western Pacific Ocean, and in the Central Indian Ridges (Kairei Field), the Indian Ocean, we collected by means of DSVs *Shinkai 2000*, *Shinkai 6500* and a grab sampler deployed from RV *Kairei* a variety of samples, such as hydrothermal vent plumes, chimney structures, hydrothermal vent animals (tube-dwelling polychaetes, tube worms, vent mussels and shrimps), seafloor microbial mats and sediments, and ambient seawater. Properties of the representative samples are summarized in Table 1. The subsamples of water and slurries of chimneys, sediments, animals and the pumice stuffed into 'in situ colonization systems' (ISCSs) were anaerobically prepared onboard under an atmosphere of nitrogen. The chimney structure was subsampled into three different sections (surface layer of the chimney, inside structure and vent orifice surface) as described by Takai et al. [10]. The sediments recovered directly from the seafloor within 15 cm length of push core or subsequently from the sediments in the grab sampler box were subsampled into two different sections (surface and bottom layers corresponding to 0–7 cm and 7–15 cm, respectively). Each of the subsamples of chimneys, sediments and animals (approximately 10 g each) was suspended in 20 ml of sterilized MJ synthetic seawater [11,12] containing 0.05% (w/v) sodium sulfide in a 100-ml glass bottle (Schott Glaswerke, Mainz, Germany) tightly sealed with a butyl rubber cap under a gas phase of 100% $N_2$ (100 kPa). In addition to the samples of the natural habitats in the deep-sea hydrothermal environments, an ISCS, a newly designed microbial habitat, was tested. The ISCS consisted of a stainless-steel pipe with many small holes (5 mm diameter) (Houwa-Sangyo, Yokosuka, Japan) filled with substratum of a mixture of very porous, synthetic (merchandise name Greenrite, Matsuzaki, Chiba, Japan) and natural (Matsuzaki) pumice that had been pre-sterilized by autoclave and heating at 400°C for 3 days (Fig. 1). In the substratum of pumice, a thermal-resistant temperature probe (0–400°C, 0.01°C accuracy) was inserted and fixed, and periodically measured data were accumulated in a thermorecorder resistant to high pressure (up to 60 MPa; SN02X901, Sanyo-Sokki, Tokyo, Japan) connected by a thermal-resistant line (Sanyo-Sokki) (Fig. 1). The pipes containing substratum were inserted into a vent orifice having hydrothermal emission or placed on a vent diffusing emission, and then incubated for a defined peri-

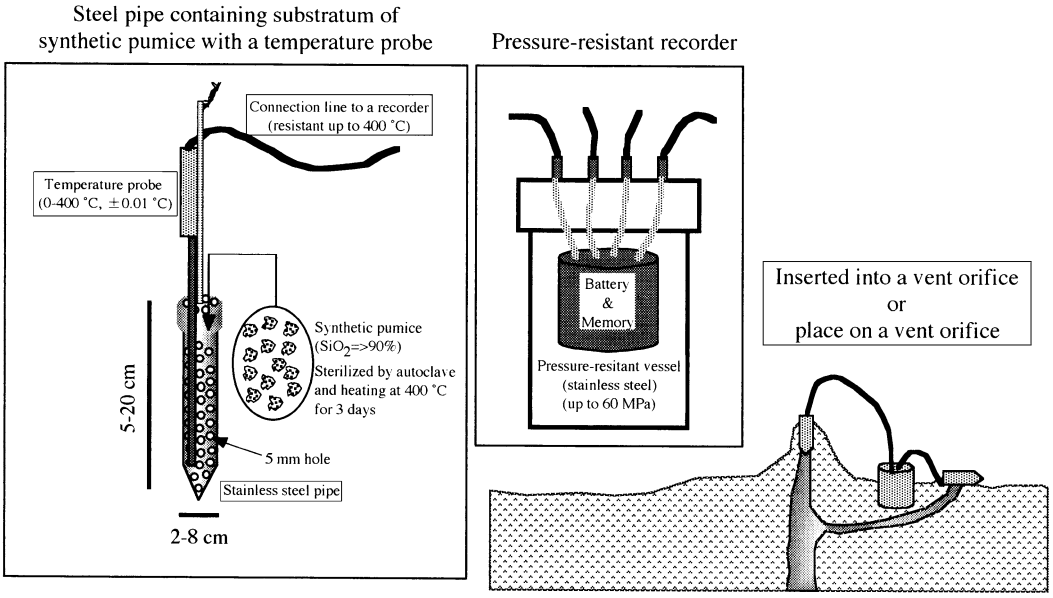


Fig. 1. A schematic figure of the ISCS. The ISCS consists of two parts. A stainless-steel pipe with many small holes contains substratum of very porous, synthetic pumice. In the substratum of synthetic pumice, a thermal-resistant temperature probe is inserted and fixed. This part is connected to a thermorecorder resistant to high pressure (up to 60 MPa) by a thermal-resistant line (up to 400°C). It was deployed in a flow of hydrothermal emission or placed on the vent diffusing emission, and then incubated for a defined period.

od. After incubation, it was recovered by the submersible. The slurry of the substratum was prepared as described above. The in situ temperatures of the vent fluid, sediments and animal colonies and of the ISCS were measured using a self-recording thermometer RMT-0-400 (Rigo-Sha, Tokyo, Japan). Physical properties such as pH and oxidation–reduction potential (ORP) of the sediments were measured onboard with a digital thermometer and multiprobe (Horiba, Kyoto, Japan).

### 2.2. Enrichment and purification

The subsamples of the water samples and slurries were immediately inoculated into MMJHS medium (described below). The cultures were incubated onboard at 25, 37, 55, 70, 85 and 95°C in dry ovens. Most tubes of MMJHS medium became turbid after 2–7 days and predominantly contained highly motile, bent, flexible rods, while several tubes contained non-motile irregular coccoids. To obtain pure cultures, the dilution-to-extinction technique was performed in the laboratory under the same condition as enrichment [13].

### 2.3. Culture conditions

The enrichment and purification were conducted in MMJHS medium, which was MMJ medium [14] supplemented with 10 mM sodium thiosulfate and 3% (w/v) elemental sulfur, but with 10 mM sodium nitrate in the absence of headspace $O_2$. Sodium sulfide was not included in the MMJ medium. Three types of headspace containing (i) 80% $H_2$ and 20% $CO_2$ (3 atm), (ii) 79% $H_2$, 20% $CO_2$ and 1% $O_2$ (3 atm) and (iii) 75% $H_2$, 15% $CO_2$ and 10% $O_2$ (3 atm) were conducted for culturing anaerobic nitrate-reducing, microaerobic and aerobic $H_2$-oxidizers (HOX) and S-oxidizers (SOX), respectively. To prepare MMJHS medium, 50 mg of sodium selenite and 30 mg of sodium tungstate were dissolved in 1 l of MJ synthetic seawater [11,12], and the pH of the medium was adjusted to around 7.0 with NaOH before autoclaving. After autoclaving, concentrated solutions of vitamins [15], $NaHCO_3$, sodium thiosulfate and suspensions of elemental sulfur were added to the medium. In the absence of headspace $O_2$, sodium nitrate was added. These solutions were separately sterilized by autoclaving except for the vitamin solution, which was filter-sterilized. The pH of the medium was adjusted with $H_2SO_4$ or NaOH in an anaerobic chamber under 90% $N_2$ and 10% $H_2$ if necessary. The medium was dispensed at 20% of the total tube volume and was tightly sealed with a butyl rubber stopper under a gas phase described above. The cultures were incubated in a dry incubator at the designated temperature.

### 2.4. Light and electron microscopy

Cells were routinely examined under a phase-contrast Olympus BX51 microscope with the Olympus Camedia C3030 digital camera system. Transmission electron microscopy of negatively stained cells was carried out as described by Zillig et al. [16]. Cells grown in MMJHS medium in the mid-exponential phase of growth were negatively stained with 2% (w/v) uranyl acetate and examined under a JEOL JEM-1210 electron microscope at an accelerating voltage of 120 kV.

### 2.5. Isolation of DNA

Genomic DNA of each of the isolates was prepared from 2 ml of culture in the mid-exponential phase of growth. Cells were harvested by centrifugation and the pellets were rinsed with the sterilized MJ synthetic seawater. The pellets were then resuspended with 20 μl of sterilized, distilled and deionized water (DDW). The resuspended cultures were applied to Soil DNA Kit Mini Prep (MO BIO Laboratories, Solana Beach, CA, USA) according to the manufacturer's suggested protocol.

### 2.6. Amplification of 16S rRNA genes and sequence determination

The 16S rRNA genes (rDNA) were amplified by the polymerase chain reaction (PCR) using Bac27F and 1492R primers [17,18]. Using approximately 1.5 kb of PCR product as a template, the partial sequence of the rDNA was directly determined in both strands using the dideoxynucleotide chain termination method with a DNA sequencer Model 3100 (Perkin Elmer/Applied Biosystems, Foster City, CA, USA). The sequence similarity among the isolates was analyzed using the FASTA program included in the DNASIS software (Hitachi Software, Tokyo, Japan). The rDNA sequence was also analyzed using the gapped-BLAST search algorithm [19,20] to estimate the degree of similarity to other bacterial 16S rDNA sequences.

### 2.7. Data analysis

The partial sequences of the rDNA from the new isolates were manually realigned to 16S rDNA data from the Ribosomal Data Project II (RDP-II) [21] based on the alignments determined using the Sequence Aligner program of RDP-II. Phylogenetic analyses were restricted to nucleotide positions that could be unambiguously aligned. Evolutionary distance matrix analysis (using the Kimura two-parameter method, the least-squares distance method and a transition/transversion rate of 2.0) and neighbor-joining analysis were performed using the PHYLIP package (version 3.5; obtained from J. Felsenstein, University of Washington, Seattle, WA, USA). Bootstrap analysis was performed to provide confidence estimates for phylogenetic tree topologies.

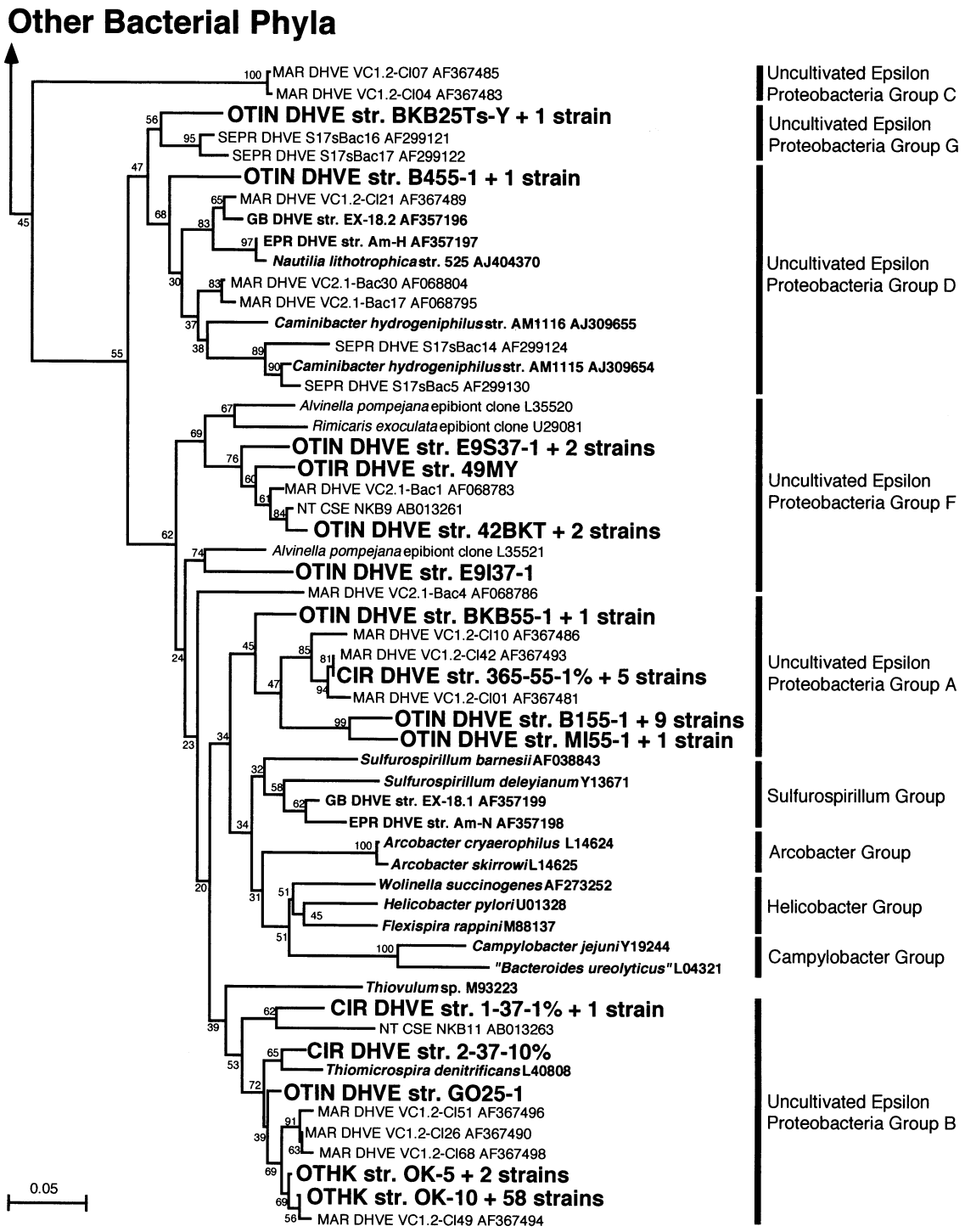


Fig. 2. Phylogenetic tree of representative 16S rRNA gene sequences from cultivated strains and deep-sea hydrothermal vent clones within ε-Proteobacteria. Bootstrap analysis was performed with 100 resampled data. Bold names show the cultivated strains and larger names represent the isolates in this study. Classification of the deep-sea hydrothermal vent rDNA clones is according to Corre et al. [6] except for the Uncultivated ε-Proteobacteria Group G, which was originally described as the South Pacific Clone Group II. Abbreviations are as follows: DHVE, deep-sea hydrothermal vent environment; MAR, Mid Atlantic Ridge; OTIN, Okinawa Trough Iheya North; SEPR, South East Pacific Rise; GB, Guaymus Basin; EPR, East Pacific Rise; OTIR, Okinawa Trough Iheya Ridge; NT, Nankai Trough; CSE, cold seep environment; CIR, Central Indian Ridge; OTHK, Okiwana Trough Hatoma Knoll. The sequences from the representative strains are available through DDBJ under accession numbers as follows: str. BKB25Ts-Y, AB091298; str. B455-1, AB091297; str. E9S37-1, AB091300; str. 49MY, AB091292; str. 42BKT, AB091292; str. E9I37-1, AB091299; str. BKB55-1, AB091294; str. 365-55-1%, AB091303; str. B155-1, AB091304; str. MI55-1, AB091296; str. 1-37-1%, AB091301; str. 2-37-10%, AB091302; str. GO25-1, AB091295; str. OK-5, AB088432; str. OK-10, AB088432.

## 3. Results and discussion

### 3.1. Sample collection and description

From deep-sea hydrothermal vent systems in the Okinawa Trough, the Western Pacific Ocean, and in the Central Indian Ridge, the Indian Ocean, a variety of samples was collected by means of DSVs *Shinkai2000*, *Shinkai6500* and a grab sampler deployed from RV *Kairei* (Table 1). These samples represented typical microbial habitats occurring in the deep-sea hydrothermal environments and varying in physical conditions such as temperature (4 to $>300$°C), hydrostatic pressure (10–25 MPa) and state of habitats (benthic and planktonic), geological background (back-arc basin and mid-ocean ridge) and geographical location (the Western Pacific and the Central Indian oceans). In addition, a newly designed habitat of solid surface for colonization of the microbial communities was prepared by deployment of a self-temperature-recording ISCS (Fig. 1). It was inserted in a flow of hydrothermal emission or placed on emission diffusing from a vent, and then incubated for a certain period. Unfortunately, in these experiments, the temperature data of the ISCS during the deployment were not retrieved because of technical problems (melting of the connection lines by vent emission). However, the short-term temperature shift (for 10–30 min) and the mean temperature were determined by another self-recording thermometer (Table 1). The result strongly suggested that the temperature of the ISCS deployed in the vigorous hydrothermal vent emission inside the vent orifice had been stable and equilibrated with that of the vent emission during the deployment. However, this may not always imply that the microorganisms cultivated from the ISCS are derived from the superheated vent emission. The microorganisms may still be contaminants from the ambient microhabitats (chimney and seawater) that entered the system during the recovery of the ISCS.

### 3.2. Isolation and phylogenetic analysis

The subsamples of the water samples and slurries were inoculated onboard into MMJHS medium and incubated at 25, 37, 55, 70, 85 and 95°C. Most tubes of MMJHS medium became turbid after 2–7 days and predominantly contained bent, flexible motile rods, while several tubes contained non-motile irregular coccoids.

We isolated a total of 127 strains by the extinction-to-dilution technique from enrichment cultures at all temperatures tested. Most of the isolates had similar morphological properties, cells being highly motile, slightly bent, flexible rods with a single polar flagellum, while some isolates were motile, straight short to long rods with a flagellum. One strain, designated as str. 49MY, was an irregular coccoid, in which motility was not observed. We extracted genomic DNA from all isolates, amplified the 16S rDNA sequences and determined the partial sequences in both strands. The sequence similarity and the phylogenetic analyses of the rDNA sequences revealed that 30 strains obtained from enrichment cultures at 70, 85 and 95°C were closely related (more than 97%) with members of the order Aquificales, such as *Aquifex* and *Persephonella* species, whereas 97 isolates at 25, 37 and 55°C were affiliated within the ε-Proteobacteria. Based on the phylogenetic tree including the new isolates, the previously described strains and the deep-sea hydrothermal vent clones (Fig. 2), a great phylogenetic diversity of ε-Proteobacteria was successfully isolated. Most of these represent previously uncultivated phylotypes (the first cultivated members within the Uncultivated ε-Proteobacteria Groups A, B, F and G, as well as some novel members of Group D) (Fig. 2). The phylogenetic distribution of the representative strains was not evidently correlated with their growth preference to primary electron acceptor and head space $O_2$ concentrations and with the geographical location of isolation, while the growth temperature for isola-

Table 2
Temperature for isolation and primary electron acceptor of representative strains of ε-Proteobacterial isolates

| Phylogenetic group | Representative strains | Isolation temperature (°C) | Primary electron acceptor[a] |
|---|---|---|---|
| Uncultivated ε-Proteobacteria group A | str. BKB55-1 | 55 | nitrate |
| | str. 365-55-1% | 55 | $O_2$ (1%) |
| | str. B155-1 | 55 | nitrate |
| | str. MI55-1 | 55 | nitrate |
| Uncultivated ε-Proteobacteria group B | str. 1-37-1% | 37 | $O_2$ (1%) |
| | str. 2-37-10% | 37 | $O_2$ (10%) |
| | str. GO25-1 | 25 | nitrate |
| | str. OK-5 | 25 | $O_2$ (10%) |
| | str. OK-10 | 25 | $O_2$ (10%) |
| Uncultivated ε-Proteobacteria group D | str. B455-1 | 55 | nitrate |
| Uncultivated ε-Proteobacteria group F | str. E9S37-1 | 37 | nitrate |
| | str. 49MY | 25 | $O_2$ (1%) |
| | str. 42BKT | 25 | $O_2$ (1%) |
| | str. E9I37-1 | 37 | nitrate |
| Uncultivated ε-Proteobacteria group G | str. BKB25Ts-Y | 25 | elemental sulfur[b] |

[a]The potential electron acceptor in the medium used for enrichment and isolation.
[b]Production of hydrogen sulfide was observed.

tion was somewhat correlated with the phylogenetic affiliation of the isolates (Table 2). All strains grown at 55°C belonged to Groups A and D together with the previously described thermophiles *C. hydrogeniphilus* [8] and *N. lithotrophica* [9] (Fig. 2). These results indicate that the deep-sea hydrothermal vent ε-Proteobacteria might have a cosmopolitan distribution in the global deep-sea hydrothermal environments and be comprised of members that range from psychrophilic to thermophilic, consistent with their distribution in a broad temperature range of habitats.

Successful cultivation of novel ε-Proteobacteria greatly expands our view of the physiological diversity of previously uncultivated, chemolithoautotrophic ε-Proteobacteria from deep-sea hydrothermal environments. Further taxomic and physiological characterization of the representative strains having novel lineages is now underway. To simplify the experimental procedure in this study, the potential energy sources of $H_2$, elemental sulfur and thiosulfate were simultaneously used for both enrichment and isolation, and sulfide was not tested for the enrichment cultures. Furthermore, none of the potential electron acceptors other than $O_2$ and nitrate such as iron (III), manganese (IV), sulfite, selenate and arsenate was incorporated in the enrichment strategy. This may have resulted in the incomplete characterization of energy metabolisms among the isolates. Ongoing characterization has revealed that most isolates are able to utilize both $H_2$ and various sulfur compounds as electron donors and both nitrate and $O_2$ as electron acceptors, but some are sulfur- and thiosulfate-oxidizing, strictly microaerophilic (e.g. str. OK-5 and str. OK-10) and some are $H_2$-dependent sulfur-reducers (e.g. str. BKB25Ts-Y). The physiological traits implicate the remarkable relatedness of energy metabolisms between deep-sea hydrothermal vent Aquificales and ε-Proteobacteria. The diversity of the cultivated deep-sea hydrothermal chemolithoautotrophs of Aquificales [22–24] and ε-Proteobacteria must provide a key to elucidate energy flux and biogeochemical cycling of carbon, nitrogen and sulfur.

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