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Analysis of Dissimilatory Sulfite Reductase and 16S rRNA Gene Fragments from Deep-Sea Hydrothermal Sites of the Suiyo Seamount, Izu-Bonin Arc, Western Pacific

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This study describes the occurrence of unique dissimilatory sulfite reductase (DSR) genes at a depth of 1,380 m from the deep-sea hydrothermal vent field at the Suiyo Seamount, Izu-Bonin Arc, Western Pacific, Japan. The DSR genes were obtained from microbes that grew in a catheter-type in situ growth chamber deployed for 3 days on a vent and from the effluent water of drilled holes at 5°C and natural vent fluids at 7°C. DSR clones SUIYOdsr-A and SUIYOdsr-B were not closely related to cultivated species or environmental clones. Moreover, samples of microbial communities were examined by PCR-denaturing gradient gel electrophoresis (DGGE) analysis of the 16S rRNA gene. The sequence analysis of 16S rRNA gene fragments obtained from the vent catheter after a 3-day incubation revealed the occurrence of bacterial DGGE bands affiliated with the *Aquificae* and γ- and ε-*Proteobacteria* as well as the occurrence of archaeal phylotypes affiliated with the *Thermococcales* and of a unique archaeon sequence that clustered with "*Nanoarchaeota*." The DGGE bands obtained from drilled holes and natural vent fluids from 7 to 300°C were affiliated with the δ-*Proteobacteria*, genus *Thiomicrospira*, and *Pelodictyon*. The dominant DGGE bands retrieved from the effluent water of casing pipes at 3 and 4°C were closely related to phylotypes obtained from the Arctic Ocean. Our results suggest the presence of microorganisms corresponding to a unique DSR lineage not detected previously from other geothermal environments.

Deep-sea hydrothermal vent sites exhibit much microbial diversity: due to their sharp physical and chemical gradients, they harbor hyperthermophiles, mesophiles, psychrophiles, chemolithotrophs, heterotrophs, anaerobes, microaerobes, and aerobes (29). The use of molecular techniques of the 16S rRNA gene, combined with the physiological characterization of isolated strains, has allowed researchers to study the phylogenetic diversity and ecosystems at deep-sea hydrothermal vents (17, 18, 19, 35, 45, 48, 49). Furthermore, molecular ecological approaches to studying the 16S rRNA gene have revealed the presence of phylogenetically novel archaeal and bacterial sequences at these sites (44, 50).

Sulfate-reducing prokaryotes (SRP) that obtain energy from dissimilatory sulfate reduction play an important role in the mineralization of organic matter in organic sulfate-rich marine anaerobic sediments, in addition to being key organisms in the sulfur cycle (25, 59). Biological sulfate reduction at temperatures up to 110°C was observed in deep-sea hydrothermal vent sediments of the Guaymas Basin in the Gulf of California, Mexico (26). Recently, a strain belonging to the genus *Thermodesulfobacterium* was isolated from a vent at Guaymas Basin (24). Sequence analysis of the dissimilatory sulfite reductase (DSR) gene, coding for an enzyme that catalyzes the reduction of sulfite to sulfide during anaerobic sulfate respiration, was found to be useful in the detection of SRP within complex microbial populations. Indeed, molecular ecological approach using the DSR gene has even been successfully used to detect SRP in complicated habitats, such as estuarine and marine sediments (12, 27, 53), hypersaline microbial mats (33), deep-sea hydrothermal vent polychaete annelids (11, 13), terrestrial hot springs (14, 37), a uranium tailing site (8), and a hydrothermal subsurface mine (3, 38). However, relatively little is known about the phylogenetic relationships between the DSR genes from deep-sea hydrothermal vent environments and those from other terrestrial geothermal environments, such as high-temperature hot springs and subsurface habitats.

The Suiyo Seamount is an active submarine volcano located on the volcanic front of the Izu-Bonin Arc, Western Pacific, Japan (61). Vigorous hydrothermal activity has occurred on the caldera floor atop the west peak of the Suiyo Seamount (58). The caldera floor is predominantly covered with sandy sediment and hydrothermal precipitations and lacks any evidence of muddy pelagic sediment. Organic geochemical stud-

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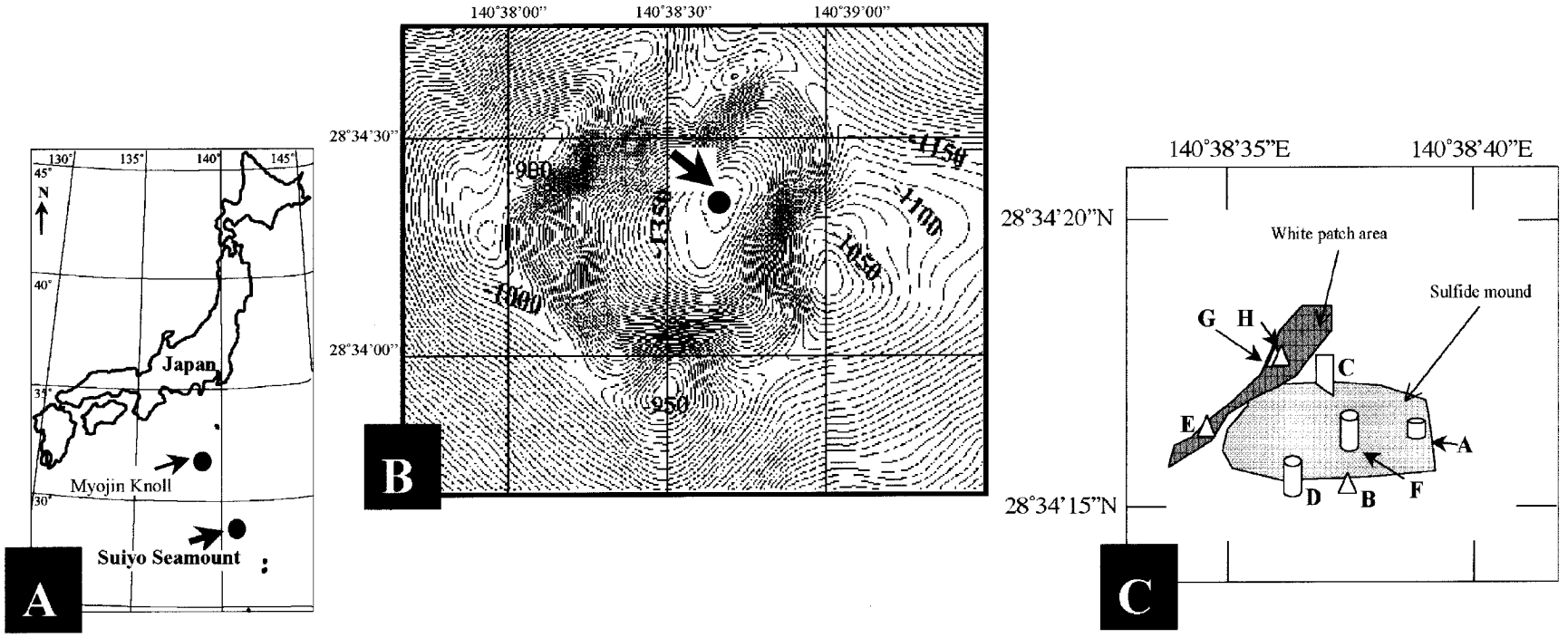

FIG. 1. (A) Map of the Western Pacific, showing the locations of the Suiyo Seamount and the Myojin Knoll. (B) Map of the Suiyo Seamount, Izu-Bonin Arc, Western Pacific, Japan (28°34′N, 140°38′E). Samples were taken from a depth of 1,380 m. (C) Map showing sampling sites A to H.

TABLE 1. Environmental samples used in this study

Site[a]	Dive no.	Filter no.	Location	Depth (m)	Filter temp[b] (°C)	Filter vol (liter)	Chemical analysis[c]				Sample
							pH	Mg concn (mM)	Si concn (μM)	H_2S concn (mM)	
A	HY30	F1	28°34′16.9″N 140°38′38.0″E	1,385	33–55	0.8	5.2	38.0	3.8	0.51	Effluent water from drilled hole (APSK07)
B	HY32	F2	28°34′15.2″N 140°38′37.5″E	1,384	299–300	4.0	3.7	2.1	12.2	1.50	Effluent water from hydrothermal vent
C	HY25		28°34′17.0″N 140°38′37.0″E	1,384	ND[d]	ND	ND	ND	ND	ND	Catheter-type in situ growth chamber placed on hydrothermal vent
D	1383	F-1	28°34′15.2″N 140°38′36.6″E	1,380	3 (maximum, 4)[e]	6.5	6.6	51.8	0.43	0.74	Effluent water from casing pipe (APSK09)
E	1385	F-1	28°34′16.9″N 140°38′38.8″E	1,366	25–36	14.0	7.2	53.1	0.2	ND	Diffuse flow from white patch area
F	1386	F-1	28°34′16.1″N 140°38′37.5″E	1,380	4	15.5	7.1	52.7	0.17	ND	Effluent water from casing pipe (APSK10)
G	1388	F-2	28°34′17.4″N 140°38′36.3″E	1,376	310	1.8	4.0	7.1	11.1	1.40	Effluent water from hydrothermal vent
H	1389	F-1	28°34′17.4″N 140°38′36.0″E	1,383	3–50	ca. 20	6.2	49.7	0.71	0.03	Diffuse flow from white patch area

[a] Site locations are shown in Fig. 1C.
[b] The temperatures were measured in situ while the water was filtered to collect microorganisms. Fluid temperature occasionally fluctuated due to mixing with ambient seawater.
[c] Samples for chemical analysis were collected by the same sampling procedure at each site [J. Ishibashi, Y. Morimoto, Y. Umeki, F. Kouzuma, T. Toki, U. Tsunogai, K. Namba, M. Utsumi, T. Yamanaka, H. Chiba, and K. Okamura, EOS Trans. AGU, 83(41), abstr. v11c-05, 2002].
[d] ND, not determined.
[e] The temperature was measured when the water was filtered for chemical analyses.

ies on its surface sediments have revealed only trace amounts of long-chain fatty acids originating from a higher plant wax of terrestrial origin (T. Yamanaka, H. Naraoka, F. Kitajima, T. Naito, K. Marumo, and T. Urabe, 2002 Ocean Sci. Meet., abstr. OS32O-04, 2002). This is to be expected, since the Suiyo Seamount is located about 1,000 km from land. It also lacks pelagic sediment, since at less than one million years old, it formed too recently for sediment accumulation on a geological time scale.

In this study, we first report unique α-subunit DSR-deduced amino acid sequences from the deep-sea hydrothermal sites of the Suiyo Seamount. In order to compare phylogenetic relationships between the DSR and 16S rRNA gene sequences and to investigate the temperature-related distribution of microorganisms, we describe the microbial community structure in several samples, including effluent water from casing pipes at temperatures of 3 or 4°C and from a hole drilled with a tethered marine rock drill at 33 to 55°C, natural vent fluids at various temperatures (7 to 300°C), and the substratum of a catheter-type in situ growth chamber (vent catheter) placed on the hydrothermal vent.

MATERIALS AND METHODS

Sample collection. Water samples at various temperatures at a depth of approximately 1,380 m were collected from the natural vents and drilled holes in the hydrothermal sites of the Suiyo Seamount, Izu-Bonin Arc, Western Pacific, Japan (28°34′N, 140°38′E) (Fig. 1 and Table 1). A remotely operated vehicle (ROV), the *Hakuyo* 2000, was used during an SR02 cruise of the mother ship *Shinryumaru* (3 to 15 August 2002). A manned research submersible, *Shinkai* 2000, was used during an NT02-09 cruise of the R/V *Natsushima* (22 August to 18 September 2002). To collect microorganisms, a peristaltic pump was used in situ to pump water through a 0.2-μm-pore-size, 142-mm-diameter filter (Supor-200; Pall Corporation, Inc., Ann Arbor, Mich.) attached to the vehicles (drawing speed about 0.15 liter/min). At each site, water samples for chemical analyses were pumped into the collection syringe and filter assembly. A portion of the filter was aseptically placed into a sterilized 2-ml screw-cap microcentrifuge tube and then frozen at −20°C on the ship until processed.

Steel casing pipes, APSK07 (length, approximately 1,600 mm; outside diameter, 70 mm) at site A, titanium casing pipes, APSK09 (600 mm above the sea bottom and approximately 2,600 mm below the surface; outside diameter, 70 mm) at site D, and APSK10 (450 mm above the sea bottom and approximately 1,200 mm below the surface; outside diameter, 70 mm) at site F, were inserted into the holes (at depths of 2,690 mm at site A, 8,992 mm at site D, and 7,035 mm at site F) bored with a tethered marine rock drill (the so-called benthic multicoring system [BMS]) to approach the subvent biosphere directly beneath the seafloor during the 2001 and 2002 BMS cruises (site A, 18 to 27 June 2001; sites D and F, 15 to 23 July 2002). The temperatures of effluent water from casing pipes APSK07, APSK09, and APSK10 after insertion were 156, 6, and 65 to 89°C, respectively. Steel casing pipe APSK07 had been destroyed by corrosion at sampling time.

A vent catheter of stainless steel pipe (length, 500 mm; inner diameter, 20 mm) with tipping porous glasses wrapped with titan mesh (length, 100 mm) was designed for in situ collection and incubation of microbes in venting fluid below the seafloor using porous inorganic grains as a substratum (18a). The apparatus was sterilized by autoclaving and transferred to the seafloor in a plastic cylinder containing filtered salt water to minimize contamination. It was inserted into a vent orifice at site C using a manipulator of the *Hakuyo* 2000 and left there for 3 days. After the apparatus was recovered (measures were taken to prevent contamination from the surrounding seawater during its ascent), a portion of porous grain samples that had changed from white to gray was aseptically placed into a sterilized 2-ml screw-cap microcentrifuge tube and frozen at −20°C aboard the ship until processed.

Chemical analysis. Concentrations of major chemical species were analyzed using the separate aliquots obtained by the same sampling procedure in order to characterize the signatures of the samples obtained. Immediately after sample recovery, the pH and hydrogen sulfide concentration were determined onboard using an electrode and the colorimetric technique (9), respectively. Samples were filtered with a 0.45-μm-pore-size filter and stored in a refrigerator. The silica concentration was also analyzed onboard by the colorimetric method (16) within 24 h. Magnesium concentration was analyzed after a 200-fold dilution using inductively coupled plasma atomic emission spectroscopy in an onshore laboratory. Both magnesium and silica concentrations were conventionally employed as indicators for mixing between pure hydrothermal fluid and ambient seawater (54). Magnesium is thought to be absent in high-temperature hydrothermal fluid. Silica concentrations varied from 0.12 μmol/kg in ambient seawater to 13.0 mmol

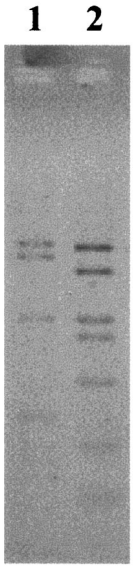

FIG. 2. *Alu*I restriction patterns of two clone families identified in the clone library of DSR gene fragments retrieved from Suiyo Seamount. Lane 1, clone family A (SUIYOdsr-A); lane 2, clone family B (SUIYOdsr-B).

kg^{-1} in pure hydrothermal fluid (hydrothermal end member) from the Suiyo hydrothermal field. Hydrogen sulfide concentration of the hydrothermal end member was estimated as 2.0 mmol kg^{-1} [J. Ishibashi, Y. Morimoto, Y. Umeki, F. Kouzuma, T. Toki, U. Tsunogai, K. Nanba, M. Utsumi, T. Yamanaka, H. Chiba, and K. Okamura, EOS Trans. AGU 83(47), abstr. V11C-05, 2002].

DNA extraction. Nucleic acids of microorganisms were extracted by a bead-beating, phenol-chloroform extraction procedure as follows. The filter and crumbled porous glasses were mixed with 850 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), 850 μl of chloroform-isoamyl alcohol (24:1 [vol/vol]), 30 μl of 20% sodium dodecyl sulfate, and 0.5 g of glass beads (with diameters of 0.1 and 0.05 mm). After the bead-beating step and centrifugation, the aqueous phase was transferred to a new tube and extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]). A 0.6 volume of isopropanol was added to the aqueous phase that had been transferred to a new tube. After centrifugation, the precipitated DNA was rinsed with 1 ml of 70% (vol/vol) ethanol (−20°C), dried, resuspended in double-distilled water (RNase- and DNase free), and then stored at −20°C.

Amplification and cloning of DSR gene fragments. DSR gene fragments were amplified with a primer pair, DSR1F and DSR4R, as described previously (56). Amplification was performed with a DNA thermal cycler (PCR EXPRESS; HYBAID, Franklin, Mass.) as follows: 30 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 54°C, and 3 min at 72°C. The reaction was completed by a final extension step of 10 min at 72°C. When there were several nonspecific bands, a touchdown PCR was performed (annealing temperature from 65 to 55°C for 20 cycles). When there was no specific band, the PCR products were reamplified at 10, 20, and 30 cycles with 1 μl of the amplicons as template DNA to obtain DSR gene fragments. For the DSR PCR amplification, 1× EX *Taq* buffer (Takara Shuzo, Kyoto, Japan), 1 to 100 ng of template DNA, 250 μM (each) of the four deoxynucleoside triphosphates, 25 pmol (each) primer, and 2.5 U of EX *Taq* DNA polymerase (Takara) were combined in a final volume of 50 μl. The DSR gene fragments were analyzed by electrophoresis in 1.1% (wt/vol) agarose S gels (Nippon Gene) containing ethidium bromide (1 μg ml^{-1}). The DSR gene products were cloned as described previously (38), and insert-containing clones reamplified with the vector primers M13 reverse and M13 forward were screened by restriction fragment length polymorphism analysis using restriction enzyme *Alu*I as described previously (38). By using a CONCERT Rapid PCR purification kit (Invitrogen, Carlsbad, Calif.), the PCR insert-containing amplicons were purified for use as the template DNA for sequencing.

TABLE 2. Abundance of clone families in the library of DSR gene fragments retrieved from Suiyo Seamount

Clone family	No. of clones (% of clones) at sample site:		
	C	F	H
SUIYOdsr-A	11 (52)	8 (30)	0 (0)
SUIYOdsr-B	10 (48)	19 (70)	35 (100)

Amplification and DGGE of 16S rRNA gene fragments. The bacterial and archaeal 16S rRNA gene fragments were amplified as described previously (7). A touchdown PCR was performed for primer pair Eub341F (with GC clamp) and 907R (annealing temperature decreased from 65 to 55°C in 20 cycles) and for primer pair Arch344F (with GC clamp) and Arch915R (annealing temperature decreased from 71 to 61°C in 20 cycles). PCR products were subjected to DGGE analysis, which was performed with D-code systems (Bio-Rad Laboratories, Hercules, Calif.) with 1.5-mm-wide gels as described previously (35). PCR products were applied directly to 6% (wt/vol) polyacrylamide gels with denaturing gradients from 20 to 60% (100% denaturant is 7 M urea and 40% [vol/vol] deionized formamide). After PCR products of the second amplification were electrophoresed again in a DGGE to check the purity of the bands, the PCR amplicons were purified with a CONCERT Rapid PCR purification kit (Invitrogen) to use as the template DNA for sequencing.

16S rRNA and DSR gene fragment sequencing and phylogenetic analysis. Sequencing reactions were performed using the ABI PRISM Big Dye Terminator Cycle sequencing kit and an ABI model 310 automated sequencer (Applied Biosystems) according to the manufacturer's instructions. 16S ribosomal DNA (rDNA) fragments from DGGE bands were sequenced using primers 341F and 907R. Partial sequences of DSR amplification products were sequenced by using primer DSR1F and vector primers M13 reverse and M13 forward for clones. The sequences from DGGE bands and the deduced amino acid sequences of α-subunits of DSR genes were entered into the BLAST (2) and FASTA programs (32) of the National Center for Biotechnology Information and the DNA Data Bank of Japan (DDBJ) in order to identify phylogenetic relationships. Sequence alignments with portions of the 16S rRNA and deduced DSR amino acid sequences of reference prokaryotes from DDBJ/EMBL/GenBank were performed by using the CLUSTAL W program (52), and matrices of evolutionary distance were constructed by the neighbor-joining method (47). Phylogenetic trees were visualized with TreeView software (41). Bootstrap resampling analysis of 1,000 replicates was performed to estimate the confidence of tree topologies. Maximum-parsimony analysis was accomplished using MEGA2 program (31). The RNA secondary structure was predicted by using the free-energy minimization algorithm with Genetyx-Mac software (Software Development Co., Ltd., Tokyo, Japan).

Nucleotide sequence accession numbers. The 16S rRNA and DSR gene sequences were submitted to DDBJ/EMBL/GenBank and have been assigned the following accession numbers: AB096615 to AB096630 (16S rRNA genes) and AB095362 and AB095363 (DSR genes).

RESULTS AND DISCUSSION

Environmental characteristics. During the 3 days the vent catheter was set up, the color of its porous glass turned black due to the buildup of FeS and other sulfide compounds on the inside surface of the stainless steel pipe at site C where the maximum fluid temperature was 279°C. The temperature of effluent water from a drilled hole (APSK07) at site A and that of a diffusive flow from a white patch area at sites E and H were lower than those of hydrothermal fluids at sites B and G (Table 1). The water at sites A, E, and H seemed to be brackish due to mixing with ambient cold seawater. The temperature of effluent water from the casing pipes (APSK09 at site D, APSK10 at site F) was the same as the ambient cold seawater (4°C). Judging from the chemical data of magnesium, silica, and sulfide concentrations, it is likely that effluent waters at sites A, B, and G were mixed with hydrothermal water of the same origin and ambient seawater. The sulfide concentration at site H was markedly low, despite the mixed hydrothermal water.

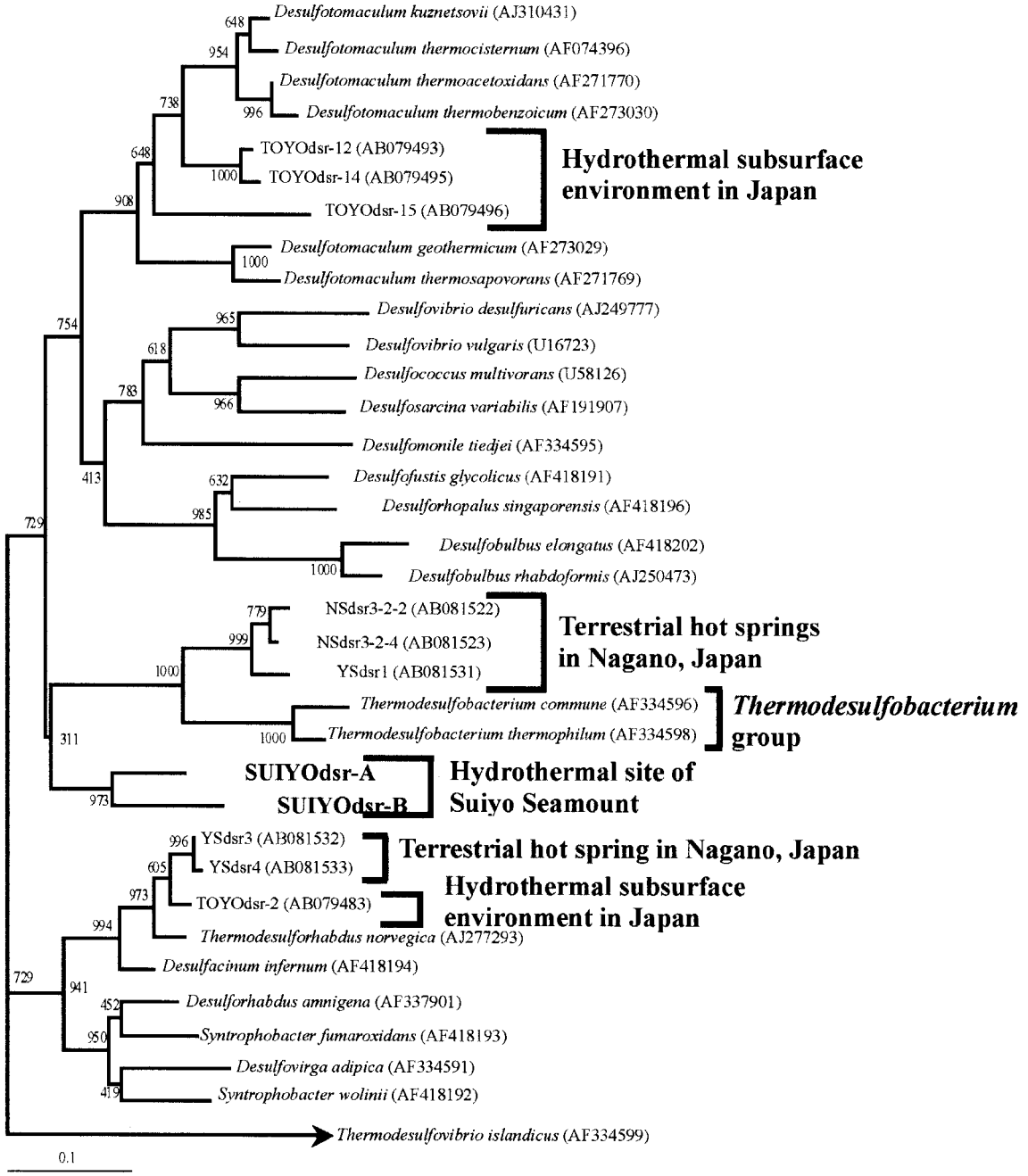

FIG. 3. Phylogenetic relationships of DSR α-subunit fragments (approximately 230 deduced amino acid sequences) obtained from the Suiyo Seamount (SUIYOdsr-A and SUIYOdsr-B) as determined by neighbor-joining analysis. The bar labeled 0.1 represents an estimated 10% sequence divergence. Numbers beside branching points indicate bootstrap values determined from 1,000 iterations.

Clone library characterization. Gene fragments encoding DSR (ca. 1.9 kb) were detected in the vent catheter after a 3-day incubation at site C and in the water samples at sites F and H. The DSR-PCR products from sites C and F together with those from site H were obtained with touchdown PCR amplification and another PCR for 10 cycles after the first PCR, respectively. No DSR-PCR product was amplified from the negative-control samples using DSR primers. A clone library of the PCR products of DSR gene fragments was used to investigate the diversity of the DSR genes of SRP in the samples at sites C, F, and H. A total of 83 clones were put into two clone families designated SUIYOdsr-A and SUIYOdsr-B on the basis of *Alu*I restriction fragment banding patterns (Fig. 2). The frequency of the DSR clone family SUIYOdsr-A was higher at site C than at other sites (Table 2). However, PCR-dependent techniques have several pitfalls and potential biases (55). PCR biases may have occurred in the present study due to our use of touchdown PCR amplification and reamplification of the first PCR products from hydrothermal water. To clarify the temperature-related SRP in deep-sea hydrothermal

Sulfate reducers and Suiyo DSR clones	Amino acid positions 254 280
δ-Proteobacteria-like	
SUIYOdsr-A	YIGGELKPNAGAHSDRDWGPFDIQKEV
SUIYOdsr-B	YVGGELKPNAGAHSDRDWGPFDIEKEV
NSdsr3-2-2	YVAGELKPNAGAHSDRDWGPFDIKKEV
YSdsr1	YVAGELKPNAGAHSDRDWGPFDIKKEV
Thermodesulfobacterium commune	YVRGELKPNAGAFSDRDWGPFDIKKEV
Desulfosarcina variabilis	YVGGEFAPNGGAHSGRNWGAFDIQKEV
Desulfotomaculum kuznetsovii	YIAGDIVPRGGFHKGRDWGKVDIQKEV
TOYOdsr-8	YIEGDILPSGGAHNGRDWGKFDIKKEV
Desulfomonile tiedjei	YVGGELKPNAGAHSSRDWGKFDIIAEV
Desulfovirga adipica	YVGASCCPMXGAGG-TEKRAFSITEEV
Thermodesulforhabdus norvegica	YVAGELVPNGGAHG-FEKRPLDIQAEV
TOYOdsr-2	YVGGELVPHGGADG-SEKRPLDIQAEV
Gram-positive SRB	
Desulfitobacterium dehalogenans	YVAEV----------XNIQAVV
Desulfosporosinus orientis	YVSEG----------FDIQEMV
Archaea	
Archaeoglobus fulgidus	YASW----------MDIENEV
Archaeoglobus profundus	YAKAG----------VDIEKQV
Phylum *Nitrospirae*	
Thermodesulfovibrio yellowstonii	YAKKG----------MNIQEEI
Thermodesulfovibrio islandicus	YAKKG----------MNIQEEI

FIG. 4. Deduced amino acid alignment of the α-subunits of DSR genes showing that SUIYOdsr-A and SUIYOdsr-B have insertions between positions 259 and 273 of the α-subunits of DSR genes. Amino acid positions correspond to those of the α-subunit of the *Desulfovibrio vulgaris* DSR gene (28).

vents, it is essential to develop a novel quantitative technique for DSR genes based on sequence information and the accumulated DSR gene sequence data for several deep-sea hydrothermal systems.

Phylogenetic analysis of DSR gene fragments. The DSR clones retrieved from the hydrothermal site at the Suiyo Seamount represent a separate but somewhat related lineage with several DSR clones predominantly retrieved from Japanese terrestrial hot springs (37) and with the DSR-deduced amino acid sequences of the *Thermodesulfobacterium* group, although this position was not strongly supported by a high bootstrap value (Fig. 3). By using the FASTA programs, the designated DSR clones, SUIYOdsr-A and SUIYOdsr-B, were found to be less closely related to cultivated species (their closest relative being *Desulfotomaculum thermosapovorans* [similarity values, 81 and 75%, respectively]). In addition, these clones were less closely related to environmental clones retrieved from geothermal environments (>75% nucleotide similarity) (3, 12, 14, 37, 38). As shown in Fig. 4, SUIYOdsr-A and SUIYOdsr-B had a major insertion between positions 259 and 273 of α-subunits of the DSR gene (numbered according to the numbering system for *Desulfovibrio vulgaris* (28), as did δ-*Proteobacteria*-like SRB as previously described by Klein et al. (30). These results suggest the presence of a unique SRP at the deep-sea hydrothermal sites of the Suiyo Seamount.

No DSR clone related to the α-subunits of DSR genes of the hyperthermophilic marine sulfate reducers *Archaeoglobus fulgidus* and *Archaeoglobus profundus* (30) was found in the present study. Similarly, on the basis of 16S rRNA gene sequence analysis, no *Archaeoglobales*-like phylotype was found for any samples, although such phylotypes have been found in an in situ growth chamber placed at a Mid-Atlantic Ridge hydrothermal vent (44) and in a black smoker chimney at Myojin Knoll, Izu-Ogasawara Arc, Japan (50). However, Takai and Horikoshi (50) reported the small number of microbes and the dearth of archaeal clones that contained an *Archaeoglobales*-like phylotype compared to that of bacterial clones in a black smoker chimney at the Suiyo Seamount. The *Archaeoglobales*-like phylotype seems to be a minor population below the detection limit of the methods used during the current investigations.

Phylogenetic analyses of bacterial 16S rDNA fragments. To compare phylogenetic relationships based on the DSR genes to those based on the 16S rRNA gene sequences and to investigate the temperature-related distribution of members of the domain *Bacteria*, we analyzed the DGGE profiles of PCR-amplified 16S rDNA fragments with a primer set for the domain. There were major differences in the profiles of DGGE bands for samples from the vent catheter (site C), the casing pipes (sites D and F), and the natural vents at other sites (Fig. 5 and Table 3). No PCR product was amplified from the negative-control samples by using bacterial primers.

The SUIYO-E8, -E9, and -E10 DGGE bands obtained from the vent catheter were affiliated with the phylum *Aquificae*. The *Aquificae*-like phylotypes, SUIYO-E8 and -E9, were closely related to a thermophilic hydrogen-oxidizing bacterium, *Persephonella hydrogenophila*, isolated from a hydrothermal vent chimney at the Suiyo Seamount (36). Recently, the thermophilic, strictly chemolithoautotrophic, microaerophilic, and hydrogen-oxidizing strains of the genus *Persephonella* have been obtained from deep-sea hydrothermal vent sites in the Pacific Ocean and Guaymas Basin (17). Their optimum temperature for growth was approximately 70°C (17, 36). The *Aquificae*-like phylotype SUIYO-E10 was closely related to the extremely thermophilic, chemolithoautotrophic, nitrate-reducing bacterium, *Thermovibrio ruber* (22). The optimum temperature for its growth was approximately 75°C. The presence of DGGE bands within the *Aquificae* suggests that there had been a period of optimum temperature for in situ growth of *Aquificae*-like microbes in the vent catheter, despite a measured temperature of 279°C.

The SUIYO-E7 DGGE band was related to several uncultured ε-*Proteobacteria* clones retrieved from sediments at the Guaymas Basin (51) and from mucous secretions of a hydrothermal vent polychaete (1). Recent studies of the ε-*Proteobacteria* 16S rRNA gene indicate a high bacterial diversity at deep-sea hydrothermal vents (1, 6, 10, 34, 43, 44, 49, 51). Furthermore, bacteria within the ε-*Proteobacteria* subclass that oxidize hydrogen or sulfur compounds (elemental sulfur or thiosulfate) have been isolated from deep-sea hydrothermal vent samples (49). Although it is impossible to determine the function (ferrous and/or sulfur-utilizing) of the ε-*Proteobacteria* phylotype SUIYO-E7 without more detailed physiological data, such ε-*Proteobacteria*-like microbes may contribute to iron or sulfur metabolism within the vent catheter at deep-sea hydrothermal vents.

DGGE bands SUIYO-E11 and -E12, which were closely related to phylotypes obtained at a depth of 131 m in the Arctic Ocean (4), were observed only for organisms isolated from casing pipes at sites D and F. The maximum temperatures measured at sites D and F were 4 and 5°C, respectively,

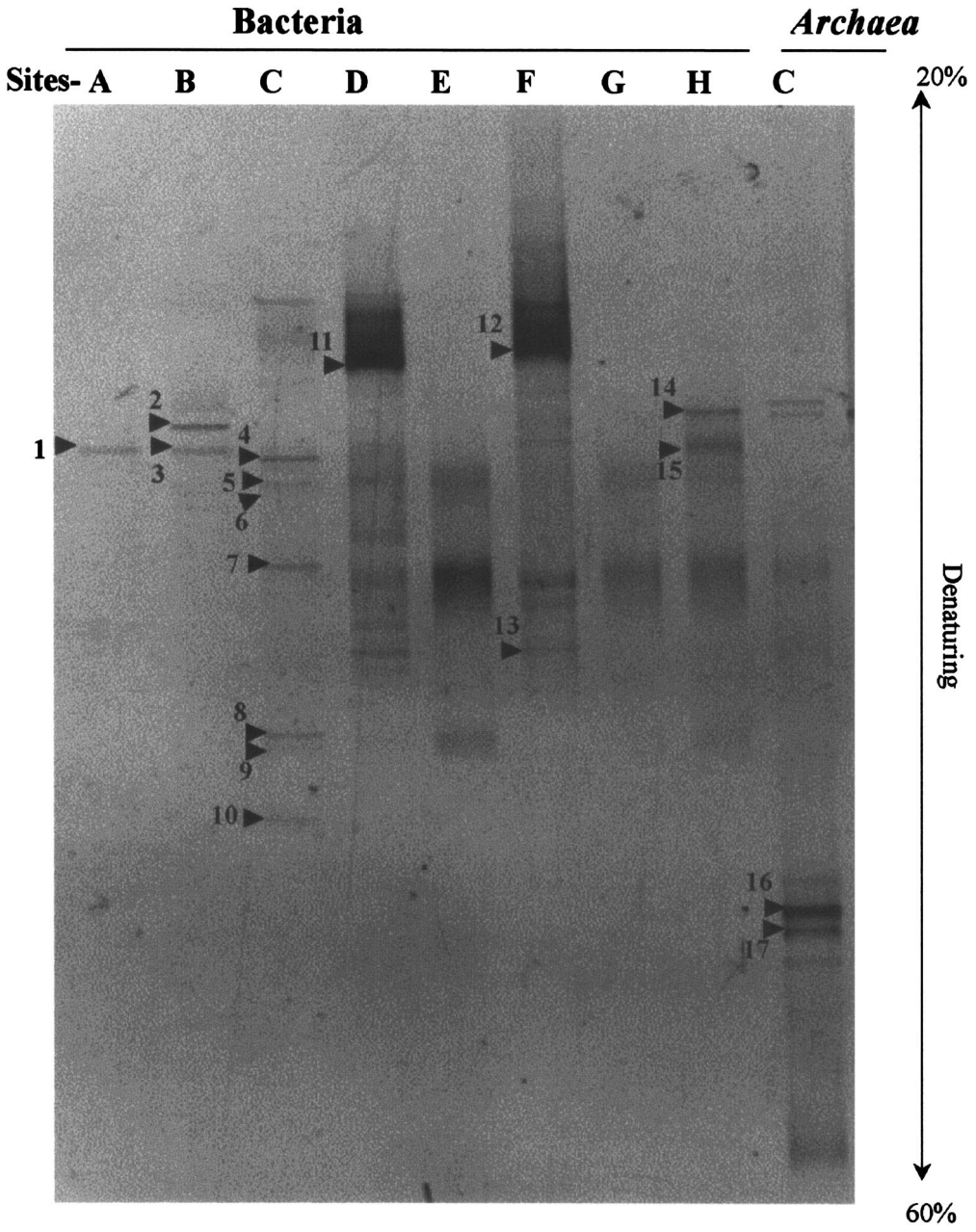

FIG. 5. DGGE profiles of 16S rDNA genes derived from filtered hydrothermal water from drilled holes (lanes A, D, and F), natural vents (lanes B, E, G, and H), and the substratum of catheter-type in situ growth chamber (lane C) with primer sets for the domain *Bacteria* and *Archaea*. The bands labeled with numbers were sequenced. Bands: 1, SUIYO-E1; 2, SUIYO-E2; 3, SUIYO-E3; 4, SUIYO-E4; 5, SUIYO-E5; 6, SUIYO-E6; 7, SUIYO-E7; 8, SUIYO-E8; 9, SUIYO-E9; 10, SUIYO-E10; 11, SUIYO-E11; 12, SUIYO-E12; 13, SUIYO-E13; 14, SUIYO-E14; 15, SUIYO-E15; 16, SUIYO-A16; 17, SUIYO-A17.

whereas the other site temperatures were above 18°C. It is likely that these DGGE bands were derived from the psychrophilic microbes growing in the casing pipe at sites D and F.

The DGGE bands SUIYO-E1 and -E2 matched the sequence of the *Thiomicrospira* sp. strain MA2-6 (35). Sulfur-oxidizing bacteria of the genus *Thiomicrospira* and related phylotypes have been frequently isolated from deep-sea hydrothermal vent samples (5, 23, 35, 46, 60). Muyzer et al. (35) demonstrated that *Thiomicrospira* spp. were dominant community members in hydrothermal vent sites at the Mid-Atlantic Ridge. Similarly, it is likely that members of the *Thiomicrospira* species played a key role in the sulfur oxidation of hydrothermal sites in the Suiyo Seamount.

The DGGE band SUIYO-E14 was closely related to an obligately anaerobic and phototrophic bacterium, *Pelodictyon luteolum*, that utilizes sulfide and sulfur as an electron donor (15). The SUIYO-E14 band was found only in the sample from site H where sulfide was depleted in hydrothermal water. Nis-

TABLE 3. 16S rDNA sequence similarity between sequences from samples and related taxa

Phylogenetic group (DDBJ/EMBL/GenBank accession no.)	% Similarity	DGGE band	Presence of the DGGE band at sampling site: C (279°C)	G (310°C)	B (300°C)	A (55°C)	E (25–36°C)	H (3–50°C)	D (3°C)	F (4°C)
Bacteria										
Aquificae										
Persephonella hydrogenophila (AB086419)	97	SUIYO-E8, E9	+							
Thermovibrio ruber (AJ316619)	95	SUIYO-E10	+							
"*Chlorobia*"										
Pelodictyon luteolum (Y08107)	98	SUIYO-E14						+		
α-*Proteobacteria*										
Methylobacterium sp. strain SY-2 (AJ278347)	99	SUIYO-E13							+	+
γ-*Proteobacteria*										
Thiomicrospira sp. strain MA2-6 (L40811)	100	SUIYO-E1, -E3			+	+				
Arctic96AD-3 (AF354607)	92	SUIYO-E5, -E6	+							
	98	SUIYO-E12								+
Arctic96AD-9 (AF354608)	97	SUIYO-E11							+	
Strain BD1-1 (AB015514)	89	SUIYO-E4	+							
δ-*Proteobacteria*										
Bdellovibrio sp. strain JS10 (AF084863)	92	SUIYO-E2			+					
Ethylbenzene-degrading consortium DGGE band C (AB062689)	97	SUIYO-E15						+		
ε-*Proteobacteria*										
Clone P. palm C/A 26 (AJ441213)	92	SUIYO-E7	+							
Archaea										
Crenarchaeota										
Thermococcus sp. strain MZ11 (AY017179)	94	SUIYO-A16	+							
Not determined	82	SUIYO-A17	+							

bet et al. (40) hypothesized that deep-sea hydrothermal vent systems appear to support the growth of phototrophic microorganisms due to the potential excitation of bacteriochlorophyll (BChl) *a* and *b* by black-body emission. However, members of the genus *Pelodictyon* contain BChl *c*, *d*, or *e* as their major photosynthetic pigments (15). Likewise, a thermophilic green sulfur bacterium, *Chlorobium tepidum*, whose optimum growth temperature is 47 or 48°C, contains BChl *c* as its major photosynthetic pigment (57). Therefore, it is difficult to determine the in situ function of the microorganism corresponding to the SUIYO-E14 band without more detailed physiological data.

The SUIYO-E15 band was closely related to the mesophilic ethylbenzene-degrading sulfate-reducing bacterium in an ethylbenzene-degrading consortium (39). The microorganisms corresponding to the SUIYO-E15 band appear to be the mesophilic bacteria. Although the occurrence of a DSR gene related to the *Desulfobacter*-like DSR genes (42) was anticipated, no DSR gene was related to the SRP in the δ-*Proteobacteria*. PCR biases may have occurred in the present study.

Phylogenetic analyses of archaeal 16S rDNA fragments. Archaeal PCR-amplified 16S rDNAs were obtained from sites C to H. However, the DGGE bands successfully sequenced were the two DGGE bands, designated SYUIYO-A16 and -A17, that were obtained only from the vent catheter at site C. This gap is most likely due to the use of the chamber. No PCR product was amplified from the negative-control samples using archaeal primers.

The SUIYO-A17 DGGE band was less closely related to cultivated species and environmental clones belonging to *Archaea* (Table 3). The SUIYO-A17 DGGE band formed a separate but closely associated lineage with "*Nanoarchaeum equitan*," as represented by a nanosized hyperthermophilic symbiont (20), and with the nanoarchaeotal clones CU-1 and OP-9 (21) (Fig. 6). This position was strongly supported by high bootstrap values above 701 (neighbor-joining method) and 82 (maximum-parsimony method), respectively. The SUIYO-A17 DGGE band did not cluster with several "ancient archaeal group" clones representing deeply rooted lineages in the *Crenarchaeota* that had been found in black smoker chimneys at Myojin Knoll and the Suiyo Seamount (50). Moreover, two secondary structural features of the 16S rRNA of the SUIYO-A17 DGGE band were investigated to confirm its affiliation with *Archaea* (Fig. 7). This sequence contains the conserved characteristic archaeal secondary structures, structures 17 and 18, which correspond to the helix number (20). These results indicate the potential presence of as-yet-uncultivated *Archaea* at the deep-sea hydrothermal sites of the Suiyo Seamount. However, the branching position of the cluster consisted of "*Nanoarchaeum equitans*," and the SUIYO-A17 position was not strongly supported by high bootstrap values. Therefore, no accurate branching position of the cluster in the domain *Archaea* could be determined. Further studies, as well as microscopy observations with fluorescence-labeled oligonucleotide probes and culture-based approaches, should be undertaken to clarify whether there is a nanosized hyperthermophilic symbiont corresponding to SUIYO-A17 in the deep-sea hydrothermal vents of the Suiyo Seamount.

Conclusions. Although there was no evidence of phylogenetic congruence between DSR and 16S rRNA tree topology, the present analysis of DSR gene sequences indicated a notable cluster from the deep-sea hydrothermal sites of the Suiyo

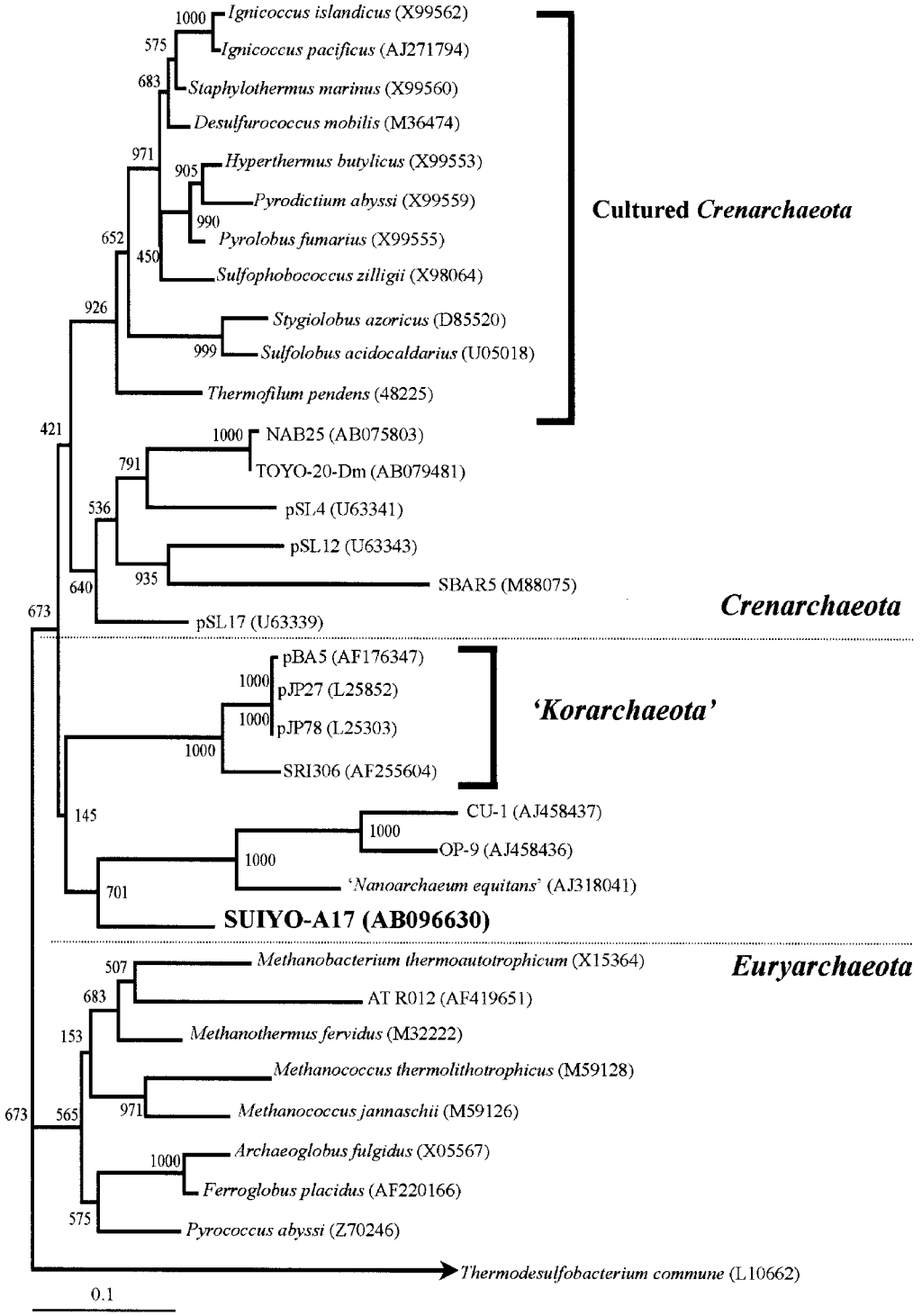

FIG. 6. Phylogenetic relationships of archaeal 16S rDNA fragments (approximately 450 bp) obtained from Suiyo Seamount (in bold type) as determined by neighbor-joining analysis. The bar labeled 0.1 represents an estimated 10% sequence divergence. Numbers beside branching points indicate bootstrap values determined from 1,000 iterations.

Seamount, Izu-Bonin Arc, Western Pacific, Japan. The occurrence of DSR genes may reveal a potential dissimilatory sulfate reduction in the hydrothermal vent sites. Furthermore, 16S rRNA gene analysis provided the temperature-related distribution of the microbial community structure in this hydrothermal site. Further efforts to develop tools for the quantification of DSR gene and its mRNA combined with physiological characterizations (sulfide production and sulfate reduction rate) should provide the temperature-related distribution of the SRP and their role in the sulfur cycle in deep-sea hydrothermal vents.

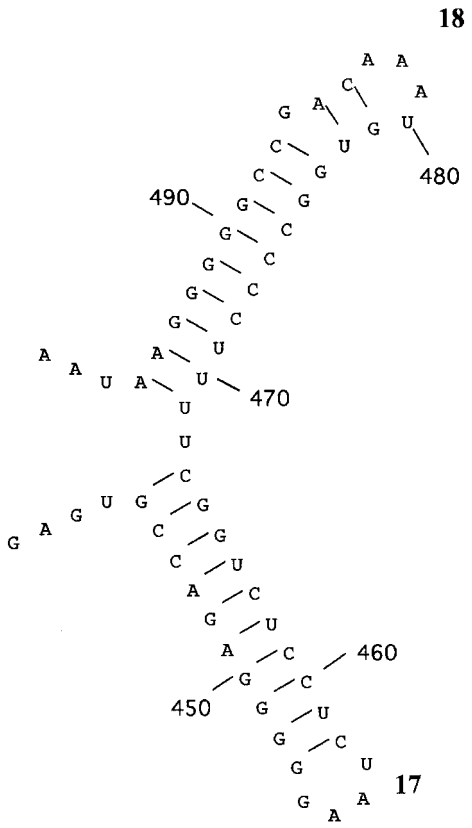

FIG. 7. Conserved characteristic archaeal secondary structures in the region between positions 440 to 498 (*Escherichia coli* numbering). Boldfaced numbers 17 and 18 correspond to the helix number (20).

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