Archaeal Diversity and Distribution along Thermal and Geochemical Gradients in Hydrothermal Sediments at the Yonaguni Knoll IV Hydrothermal Field in the Southern Okinawa Trough^{∇†}

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Received 24 April 2009/Accepted 11 December 2009

A variety of archaeal lineages have been identified using culture-independent molecular phylogenetic surveys of microbial habitats occurring in deep-sea hydrothermal environments such as chimney structures, sediments, vent emissions, and chemosynthetic macrofauna. With the exception of a few taxa, most of these archaea have not yet been cultivated, and their physiological and metabolic traits remain unclear. In this study, phylogenetic diversity and distribution profiles of the archaeal genes encoding small subunit (SSU) rRNA, methyl coenzyme A (CoA) reductase subunit A, and the ammonia monooxygenase large subunit were characterized in hydrothermally influenced sediments at the Yonaguni Knoll IV hydrothermal field in the Southern Okinawa Trough. Sediment cores were collected at distances of 0.5, 2, or 5 m from a vent emission (90°C). A moderate temperature gradient extends both horizontally and vertically (5 to 69°C), indicating the existence of moderate mixing between the hydrothermal fluid and the ambient sediment pore water. The mixing of reductive hot hydrothermal fluid and cold ambient sediment pore water establishes a wide spectrum of physical and chemical conditions in the microbial habitats that were investigated. Under these different physico-chemical conditions, variability in archaeal phylotype composition was observed. The relationship between the physical and chemical parameters and the archaeal phylotype composition provides important insight into the eco-physiological requirements of uncultivated archaeal lineages in deep-sea hydrothermal vent environments, giving clues for approximating culture conditions to be used in future culturing efforts.

Deep-sea hydrothermal activity results in diverse physical and chemical environments for the resident microbial communities. Using cultivation techniques and culture-independent molecular analyses, diverse lineages of archaea and bacteria have so far been observed from chimney structures, retrieved *in situ* colonization systems settled in or on the hydrothermal conduit, microbial mats, sediments, and chemosynthetic macrofaunal bodies (19, 35, 62). Especially in the domain *Archaea*, most of lineages derived from hydrothermal environments have not yet been cultivated, and little is known about their physiological and metabolic traits.

Environmental conditions of the habitat for a particular uncultivated archaeal lineage permit us to speculate about the physiological and metabolic traits of the archaea. For instance, the acidophilic and thermophilic archaeon "*Aciduliprofundum boonei*," representing the previously uncultivated deep-sea hydrothermal vent euryarchaeotic group I (DHVEG I) subgroup 2 (DHVE2), has been isolated from a chimney habitat in the Lau Basin (49). In fact, before the cultivation of *A. boonei*, the DHVE2 was assumed to consist of thermophilic and acidophilic heterotrophs because their habitats had similar characteristics (13, 48, 60, 68). In order to elucidate the distribution patterns of the functionally unknown microbial components in response to the dynamically varying physico-chemical conditions, hydrothermally influenced sediments are considered better study targets than hot vent chimney structures to determine the eco-physiological roles of uncultivated microbes. This is because, unlike vent chimneys, sedimentary habitats affected by subsurface hydrothermal fluid are expected to have more moderate physico-chemical gradients from mixing of hydro-

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† Supplemental material for this article may be found at <http://aem.asm.org/>.

[∇] Published ahead of print on 18 December 2009.

thermal fluid and ambient seawater due to the relatively lower heat convection and hydrothermal fluid penetration. Several studies have already examined the phylogenetic diversity of archaea and bacteria in hydrothermal sediments from the Guaymas Basin (7, 66), the Rainbow vent field in the Mid-Atlantic Ridge (39), and the Iheya Ridge and the Yonaguni Knoll IV in the Okinawa Trough (14, 57). However, only the relationship between the distribution pattern of microbial components and the physico-chemical conditions of these environments has been addressed.

The Yonaguni Knoll IV hydrothermal field located at the southern end of the Okinawa Trough is characterized as having thick sediment, several Cl-enriched black smoker sites, and numerous vapor-enriched clear fluid sites (25, 56). The geochemical characterization of these hydrothermal fluids revealed that hydrothermal fluids undergo phase separation under the seafloor (25, 56). Furthermore, the emission of liquid CO₂ droplets has been reported, and occurrence of subseafloor CO₂ hydrate is assumed to have arisen in response to pore water chemistry in the sediments at liquid CO₂ emission sites (14, 25). According to pore water chemistry, it seems likely that these vapor-enriched hydrothermal fluids permeate the sediments around hydrothermal vent sites, and the subseafloor formation-dissociation processes of gas hydrates produce a variety of hydrothermally affected sedimentary habitats (25).

In this study, we focused on the “abyss vent” site, which is characterized by 90°C hydrothermal emissions that discharge directly from the seafloor sediments (56). Sediment cores (>25 cm in length) were taken at horizontal distances of 0.5, 2, and 5 m from the hydrothermal emission while the *in situ* temperature of sediments was measured simultaneously. Vent fluids and interstitial water chemistry of the sediments were characterized along vertical and horizontal gradients of subseafloor mixing zones. Microbial distributions, particularly of archaea, were ascertained by culture-independent molecular analyses targeting the small subunit (SSU) rRNA gene and, *mcrA* (gene for methyl coenzyme A [CoA] reductase subunit A) and archaeal *amoA* (gene for ammonia monooxygenase large subunit). Molecular analyses for the functional genes, *mcrA* and *amoA*, are expected to indicate diversity and abundance of methanogens, anaerobic methanotrophs, and archaeal ammonium oxidizers that utilize hydrogen, methane, and ammonium, respectively, in hydrothermal fluids as electron donors. In addition, we inferred the phylogenetic diversity and distribution patterns of the bacterial SSU rRNA genes that provide insight into the potential metabolic characteristics and microbial ecosystems in each habitat.

MATERIALS AND METHODS

Site description, sampling of hydrothermal fluids and sediments, and deployment of ISCSs. The deep-sea hydrothermal area on the Yonaguni Knoll IV is located at the southern end of the Okinawa Trough (24°50′ to 24°51′N, 122°51.5′ to 122°52.5′E) (25). We conducted two cruises YK03-05 (July 2003) and YK04-05 (May 2004) on the *R/V Yokosuka* in order to investigate this hydrothermal field with the manned submersible *Shinkai 6500*. The collection of hydrothermal fluids and deployment of the *in situ* colonization system (ISCS) devices with a self-temperature recording system (STR-ISCS), described previously (59), was focused on the diffuse flow of a clear smoker we called the abyss vent. Samples used for this study are described in Table S1 in the supplemental material. Hydrothermal fluid diffusing from the abyss vent (sample identifier [ID] 762W2) was collected using a gas-tight WHATS (water hydrothermal-fluid *Atsuryoku* tight sampler) fluid sampler (52, 69, 70) during the YK03-05 cruise, and

in situ temperatures of the fluid were measured by a self-recording thermometer. The STR-ISCS was deployed in the hydrothermal fluid conduit for 4 days. Sediment cores were collected at distances of 0.5, 2, and 5 m from the diffusing emission by push corers during the YK03-05 and YK04-05 cruises (Table S1). At a point 5 m from the hydrothermal emission, two replicate cores were taken for microbiological and geochemical analysis. The *in situ* temperatures of sediments of these cores were determined by vertically allayed multipoint temperature probes. Ambient seawater (sample ID 816NW) in the hydrothermally active area was collected using a Niskin bottle (General Oceans) during the YK04-05 cruise.

Sediment cores were divided from top to bottom into 5-cm sections using either sterilized, top-cut 50-ml syringes or spatulas. Subsamples for DNA extraction were placed in plastic tubes and stored at −80°C. The pumice stuffed in ISCS was stored at −80°C for DNA extraction. The vent emission in gas-tight WHATS bottles (approximately 150 ml) and the ambient deep seawater in the Niskin bottle (approximately 1 liter) were filtered using 0.22-μm-pore-size cellulose acetate filters of 22- and 45-mm diameters, respectively, and stored at −80°C for DNA extraction.

Geochemical analysis of hydrothermal fluid and interstitial waters. The filtered water samples described above were subjected to geochemical analysis. In addition to the hydrothermal fluid and ambient seawater, interstitial water was extracted from the sediments after the method described by Nakaseama et al. (37). Geochemical analyses of water samples were conducted following Gieskes et al. (9) with slight modifications (37). Silica and ammonia concentrations were determined onboard by colorimetric techniques (9), and sulfate concentrations were determined by ion chromatography after a 300-fold dilution. Analytical errors associated with these chemical analyses, estimated using replicates, were within ±3%. Concentrations of CO₂ and CH₄ and the stable carbon isotopic ratio of CO₂ and CH₄ dissolved in hydrothermal fluids and interstitial water samples were analyzed as described in Tsunogai et al. (70).

Nucleic acid extraction. Total DNA was extracted from sediments and pumice material contained in the ISCS using an Ultra Clean Soil DNA Purification Mega Kit or Ultra Clean Soil DNA Purification Kit (Mo Bio Laboratories) and was further purified using MagExtractor-PCR and Gel Clean-up kit (Toyobo Inc.). The DNA from the microbial fraction separated by filtration from the hydrothermal fluid and the ambient seawater was extracted with an Ultra Clean Microbial DNA Kit (Mo Bio Laboratories).

Amplification of the archaeal and bacterial SSU rRNA genes, *mcrA*, and archaeal *amoA* and clone analysis. Archaeal and bacterial SSU rRNA genes were amplified from extracted DNA by PCR using LA *Taq* polymerase with GC buffer (Takara Bio Inc.). The oligonucleotide primers for PCR amplification were Arch21F (TTCCGGTTGATCCYGCCGGA) and Arch958R (YCCGGCG TTGAMTCCAATT) or U1492R (ASGGTACCTTGTTACGACTT) for archaeal SSU rRNA genes and B27F (AGAGTTTGATCCTGGCTCAG) and U1492R for bacterial SSU rRNA genes (6, 26). PCR amplification was performed with a thermal cycler GeneAmp 9700 (Perkin-Elmer) using DNA amplification conditions of 35 cycles at 96°C for 25 s, 50°C for 45 s, and 72°C for 120 s for archaeal SSU rRNA genes and conditions of 20 to 30 cycles of 96°C for 25 s, 54°C for 45 s, and 72°C for 120 s for bacterial SSU rRNA genes. The gene fragments of *mcrA* and *amoA* were also amplified with Ex *Taq* polymerase (Takara Bio Inc.) using primers ME1F (CGMATGCAATHGGWATGTC) and ME2R (TCATKGCRTAGTTDGGRTAGT) for *mcrA* (10) and Arch-amoAF (STAATGGTCTGGCTTAGACG) and Arch-amoAR (GCGGCCATC CATCTGTATGT) (8) for *amoA* under the following DNA amplification conditions: for *mcrA*, 40 cycles at 96°C for 25 s, 40°C for 45 s, and 72°C for 60 s; for *amoA*, 40 cycles at 96°C for 25 s, 53°C for 60 s, and 72°C for 60 s.

The amplified gene fragments were cloned into pCRII vector (Invitrogen), and clone libraries were constructed. The inserts were directly sequenced by the dideoxynucleotide chain termination method using a dRhodamine sequencing kit (PE Applied Biosystems) in accordance with the manufacturer's instructions.

The primers Arch21F and Bac27F were used for the initial single-strand sequencing of the archaeal and bacterial rRNA genes, respectively. Sequence similarity among all of the single-stranded SSU rRNA gene sequences (approximately 0.5 to 0.7 kb) was analyzed by the FASTA-composing algorithm run on DNASIS software (Hitachi Software). In the clone analysis, archaeal and bacterial SSU rRNA gene sequences showing ≥97 and ≥96% identity, respectively, were assigned to the same phylogenetic clone type (phylotype). Representative SSU rRNA gene clones from each phylotype were subjected to additional sequencing, and approximately 0.8 to 1.0 kb of sequence was determined from both strands. In *mcrA* and *amoA* clone analyses, primers M13M4 and M13RV (Takara Bio Inc.) were used to determine the full-length sequences. Sequences showing ≥96% identity for both gene fragments were assigned to the same phylotype.

Quantification of the SSU rRNA genes, *mcrA*, and archaeal *amoA*. Quantification of the archaeal and whole prokaryotic SSU rRNA gene population was performed according to a previously published method (58). The primers and probes used were Arch349F (GYGCASCAGKCGMGAAW), Arch806R (GGA CTACVSGGGTATCTAAT), and Arch516F (TGYCAGCCGCCGCGGTAAH ACCVGC) for the archaeal SSU rRNA gene and Uni340F (CCTACGGGRBG CASCAG), Uni806R (GGACTACNNGGGTATCTAAT), and Uni516F (TGY CAGCMGCCGCGGTAAHACVNRS) for the whole prokaryotic SSU rRNA gene (58). Quantification of the ANME *mcrA* group c-d was conducted with modified primers and a probe from a previous study (44). The primers and probe used in this study were cd_F2-2 (GCTCTACGACCAGATMTGGCTYGG), cd_R4 (CCGTAGTAYGTGAAGTCRTCCAGCACw), and cd_3 (CTGCGTR AATCCGACACCRCTGACATGTA). A mixture of *mcrA* clones acquired in this study (p816_M_1.01 and p763_M_1.03) was used as a PCR template for obtaining standard curves. For quantification of the *mcrA* gene, the PCR was conducted using Ex *Taq* polymerase (Takara Bio Inc.) with primer and probe concentrations of 0.8 and 0.4 pmol μL^{-1} , respectively. The amplification conditions consisted of preheating at 96°C for 2 min followed by 50 cycles of 96°C for 25 s and 65°C for 6 min. For quantification of the archaeal *amoA* gene, SYBR Premix Ex *Taq* II (Takara Bio Inc.) with MgCl₂ (final concentration, 5 mM) was used for amplification with primers Arch-amoAF and Arch-amoAR (8) (each at a final concentration of 0.4 pmol μL^{-1}). Amplification conditions consisted of preheating at 95°C for 30 s followed by 40 cycles of 95°C for 5 s, 50°C for 30 s, and 64°C for 34 s. A mixture of *amoA* clones (p763_AMO_1.02, p763_AMO_1.03, p763_AMO_1.21, and p763_AMO_4.21) acquired in this study was used as a template for PCR to obtain standard curves. All quantitative PCR analyses were performed using a 7500 Real Time PCR System (PE Applied Biosystems). The numbers of all three genes in each sample were determined as averages of the duplicate or triplicate analyses.

Phylogenetic analysis. Representative sequences of the SSU rRNA gene phylogenies, as well as sequences of the *mcrA* and *amoA* clones, were subjected to a similarity search against the DDBJ/EMBL/GenBank databases using the FASTA (<http://fasta.ddbj.nig.ac.jp/top-j.html>) and BLAST (<http://blast.ddbj.nig.ac.jp/top-j.html>) search programs, respectively. Representative SSU rRNA gene sequences were aligned and phylogenetically classified into certain taxonomic units using ARB, version 20030822 (30). The SSU rRNA gene, *mcrA*, and *amoA* sequences were automatically aligned with closely related nucleotide sequences using the CLUSTALX program, version 1.81(67), and ambiguously aligned regions were manually edited. Phylogenetic trees were constructed by the neighbor-joining method using CLUSTALX, version 1.81. Bootstrap analysis using 100 replicates was performed in order to estimate confidence levels in the tree topology.

Nucleotide sequence accession numbers. The SSU rRNA gene sequences of cultured and uncultured organisms determined in this study were deposited at the DDBJ/EMBL/GenBank nucleotide sequence databases under the following accession numbers: AB235329, AB235338, AB235339, AB235342, AB369283, AB295464 to AB295469, AB301972 to AB302043, AB305328 to AB305613, and AB432988 to AB432996.

RESULTS

In situ temperature and geochemical features of hydrothermal fluid and sediment interstitial water. The maximum temperature of the flow diffusing from the abyss vent was 90°C, and the concentrations of CH₄, CO₂, SO₄²⁻, SiO₂, and NH₄⁺ in the fluids obtained using the WHATS water sampler were 1.2 mmol kg⁻¹, 93.4 mmol kg⁻¹, 24.1 mmol liter⁻¹, 2.63 mmol liter⁻¹, and 1.05 mmol liter⁻¹, respectively. SO₄²⁻, SiO₂, and NH₄⁺ concentrations in ambient seawater were 27.7, 0.11, and 0.02 mmol liter⁻¹, respectively (56). The temperature profiles in the sediments at distances of 0.5 and 2 m from the hydrothermal vent exhibited approximately linear increases with depth (Fig. 1A). Based on the linear thermal gradient at the core 760M3 site (at a distance of 2 m from the vent emission), the temperature of the core bottom (50 cm below the seafloor [cmbsf]) was estimated to be approximately 35°C (Fig. 1A). Concentrations of SiO₂, CH₄, SO₄²⁻, CO₂, NH₄⁺, and the stable carbon isotopic ratio of CH₄ and CO₂ in the interstitial water of sediment cores are shown in Fig. 1.

Observable degassing was noted during recovery of sediment cores obtained at distances of 0.5 and 2 m from the hydrothermal emission (cores 816M1 and 760M3) and even onboard after retrieval of the cores (see Fig. S1 in the supplemental material). The sediments in core 816M1 consisted of soft gray silt, while those of core 760M3 consisted of dark-gray silt with occasional large sulfur particles (<2 cm). Even so, despite degassing and the presence of void structures, the structure of the cores was relatively preserved (Fig. S1). Sediments sampled 5 m from the emission (cores 763MY and 763MW) were comprised of light-brown silt at the surface and of dark-brown silt in the deeper zone. All the cores were stratigraphically simple and did not have any distinctive layers.

Interestingly, although the CH₄ concentration in the pore water was quite different between cores 816M1 and 760M3, the total CO₂ (ΣCO_2) concentrations were generally similar between the cores (Fig. 1C). In addition, $\delta^{13}\text{C}(\text{CH}_4)$ also showed an interesting horizontal shift: -21.3 to -18.7‰ in core 816M1, -4.2 to 2.2‰ in core 760M3, and -41.7 to -38.4‰ in core 760MW (Fig. 1D).

The concentrations of SiO₂, CO₂, and CH₄ in the interstitial water at various depths within the core and in the hydrothermal fluid diffused from the abyss vent were plotted with respect to the horizontal distance of the core from the vent (Fig. 2). The SiO₂ concentration of the pore water appeared to decrease linearly with increasing distance from the vent, suggesting possible mixing of the diffusing hydrothermal fluid and the pore water over the distance between the site of the vent and that of core 763MW (at a distance of 5 m from the hydrothermal emission) as end members (Fig. 2). Considering the temperature and geochemistry of diffusing fluid at the abyss vent, ambient seawater, and core 763MW, the interstitial water in the deepest section of core 816M1 (69°C) was primarily composed of hydrothermal fluid, and the interstitial water in the deepest section of core 760M3 (approximately 35°C) was likely mixed with the hydrothermal fluid at a proportion of approximately 50% (Fig. 1). The hydrothermal fluid input into the pore water seemed to decrease linearly toward the shallower portions of the sediments of the cores (Fig. 1). If this assumption is applied to the CO₂ and CH₄ concentrations in the pore water, the CO₂ concentrations in cores 816M1 and 760M3 and the CH₄ concentration in core 760M3 are likely to be markedly lower than suggested by the mixing lines shown in Fig. 2.

Quantitative PCR of the SSU rRNA gene. The maximum copy number of the entire prokaryotic SSU rRNA gene per gram of sediment in cores of 816M1, 760M3, and 763MY was 1.9×10^8 at 2 cmbsf, 2.9×10^7 at 5 cmbsf, and 1.5×10^8 at 32 cmbsf, respectively (Fig. 3). In core 816M1, the copy number of the total prokaryotic SSU rRNA genes decreased with increasing depth while the copy number of the archaeal SSU rRNA gene was greatest in the sediments at 22 cmbsf (Fig. 3). In core 760M3, the copy numbers of both total prokaryotic and archaeal SSU rRNA genes decreased from the surface to a depth of 35 cmbsf before increasing once more between 35 and 45 cmbsf. In addition, although the proportion of archaeal SSU rRNA genes relative to the total prokaryotic SSU rRNA genes increased to 29% at a depth of 45 cmbsf, the proportion of archaeal SSU rRNA decreased from the surface to a depth of 35 cmbsf (21% near the surface and 1.3% at a depth of 35 cmbsf) (Fig. 3). In the sediments 5 m from the vent (core

FIG. 1. Physical and chemical characterization of sediment cores 816M1, 760M3, and 763MW. Temperatures and concentrations of silica (A), concentrations of sulfate and methane (B), concentrations of ammonium and carbon dioxide (C), and carbon stable isotopic ratio of methane and carbon dioxide (D) in interstitial waters from three cores are shown. Concentrations of these gases and ions in the vent emission (90°C) from the abyss vent were as follows: CH₄, 5,600 μmol kg⁻¹; SO₄²⁻, 24.1 mmol liter⁻¹; SiO₂, 2.63 mmol liter⁻¹; NH₄⁺, 1.05 mmol liter⁻¹; CO₂, 93.4 mmol kg⁻¹; δ¹³C_{CH₄}, -24.2 ‰; δ¹³C_{CO₂}, -9.1 ‰.

FIG. 2. Relationship between distance from the vent emission and the SiO_2 , CO_2 , and CH_4 concentrations in water samples from sediment cores. Dotted lines indicate the potential dilution rate presumed from SiO_2 concentrations.

763MY), the copy number of the whole prokaryotic SSU rRNA gene was slightly higher in deeper sediments than at the surface, with the highest proportion of the archaeal SSU rRNA gene in the entire prokaryotic SSU rRNA gene community observed at a depth of 32 cmbsf (Fig. 3).

Bacterial SSU rRNA gene clone analysis. A total of 506 bacterial SSU rRNA gene clones were sequenced from the same DNA assemblages in the sediment cores and water samples used for archaeal gene analyses. We could not obtain

indigenous bacterial SSU rRNA gene sequences from the pumice material in the ISCS. In contrast to archaeal SSU rRNA gene communities, the dominant phylogroups detected in bacterial SSU rRNA gene clone analysis belonged to cultivated groups, with several exceptions, such as the potentially thermophilic EM3 group (50) that is phylogenetically associated with the order *Thermotogales* and the JS1 group (73). The bacterial SSU rRNA gene compositions for each of the core samples are shown in Fig. 4.

Archaeal SSU rRNA gene clone analysis. A total of 415 archaeal SSU rRNA gene sequences were analyzed, and a diversity of archaeal SSU rRNA gene phylotypes were examined from the hydrothermal fluid, the ISCS deployed in the hydrothermal vent, ambient seawater, and sediments (Fig. 5). Phylogenetic placement of each phylotype is shown in Fig. 6. The detected archaeal phylotypes were assigned to the following groups, most of which have been detected in hydrothermal environments: the *Thermococcales*, *Desulfurococcales*, *Thermoproteales*, miscellaneous crenarchaeotic group (MCG) (16, 61, 66), hot water crenarchaeotic group I (HWCG I) (41), HWCG II (also known as UCIII) (41, 53), HWCG IV (also known as UCII) (17, 53), forest soil crenarchaeotic group (FSCG) (20), marine group I crenarchaeota (MGI) (6), deep-sea archaeal group (DSAG) (also known as marine benthic group B [MBG B]) (57, 71), ancient archaeal group (AGG) (57), DHVE1 (a subgroup in DHVEG I and also known as MBG D) (57, 71), DHVE2 (a subgroup in DHVEG I) (40, 57), *Methanosarcinales*, Guaymas euryarchaeal group (GEG) (7, 66), ANME I and II (12, 46, 57, 66), DHVE6 (a subgroup in DHVEG II) (40, 57), South Africa gold mine euryarchaeotic group (SAGMEG) (61), and DHVE3 (a subgroup in DHVEG II) (40, 57) (Fig. 5 and 6). Based on the phylogenetic classification and the distribution patterns described below, the previously proposed DHVE1 group was tentatively subdivided into three subgroups in this study (Fig. 6A). The phylogenetic classification of the MGI phylotypes (Fig. 6E) was based on Massana et al. (33) and Takai et al. (64).

FIG. 3. Copy numbers of total prokaryotic and archaeal SSU rRNA genes, *mcrA* from ANME group c-d, and archaeal *amoA* determined by quantitative PCR.

FIG. 4. Distributions of *Bacteria* shown by SSU rRNA gene clone analysis in hydrothermal sediments, ISCS, vent emission, and deep-seawater from the Yonaguni Knoll IV hydrothermal field. Numbers of sequenced clones in each clone library are shown in parentheses. Black dots indicate *in situ* temperatures of sediments. Abbreviations: V, N, and R in *Epsilonproteobacteria* indicate families *Thiovulgaceae*, *Nautiliaceae*, and *Thioreductoraceae*, respectively.

Clone analysis and quantitative PCR of *mcrA*. The *mcrA* gene fragments were amplified from the DNA samples extracted from the shallow sediments (>20 cmbsf) of cores 816M1 and 760M3. A total of 62 clones (15 or 16 sequences from each of the samples at depths of 2 and 12 cmbsf in core 816M1 and of 5, 15, and 25 cmbsf in core 760M3) were sequenced. All of the *mcrA* sequences were phylogenetically affiliated to the ANME *mcrA* group c-d, which is potentially derived from the ANME II (see Fig. S2 in the supplemental material). No *mcrA* fragments related to sequences from thermophilic and mesophilic methanogens or other methanotrophic groups were detected. We attempted to quantify the number of group c-d *mcrA* sequences using the previously developed method of Nunoura et al. (44). However, all the *mcrA* sequences obtained in this study had a 1-bp mismatch with a previously constructed forward primer and a 2-bp mismatch with both the reverse primer and probe for quantitative PCR. We therefore modified both primer and probe sequences and determined the copy numbers of *mcrA* group c-d in these sediment samples. In the group c-d *mcrA*, maximum copy numbers were observed at the sediment surface and decreased with increasing depth in both cores (816M1 and 760M3) (Fig. 3).

Clone analyses and quantitative PCR of archaeal *amoA*. Archaeal *amoA* was detected in sediment core 763MY taken at a 5-m distance from the hydrothermal emission and the ambient deep seawater (Fig. 3 and 7). A total of 120 archaeal *amoA* fragments from the core sediments and deep seawater were

sequenced and classified into 13 phylotypes belonging to five phylogenetic clusters (Fig. 7A). Based on the phylogenetic analysis of *amoA*, all *amoA* sequences were related to clusters that have previously been detected in seawater and ocean sediment samples (8) (Fig. 7A). Quantitative PCR of archaeal *amoA* was conducted for DNA assemblages only from sediment core 763MY.

DISCUSSION

Physical and chemical variation in hydrothermal sediments.

The degassing of CO₂ from hydrothermal sediments in the Okinawa Trough is associated with the presence of liquid CO₂ or CO₂ hydrates in the sediments but not with dissolved gaseous CO₂ (14, 25). Under the hydrostatic pressure at the seafloor in the Yonaguni Knoll IV field (13 MPa), the liquid CO₂ and CO₂ hydrates were likely stable at 31.1°C and at about 15°C, respectively (55). Thus, given the vertical temperature gradients (Fig. 1) and the vigorous degassing associated with both core recovery from the deep sea and core subsampling onboard in the cases of the cores 816M1 and 760M3 (see Fig. S1 in the supplemental material), liquid CO₂ and CO₂ hydrates could be hosted over most of the length and in the shallower sediments, respectively, of core 760M3, and liquid CO₂ also could be formed in shallower zones of core 816M1. The degassing of CO₂ could primarily explain the depleted CO₂ concentration in the pore water of cores 816M1 and 760M3. How-

FIG. 5. Distributions of *Archaea* shown by SSU rRNA gene clone analysis in hydrothermal sediments, ISCS, vent emission, and deep-seawater in the Yonaguni Knoll IV hydrothermal field. Black dots indicate *in situ* temperatures of sediments. Numbers of sequenced clones in each clone library are shown in parentheses. MGI subgroups are shown using acronyms (see Fig. 6E).

ever, highly depleted CH_4 concentrations in core 760M3 could not be explained simply by a degassing process.

The stable carbon isotopic compositions of pore water CH_4 in core 760M3 sediments were isotopically unusually heavy throughout the depths (Fig. 1D). The highly ^{13}C -enriched CH_4 in core 760M3 (-4.2 to 2.2‰) has not previously been described (14, 25) and would require extensive isotopic fractionation of CH_4 (-21.3 to -18.7‰) derived from the diffusing hydrothermal fluid. Although preferential partitioning of CH_4 into CO_2 hydrate formation has been demonstrated (54), whether the partition process induces large isotopic fractionation between CH_4 caged in the hydrates and dissolved in the pore water is still uncertain. The isotopically light CH_4 trapped in the CO_2 hydrates may escape more rapidly from sediment during degassing of core 760M3 than the residual ^{13}C -enriched CH_4 dissolved in the pore water. This is one possible explanation for the low concentrations of CH_4 with unusually ^{13}C -enriched carbon isotopic compositions found in core 760M3 and does not exclude other mechanisms.

The sulfate concentrations in pore water could be considered an index of seafloor microbial activity in the sediments. In cores 816M1 and 760M3, the interstitial water sulfate concentration decreased with increasing sediment depth. Because the diffusing hydrothermal fluid at the abyss vent contained $24.1 \text{ mmol liter}^{-1}$ of sulfate, the interstitial water sulfate concentrations lower than $24 \text{ mmol liter}^{-1}$ in the deeper zones of cores 816M1 and 760M3 could have resulted from microbial sulfate-reducing activity. The molecular signatures (SSU rRNA gene and *mcrA*) indicated the existence of anaerobic methanotrophs in the sediments of cores 816M1 and 760M3. In addition, potential sulfate-reducing counterparts, *Deltaproteobac-*

teria, were also found in the sediments (Fig. 4). However, based on a quantitative estimation of ANME archaeal populations in the whole microbial community in the sediments and the distribution patterns of ANME (Fig. 3), it is still unclear whether the function of sulfate-reducing, methane-oxidizing communities was sufficient for the observed geochemically assumed microbial sulfate reduction. It could be that the potentially mesophilic to thermophilic sulfate-reducing bacteria within the *Thermodesulfobacteriaceae* and *Deltaproteobacteria* utilize sulfate as an electron acceptor with energy sources such as H_2 and formate from the hydrothermal fluid input or organic carbon in sediments, which is suggested by bacterial SSU rRNA gene clone analysis in the deepest sediments of the 816M1 and 760M3 cores (Fig. 4).

Inferences from bacterial SSU rRNA gene clone analysis.

Bacterial SSU rRNA gene community structures may provide important insights into the relationship between the physical-chemical conditions of the habitats and the eco-physiological traits of the previously uncultivated archaeal lineages. The vertical shift in bacterial phylotype composition represents a shift in the preferences of both temperature and potential redox state of the respective bacterial components in the sediments of core 816M1, taken at a distance of 0.5 m from the hydrothermal emission site. Chemolithoautotrophic “*Thiovulgaceae*” *Epsilonproteobacteria* having anaerobic or microaerobic metabolism (4) dominated in bacterial SSU rRNA gene clone libraries from the sediment above 15 cmbsf, and the obligately anaerobic sulfate-reducing family *Thermodesulfobacteriaceae* (18, 34) was the most dominant in the bottom of core 816M1. The clonal abundance of the anaerobic and hy-

FIG. 6—Continued on following page

FIG. 6. SSU rRNA gene phylogenetic analysis of *Archaea* detected in hydrothermal sediments, ISCS, vent emission, and deep seawater from the Yonaguni Knoll IV hydrothermal field. Phylogenetic trees of *Euryarchaeota* (A), *Crenarchaeota* and deeply branching *Archaea* (B), *Thermoprotei* (C), MCG (D), and MG I (E) are based on the neighbor-joining method with 434, 424, 661, 737, and 757 homologous positions, respectively. Numbers indicate bootstrap values from 100 trials. GenBank/EMBL/DBJ accession numbers are given in parentheses. Bar, two substitutions per 100 nucleotides.

drogenotrophic sulfur reducers *Desulfurobacteriaceae* and “*Thermosulfidibacteriaceae*” (28, 43, 45, 63) also increased (Fig. 4).

A similar interpretation can also be made for cores 760M3 and 763MY. In the shallower sections of core 760M3 (above 25

cmbsf) at a distance of 1 m from the site of emission, the members of the *Chloroflexi*, *Deltaproteobacteria*, *Bacteroidetes*, and JS1 group dominated the bacterial SSU rRNA gene communities. The occurrence of these bacterial SSU rRNA genes

FIG. 7. (A) Phylogenetic analysis of archaeal *amoA* gene detected in deep seawater and sediments in the Yonaguni Knoll IV hydrothermal field using the neighbor-joining method with 485 homologous positions of nucleotides. GenBank/EMBL/DBJ accession numbers are given in parentheses. Bar, two substitutions per 100 nucleotides. (B) Distribution of archaeal *amoA* gene in deep seawater and sediments from the Yonaguni Knoll IV hydrothermal field. Numbers of sequenced clones in each clone library are shown in parentheses.

in microbial community structures has often been found in methane seep sediments and deep-seafloor sediments at the continental margins (15, 22, 23, 32, 42). However, in the deepest part of the core sediments, the *Thermosulfidibacteriaceae* (43, 45)-related sequences represent approximately 20% of the clonal abundance (Fig. 4). In the sediments of the most distantly located core (763MY), the bacterial SSU rRNA gene communities were generally similar over the length of the core and are primarily composed of the *Alpha*-, *Gamma*- and *Deltaproteobacteria* and *Chloroflexi* phylotypes, which are often reported as abundant in marine seafloor sediments (47, 72). Based on the recent progress in understanding heterotrophically driven microbial ecosystems in seafloor sediments (3, 29), it seems likely that the bacterial phylotypes found in the shallower sections of core 760M3 and along the entire length of core 763MY may be associated with heterotrophy. Conse-

quently, the abundance and distribution of bacterial phylotypes, both horizontally and vertically, in the sediments suggest that the hydrothermal fluid inputs may have a marked impact on microbial community structure and function in the deeper sediments of the peripheral area 2 m from the vent site. In addition to the hydrothermal fluid inputs in the deeper zones, which was already demonstrated by the physical and chemical characterization of the core sediments and the interstitial water, the localized abundance of putative hydrogenotrophic and autotrophic bacteria further implies the significance of H_2 from the hydrothermal fluid as a potential energy source of the microbial communities. In fact, the end members of the hydrothermal fluids in the Yonaguni Knoll IV field are the most H_2 rich in the Okinawa Trough (25), and the microbial communities in the chimney structures are characterized by a predominance of hydrogenotrophic chemolithoautotrophs (45).

Relevance between physical-chemical conditions of habitats and archaeal phylotypes. In this study, archaeal communities mainly composed of previously uncultivated lineages were obtained from sediments with different physical and chemical properties in the vertical and horizontal directions. At present, it is still difficult to explain how the archaeal phylotypes encountered at a site interact with specific environmental conditions. However, it may be possible to identify the physiological and metabolic traits of several archaeal phylotypes in distinct areas of sediment.

The population of group c-d *mcrA* genes from ANME II was observed to decrease with increasing depth in cores 816M1 and 760M3 (Fig. 3) while both sulfate and methane concentrations at the bottom of both cores are likely sufficient for the growth of ANME, as described above (Fig. 1B). Therefore, it is unlikely that methane and sulfate concentrations do not control the distribution of ANME in these cores. On the other hand, based on a comparison of the temperature profiles of the cores with those of *mcrA* populations, *mcrA* gene numbers appear to be inversely correlated with the temperature profiles of the cores, and the upper limit of temperature for the distribution of the ANME II *mcrA* genes is estimated to be around 25 to 30°C (Fig. 1 and 3). This estimate is not contradicted by results from previous incubation experiments (38) but is not supported by observation of anoxic oxidation of methane (AOM) at the higher temperatures reported previously from hydrothermal sediments in the Guaymas Basin (21, 66) and from the deep hot sediments in the Newfoundland Margin (51).

Only the MGI (“*Nitrosopumilales*”) were identified as being potential ammonia-oxidizing archaea (AOA) in core 763MY while other AOA candidates, such as the soil group crenarchaeota and HWC I (“*Nitrosocaldadales*”), were not found in any of the core sediments (Fig. 6B) (5, 11, 24, 27). Archaeal *amoA* genes were localized in core sediment 763MY (Fig. 3 and 7). Phylogenetic analyses of the MGI SSU rRNA gene and *amoA* sequences suggest that *amoA* detected in this study probably derived from the MGI, and the MGI subgroups are prevalent in their own habitats, such as deep-sea water and sediment (Fig. 6E and 7). Furthermore, although the MGI phylotypes are dominant in the archaeal SSU rRNA gene library constructed from the shallow sediments of core 763MY, the number of archaeal *amoA* genes is 2 orders of magnitude lower than the number of the archaeal SSU rRNA genes (Fig. 3). A similar gap was also observed in deep seawater in the North Atlantic, and Agogue et al. discussed the abundance of non-nitrifying MGI in this environment (1). The data presented in this study also highlight the potential abundance of non-nitrifying MGI in deep-sea sediments. However, to clarify whether this is indeed the case, obtaining pure cultures of MGI from deep-sea environments will be essential.

Together with the *Thermoproteales* and *Thermococcales* members, the HWC I, HWC II, HWC IV, MCG thermophilic group, and DHVE1 cluster 3 dominate the archaeal rRNA gene communities in cores 816M1 and 760M3. Sequences belonging to the HWC I have been obtained only from high-temperature terrestrial and marine environments (2, 5, 31, 41), and their thermophily is very probable because of their distribution pattern in hydrothermal sediments. Metagenomic analysis of the fosmid clone encoding the SSU rRNA gene from the HWC I member identified from a subsurface

geothermal stream also implied that the organism was potentially an O₂-resistant and/or an O₂-respiring thermophile (41). Since the sedimentary habitats where members of the HWC I are distributed contain members of the *Epsilonproteobacteria* and *Thermoproteales*, which are known to have variable O₂ responses and flexible metabolisms, the HWC I phylotypes detected from the sediments in this study may have similar physiological and metabolic characteristics like other bacterial and archaeal components that dominate similar niches. Thermophily of the HWC II, HWC IV, and the MCG thermophilic group has been discussed in previous studies of terrestrial hot springs (2, 65), subsurface geothermal streams (31), sulfide chimney structure (53), sulfur chimney structure (36), and hydrothermal sediments (57). Our study demonstrates the abundance of these archaeal phylotypes in deep-sea sediments affected by hydrothermal fluids, which highlights their potential for thermophilic life. Furthermore, the cooccurrence of these crenarchaeotes such as HWC I with strictly anaerobic bacteria at the bottom of core 760M3 indicates that, in addition to the aforementioned physiological adaptations to relatively high-redox environments, these archaea may be capable of strictly anaerobic metabolism. As the distribution patterns of these archaeal phylotypes in cores 816M1 and 760M3 are very similar to that of the members of the HWC I, it is possible that these archaeal groups have similar physiologies and metabolisms. The possible thermophily of the DHVE 1 cluster 3 members has not previously been recognized. However, in this study, the DHVE 1 cluster 3 phylotypes are consistently present in the sediments of cores 816M1 and 760M3 (Fig. 5). Consequently, in future studies, some members of the DHVE cluster 3 should be considered potential thermophiles.

Other archaeal phylotypes with restricted distribution in sediments are the DHVE 1 cluster 1 and the DSAG found in the shallower sections of core 760M3 and the deepest sections of core 763MY, respectively (Fig. 5). The DSAG phylotypes are frequently detected in cold-seep environments and deep-subseafloor sediments as well as the in sediments in hydrothermal fields (15, 23, 42, 57). Similarly, sequences of the DHVE 1 cluster 1 have been detected in sediments from cold-seep environments and the deep subseafloor (15, 23). Most of the sedimentary habitats where the DSAG and DHVE 1 cluster 1 phylotypes are most commonly retrieved appear to be relatively reducing (14, 15, 23, 57). While the distribution patterns of the DSAG and DHVE 1 cluster 1 in this study appear to support the findings of previous studies, our findings did not provide any additional clues regarding their physiologies and metabolisms.

On the basis of comparison of metabolisms of cultivated bacterial lineages distributed in the same environments, the HWCs and thermophilic MCG are likely hydrogenotrophic chemolithoautotrophs. Other uncultivated archaeal lineages, such as the DHVEs and DSAG, are considered to be heterotrophs. However, the physiological and metabolic traits of most archaeal phylotypes found in sediments are still uncertain. Further multidisciplinary investigations, combined with geochemical analysis, molecular ecological survey, cultivation- or enrichment-oriented tracer experiments, and metagenomic or single-cell genomic analyses are required to fully characterize the previously uncultivated archaeal lineages that are thriving in the deep-sea hydrothermal environments.

ACKNOWLEDGMENTS

We thank the operational staff of the *R/V Yokosuka* and *Shinkai 6500* on cruises YK03-05 and YK04-05 (JAMSTEC) for their assistance in collecting samples.

Part of this study was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant number 16780228).

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