

## ORIGINAL ARTICLE

# Defining boundaries for the distribution of microbial communities beneath the sediment-buried, hydrothermally active seafloor

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**Subseafloor microbes beneath active hydrothermal vents are thought to live near the upper temperature limit for life on Earth. We drilled and cored the Iheya North hydrothermal field in the Mid-Okinawa Trough, and examined the phylogenetic compositions and the products of metabolic functions of sub-vent microbial communities. We detected microbial cells, metabolic activities and molecular signatures only in the shallow sediments down to 15.8 m below the seafloor at a moderately distant drilling site from the active hydrothermal vents (450 m). At the drilling site, the profiles of methane and sulfate concentrations and the  $\delta^{13}\text{C}$  and  $\delta\text{D}$  isotopic compositions of methane suggested the laterally flowing hydrothermal fluids and the *in situ* microbial anaerobic methane oxidation. *In situ* measurements during the drilling constrain the current bottom temperature of the microbially habitable zone to  $\sim 45^\circ\text{C}$ . However, in the past, higher temperatures of  $106\text{--}198^\circ\text{C}$  were possible at the depth, as estimated from geochemical thermometry on hydrothermally altered clay minerals. The 16S rRNA gene phylotypes found in the deepest habitable zone are related to those of thermophiles, although sequences typical of known hyperthermophilic microbes were absent from the entire core. Overall our results shed new light on the distribution and composition of the boundary microbial community close to the high-temperature limit for habitability in the subseafloor environment of a hydrothermal field.**

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## Introduction

Deep-sea hydrothermal vents are extreme environments, especially with respect to the high temperatures, which create the limits of life on Earth. Several studies have further addressed the potential microbial habitats beneath the seafloor hydrothermal

vents, the so-called ‘sub-vent biosphere’, and have indicated the possible occurrence of functionally active and metabolically diverse (hyper-)thermophilic microbial communities associated with shallow subseafloor hydrothermal fluids and mineral deposits (Deming and Baross, 1993; Delaney *et al.*, 1998; Summit and Baross, 1998; Huber *et al.*, 2002, 2003). The compositions and functions of the sub-vent microbial communities have been inferred from culture-dependent and culture-independent analyses of microbial communities in (i) *in situ* growth chambers placed in hydrothermal fluid flows (Karl *et al.*, 1988; Reysenbach *et al.*, 2000; Corre *et al.*, 2001; Takai *et al.*, 2004), (ii) crustal fluids collected

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directly from the shallow seafloor environments (~10 m below the seafloor [mbsf]) via seafloor drilling or probe insertion (Cowen *et al.*, 2003; Higashi *et al.*, 2004; Huber *et al.*, 2006; Kato *et al.*, 2009; Orcutt *et al.*, 2011), (iii) hydrothermal sediments (Teske *et al.*, 2002; Dhillon *et al.*, 2005; Nunoura *et al.*, 2010; Teske *et al.*, 2014; Dowell *et al.*, 2016; Teske *et al.*, 2016) and (iv) chimney structures of active hydrothermal vents (Takai *et al.*, 2001; Schrenk *et al.*, 2003; Nakagawa *et al.*, 2005). These studies found that mesophilic, thermophilic and hyperthermophilic members of *Epsilonproteobacteria*, *Gammaproteobacteria*, *Aquificales*, *Thermococcales* and *Methanococcales* were the potentially predominant microbial components in the sub-vent biosphere. In addition, previous studies associated with international scientific ocean drilling projects have also indicated the existence of microbial cells in deep subsurface sedimentary and rocky habitats in the hydrothermal vent systems on the Juan de Fuca Ridge (Ocean Drilling Program (ODP) Leg 139 (Cragg and Parkes, 1994) and 169 (Cragg *et al.*, 2000; Summit *et al.*, 2000) and Integrated Ocean Drilling Program (IODP) Expedition 301 (Lever *et al.*, 2013)) and the Manus Basin (ODP Leg 193 (Kimura *et al.*, 2003)). However, these microbial explorations associated with scientific ocean drilling projects have not successfully provided data on the compositions and functions of potential seafloor microbial communities. In particular, it is unclear how local hydrothermal flow may influence the uneven distribution of sub-vent microbial community. Complex subsurface hydrothermal flow may limit or stimulate the sub-vent microbial activities.

The IODP Expedition 331 by the D/V *Chikyu* (Takai *et al.*, 2011) provided a good opportunity for direct investigation of the sub-vent biosphere at the Iheya North hydrothermal field in the Mid-Okinawa Trough. The Iheya North field is located in a continental-margin backarc basin, and the hydrothermal activity is highly influenced by trough-filling terrigenous sediment and knoll-covering volcanoclastic deposits (Kawagucci *et al.*, 2011). The seafloor geochemical and microbiological processes associated with buried organic matter and hydrothermal circulation produce unique hydrothermal fluid compositions that are enriched with high concentrations of methane and ammonia (Sakai *et al.*, 1990; Kawagucci *et al.*, 2011). During IODP Exp. 331, we conducted drilling and coring operations at five sites, located at 0 m (C0016), 100 m (C0013), 450 m (C0014) and 1550 m (C0017) east and 600 m (C0015) northwest of the most active hydrothermal mound (Takai *et al.*, 2011). Previously, we have reported downhole changes in the microbial community corresponding to variations in the seafloor hydrogeologic and lithostratigraphic structures that affected the recharging seawater and hydrothermal fluid input at Site C0017 (Yanagawa *et al.*, 2014). That study obtained the 16S rRNA gene

sequences of putative thermophiles only from the deepest sediments at 141 mbsf, where the *in situ* temperature was estimated to be approximately 90 °C, lower than the previously reported upper growth temperature limit of hyperthermophiles (122 °C) (Takai *et al.*, 2008). The results suggested that the deepest sediment at Site C0017 is inhabited by potential thermophilic populations and that the habitable limit of the seafloor microbial community lies at a depth greater than that of the bottom of the drilled hole (Yanagawa *et al.*, 2014).

In addition to Site C0017, this study investigated the seafloor environments at two additional drilling sites (Sites C0013 and C0014) that have higher thermal gradients and are closer (100 and 450 m, respectively) to the vigorous hydrothermal vents. We chose these vents to clarify the distribution, composition and function of microbial communities occurring adjacent to the high-temperature biosphere limit associated with high-temperature hydrothermal fluids. The drilling operations at these sites successfully penetrated the seafloor hydrothermal fluid reservoirs and created artificial hydrothermal fluid vents with temperatures of up to 311 °C (Kawagucci *et al.*, 2013a). Hence, the recovered core samples were potentially exposed to the *in situ* temperature range of 4 to >300 °C and provide important insights into the boundary microbial community and the limits of the biosphere.

## Materials and methods

### *Sampling sites and sample collection*

Seafloor drilling and coring operations were conducted at the Iheya North hydrothermal field in the Mid-Okinawa Trough during IODP Expedition 331 (Takai *et al.*, 2011). Coring and *in situ* temperature measurements were conducted at IODP Site C0013 and Site C0014 as previously described (Takai *et al.*, 2011). Sediment samples for geochemical and microbiological analyses were collected as previously described (Yanagawa *et al.*, 2013b, 2014). Details on the sample collections are provided in Supplementary Information Materials and Methods.

### *Geochemical analyses*

The carbon and hydrogen isotopic compositions of methane were determined via continuous-flow isotope ratio mass spectrometry as previously described (Umezawa *et al.*, 2009; Kawagucci *et al.*, 2013a). The oxygen isotopic compositions of hydrothermally altered mud were determined as previously described (Miyoshi, 2013). The concentration and isotopic composition of dissolved inorganic carbon (DIC) was determined as previously described (Miyajima *et al.*, 1995; Toki *et al.*, 2004; Noguchi *et al.*, 2013). Details are provided in Supplementary Information Materials and methods.

### Molecular analyses of 16S rRNA genes and functional genes

DNA was extracted from core subsamples using the PowerMAX Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). Before the physical cell disruption, the samples were incubated at 65 °C for 5 min. Then, mechanical shaking was performed for 10 min with a ShakeMaster (BioMedical Science, Tokyo, Japan). Other subsequent steps were performed according to the manufacturer's protocol. The extracted DNA was stored at –80 °C.

Quantitative real-time PCR (Q-PCR) was performed for prokaryotic and archaeal 16S rRNA genes and the functional genes of *dsrA*, *aprA* and *mcrA* as previously described (Nunoura *et al.*, 2008; Blazejak and Schippers, 2011; Yoshida-Takashima *et al.*, 2012; Breuker *et al.*, 2013). The primers, probes and amplification conditions used in this study are summarized in Supplementary Table S2.

For the clone analysis of 16S rRNA genes, the gene fragments were amplified by PCR using a universal and archaea-specific primer set (Nunoura *et al.*, 2012). The *mcrA* gene fragments were also amplified using specific primers. Details for primers and PCR amplification conditions are described in Supplementary Table S2. The amplified 16S rRNA and *mcrA* genes were cloned, sequenced and aligned as previously described (Yanagawa *et al.*, 2013b). The phylogenetic affiliations were identified using the SILVA SSU Ref 111 Database (Ludwig *et al.*, 2004) and phylogenetic trees were constructed. A detailed description is provided in Supplementary Information Materials and Methods. Sequences have been deposited in the GenBank database under accession numbers: AB824899-AB825952 for 16S rRNA gene sequences and LC061224-LC061266 for *mcrA* gene sequences.

### Cultivation tests

Serial dilution cultivations for anaerobic heterotrophs, methanogens, and sulfate reducers were performed to quantify the abundance of populations that can be cultivated in the laboratory using sediment slurries from a variety of depths. The cultivated population abundance of aerobic heterotrophs was quantified by colony-forming units on marine agar 2216 (BD). Details for cultivation conditions are provided in Supplementary Information Materials and methods and Supplementary Table S3.

### Microbial activity measurements

The potential rates of methane production and oxidation and acetate oxidation were determined through radioisotope-tracer incubation experiments (Tasumi *et al.*, 2015). The sediment slurry samples were incubated with appropriate radiotracers ( $^{14}\text{CH}_4$ ,  $\text{H}^{14}\text{CO}_3^-$  and  $[2\text{-}^{14}\text{C}]$  acetate) for 1 month at 30 and 60 °C based on the *in situ* temperatures in Supplementary Figure S1. The radioactivity of a

portion of the reaction products in the headspace was measured using a gas chromatograph (Shimadzu GC-2014, Shimadzu, Kyoto, Japan) and a high-sensitivity radioactivity detector RAGA Star (Raytest, Straubenhart, Germany), as shown in Supplementary Information Materials and methods. The potential activity was calculated based on the proportion of radioactive  $^{14}\text{C}$  product to total radioactive substrate.

## Results and Discussion

### Temperature in the sub-vent environment

Sediment core samples analyzed in this study were obtained from two holes at IODP Site C0013 (Holes C0013B and C0013D) and four holes at Site C0014 (Holes C0014B, C0014D, C0014E and C0014G). The depths of the deepest holes were 35 and 137 mbsf at Site C0013 (Hole C0013D) and Site C0014 (Hole C0014G), respectively. Although *in situ* temperatures were not measured during the drilling at Site C0013, it could be estimated from the fact that most of the plastic core liners melted several meters below the seafloor (Yanagawa *et al.*, 2013b). The melting point of the plastic is >70 °C. The subseafloor environment at Site C0014, at which most of the deep coring operations were conducted by using aluminum core liners, exhibited a steep temperature gradient of 3 °C m<sup>-1</sup> based on the temperatures measured during the drilling operation. At this site, the temperatures increased from 4.5 °C at the seafloor to >210 °C at 50 mbsf (Takai *et al.*, 2011; Supplementary Figure S1).

Hydrothermally altered mud with elemental sulfur and sulfide grit was found in shallow sediments at 0.8 and 4.5 mbsf in Holes C0013B and C0013D, respectively (Miyoshi *et al.*, 2015). The clay minerals in the hydrothermal mud yielded oxygen isotopic values of +8.0 and +9.6‰ (Miyoshi, 2013). These values indicated that the clay minerals formed at >138 °C (Supplementary Table S1). At Site C0014, the shallow sediments were not affected by hydrothermal alteration at depths above ~12 mbsf, whereas the deeper >12 mbsf sediments were hydrothermally altered (Miyoshi *et al.*, 2015). The oxygen isotopic values of the hydrothermally altered clays at 12.8 and 15.1 mbsf in Hole C0014B were +8.7 and +12.5‰, respectively (Miyoshi, 2013). This indicated that the clay formation temperatures were >106 °C at Site C0014 (Supplementary Table S1; Miyoshi, 2013). The geochemical thermometer estimate based on the oxygen isotopic compositions of hydrothermally altered clay minerals suggests that at some point in time, even the shallow sediments at Sites C0013 and C0014 have experienced high-temperature conditions that are close to the known temperature limit of life at 122 °C (Takai *et al.*, 2008). Indeed, any of the microbial cell observations, metabolic activity measurements, cultivation tests and prokaryotic 16S rRNA gene quantifications described below provided positive signatures for

the existence of microbial populations only in the near-surface sediments at Site C0013. Thus, most of the results and discussion in the following sections are described with a focus on Site C0014.

A rapid increase in the pore-water potassium (K) concentration was observed just below the seafloor at 12.1 mbsf in Hole C0014B, 11.5 mbsf in Hole C0014D and 17.7 mbsf in Hole C0014G (Supplementary Figure S2). These discontinuities in K concentrations could be explained by the possible occurrence of impermeable layers in the subseafloor environment (Figure 1) and hydrothermal fluid flows just below the layers. The distribution pattern of the hydrothermally altered mud supports this interpretation (Supplementary Table S1; Miyoshi *et al.*, 2015). In addition, because potassium was enriched in the endmember 310 °C hydrothermal fluids (80 mM) but low in the ambient 4 °C seawater (10 mM; Kawagucci *et al.*, 2011), the K concentration is an indicator of the degree of mixing between hydrothermal fluids and infiltrated seawater and/or ambient pore-water. Although the magnesium concentration is also an excellent indicator of mixing between ambient seawater and hydrothermal fluids, the pore-water magnesium concentration could be affected by not only mixing but also association and dissociation processes related to hydrothermally altered minerals (Takai *et al.*, 2011; Miyoshi *et al.*, 2015). Thus, in this study, the pore-water K concentration is likely more reliable than the magnesium concentration. Assuming a bimodal mixing between endmember hydrothermal fluid and seawater without conductive cooling, K concentrations lower than 37 mM correspond to the temperature range of sediments lower than 122 °C, the potential habitable temperature range of previously cultivated microorganisms. In other words, K concentrations higher than 37 mM indicate that the sediments are exposed to high temperatures exceeding the potentially habitable temperature range.

#### *Geochemical evidence of hydrothermal fluid input and methane oxidation*

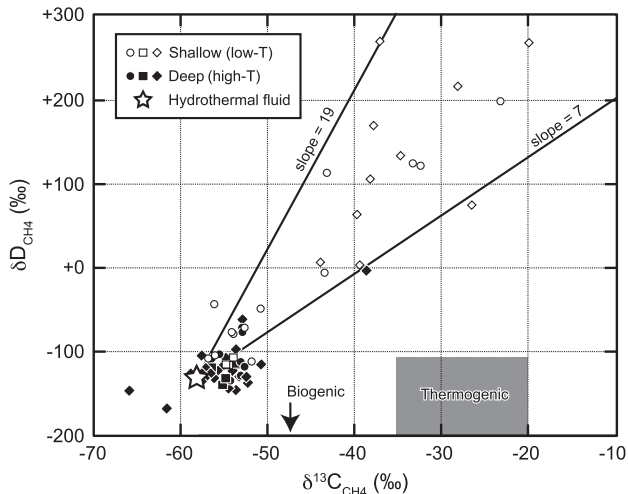
The total alkalinity and DIC concentrations featured significant peaks at shallow depths in Holes C0014B and C0014D (Supplementary Figure S2). On the basis of the stable carbon isotope compositions, this enrichment was probably caused by CO<sub>2</sub> in the

endmember hydrothermal fluids. However, the DIC concentration peaks did not match the vertical K concentration profiles in each of the holes even though both components could have been provided by the hydrothermal fluids (Supplementary Figure S2). The different profiles for these two components (DIC and K) indicated that at least two different hydrothermal fluid sources fed the different lithostratigraphic horizons, such as the depth zones of 1–8.4 mbsf and >12.1 mbsf in the case of Hole C0014B. The hydrothermal fluid input in the shallower sediments (1–8.4 mbsf in Hole C0014B and 0–2.2 mbsf at C0014D) consisted primarily of low-temperature liquid CO<sub>2</sub> and/or enriched gas components, whereas the input in the deeper zones (>12.1, >11.5 and >17.7 mbsf in Holes C0014B, C0014DE and C0014G, respectively) was derived directly from high-temperature vapor-lost fluid. Both of these fluids could result from the subseafloor phase separation and phase-partitioning processes of hydrothermal fluids (Von Damm and Bischoff, 1987).

The methane and sulfate concentrations exhibited more complex vertical variations than the DIC and K concentrations (Figure 1). The sulfate concentration exceeding the seawater level at Holes C0013B and D (Supplementary Figure S2) was probably due to the dissolution of anhydrite with decreasing temperature during the core recovery and sampling processes (Takai *et al.*, 2011). In contrast, sulfate depletion and methane enrichment (relative to seawater levels) were apparent even within the shallow depths for each hole at Site C0014 (Figure 1a). Interestingly, a clear inverse relationship existed between methane and sulfate above 14 mbsf in Hole C0014B. This indicated the lateral flows of methane-rich hydrothermal fluids in the sulfate-rich subseafloor environment. In addition to the lithostratigraphically controlled hydrothermal fluid inputs, the  $\delta^{13}\text{C}_{\text{CH}_4}$  and  $\delta\text{D}_{\text{CH}_4}$  profiles suggested the contribution of microbial community functions. The patterns in the  $\delta^{13}\text{C}_{\text{CH}_4}$  values along the vertical extent of the core were synchronized with  $\delta\text{D}_{\text{CH}_4}$  values, and isotopically positive shifts in both the  $\delta^{13}\text{C}$  and  $\delta\text{D}$  values of methane were observed only within the shallow low-temperature zones (7.8–10.7 mbsf in Hole C0014B and <15.9 mbsf in Hole C0014G), where the methane concentration was extremely low (Figures 1a and 2). These isotopically heavier shifts in methane were not directly derived from the

**Figure 1** Pore-water concentrations of methane and sulfate (Takai *et al.*, 2011) (a), the carbon and hydrogen isotopic composition of methane (b), the numbers of 16S rRNA and functional genes (c and d) and heterotrophic activity (e) in the subseafloor core samples at IODP Site C0014. Q-PCR was used to quantify numbers of 16S rRNA genes of prokaryotes (black circles) and archaea (red diamonds), and functional genes of *mcrA* (purple diamonds), *dsrA* (green triangles) and *aprA* (orange squares). The total cell counts (gray crosses) were originally reported by Takai *et al.* (2011). Heterotrophic activity was determined based on the potential activity of the anaerobic oxidation of acetate. The incubations were conducted at 30 and 60 °C based on the *in situ* temperatures in Supplementary Figure S1. The samples incubated at 60 °C were indicated with parentheses, next to each symbol. Values below the detection limit for the Q-PCR data and heterotrophic activity are plotted as open symbols on the left axes. The red-shaded layers indicate the depth range of the limits for microbes based on Q-PCR data and heterotrophic activity. These depth ranges correspond to the depths of 14.3–17.2 mbsf in Hole C0014B, 10.2–11.4 mbsf in Hole C0014D and 15.8–17.6 mbsf in Hole C0014G. The possible impermeable layers in Supplementary Figure S2 are indicated by the blue dashed lines.





**Figure 2** Cross plots of  $\delta^{13}\text{C}_{\text{CH}_4}$  and  $\delta\text{D}_{\text{CH}_4}$  values. The open star represents the previously reported high-temperature hydrothermal fluid value (Kawagucci *et al.*, 2011). Open and filled symbols represent samples obtained from shallow (low-temperature) and deep (high-temperature) zones in each hole, respectively (criteria of the depth are shown in Figure 1). Circles, squares and diamonds represent Holes C0014B, C0014DE and C0014G, respectively. Representative values for thermogenic and biogenic methane (Kawagucci *et al.*, 2013b) are shown by a shaded area and an arrow, respectively. The diagonal lines, with slopes of 7 and 19, represent the lowest and highest values of the co-variation exhibited in microbial methane oxidation.

microbial methane oxidation (Alperin *et al.*, 1988; Kessler *et al.*, 2006; Holler *et al.*, 2009; Feisthauer *et al.*, 2011).

The vertical patterns of the geochemical signals differed among the holes (Figures 1a and b; Supplementary Figure S2). The spatial heterogeneity in the seafloor pore-water geochemistry was likely affected by the local input of hydrothermal fluids, and the observed patterns, particularly in Hole C0014B, indicated vertical differences in fluid chemistry due to different sources of laterally flowing hydrothermal fluids and their mixing behavior with infiltrated seawater and ambient pore-water. In combination with the complex variations in the lithostratigraphic and physical properties of the core samples, the phase separation, flow patterns and mixing behavior of seafloor hydrothermal fluids are hypothesized to be controlled by the lithostratigraphy, such as fresh hemipelagic sediments, porous pumiceous deposits and hard impermeable layers (Takai *et al.*, 2011).

#### Abundance estimation of microbial cellular and rRNA gene populations

The microbial cell abundance at Site C0014 decreased markedly with depth from approximately  $1 \times 10^8$  cells per ml of sediment just below the seafloor to less than the detection limit of approximately  $6.5 \times 10^5$  cells per ml of sediment (Takai *et al.*, 2011; Figure 1c). A Q-PCR analysis for whole prokaryotic and archaeal 16S rRNA genes suggested

that microbial populations were present only in the shallow sediments above 14.3, 10.2 and 15.8 mbsf in Holes C0014B, C0014E and C0014G, respectively (Figure 1c). The abundance of the whole prokaryotic 16S rRNA genes with depth showed similar values and profiles to the microbial cell abundance in all of the holes. However, several shallow sediment depths in Holes C0014B and C0014G exhibited large data gaps between the microbial cell abundance and the whole prokaryotic 16S rRNA gene abundance due to the relatively higher detection limit of the microbial cell count, as shown in Figure 1c, or due to the technical limitations of the Q-PCR (Hoshino and Inagaki, 2012; Lloyd *et al.*, 2013; Morono *et al.*, 2014). The relative abundance of archaeal 16S rRNA genes increased with depth and represented approximately half of the whole prokaryotic 16S rRNA gene assemblages in the deepest zones where Q-PCR could detect 16S rRNA genes (Supplementary Figure S3).

#### Cultivation and activity measurements for heterotrophs

Successful enrichments of heterotrophs were obtained from most of the shallow sediment samples from Hole C0014B at a temperature of 30 °C, and subsequent quantitative cultivation analyses were conducted on these samples. Aerobic heterotrophs, anaerobic heterotrophs and sulfate reducers were detected at depths above 8.5, 12.2 and 3.6 mbsf, respectively (Supplementary Figure S4). In contrast, 60 °C incubations and onboard cultivation experiments for (hyper-)thermophilic heterotrophs and chemolithoautotrophs did not yield any cultivable population at any depth (Takai *et al.*, 2011). The cultivated populations ranged up to  $9.6 \times 10^5$  CFU  $\text{g}^{-1}$  sediment for aerobic heterotrophs,  $5.5 \times 10^7$  cells  $\text{g}^{-1}$  sediment for anaerobic heterotrophs and  $5.5 \times 10^2$  cells  $\text{g}^{-1}$  sediment for sulfate reducers. The 16S rRNA gene sequences of the isolated aerobic heterotrophs, anaerobic heterotrophs and sulfate reducers were highly similar to those of *Geofilum rubicundum* (100% similarity) [NR\_112717], *Clostridium* sp. S710(0)-1 (98% similarity) [GU136592] and *Desulfomicrobium norvegicum* (100% similarity) [NR\_025407], respectively (Supplementary Table S3). Potential anaerobic heterotrophic activities were also detected in the sediment samples from which the anaerobic heterotrophs were successfully cultivated (Figure 1e). The activity, defined as the oxidation of the methyl group of  $^{14}\text{C}$ -labeled acetate to  $^{14}\text{CO}_2$ , gradually decreased with increasing sediment depth. Notably, all of the estimations of cultivation-dependent viable heterotrophic populations,  $^{14}\text{C}$ -tracer heterotrophic activities and prokaryotic 16S rRNA gene quantification provided independent detectable signatures for the occurrence of a microbial community in the same depth zone of the seafloor environment (Figures 1c and e; Supplementary Figure S4).



under high-temperature conditions (Holler *et al.*, 2011). Hence, the HotSeep-1 group is thought to be responsible for high-temperature sulfate reduction, coupling with the anaerobic oxidation of methane (AOM) by a deeply branching, putatively thermophilic group of methanotrophic archaea (ANME-1-Guaymas I; Holler *et al.*, 2011). Although the sequences related to anaerobic methanotrophs (ANMEs) were found in certain depth horizons, the members closely related to ANME-1-Guaymas I did not co-exist with the bacterial HotSeep-1 group. Notably, the ANME-1-Guaymas I phylotypes were detected only at 14.3 mbsf in Hole C0014B, representing only 2% of the archaeal clone library (Supplementary Data S1).

Archaeal 16S rRNA gene amplicons were obtained from samples from the deepest zones at Site C0014, at which many positive signals for the existence of microbial communities were detected. These archaeal 16S rRNA gene amplicons were detected not by using a universal primer set but by using the archaea-specific primer set Arch530F/Arch958R (Figure 3b). The archaeal 16S rRNA gene communities in these sediments were dominated (42–97% of total archaeal sequences) by the Hot Water Crenarchaeotic Group IV (HWCGIV; also known as UCII or a subcluster of the Terrestrial Hot Spring Crenarchaeotic Group [THSCG]; Supplementary Data S1). They were also detected as the predominant population (99% of the total archaeal sequences) in the near-surface sediment at 1 mbsf in Hole C0013B, which is the only sample containing detectable 16S rRNA genes at Site C0013 (data not shown). The HWCGIV sequences have previously been reported in deep-sea hydrothermal vent habitats (Schrenk *et al.*, 2003; Nunoura *et al.*, 2010; Yoshida-Takashima *et al.*, 2012) and in a high-temperature zone at 141.1 mbsf at Site C0017 (Yanagawa *et al.*, 2014).

#### *Microbial functions of methanogenesis and AOM*

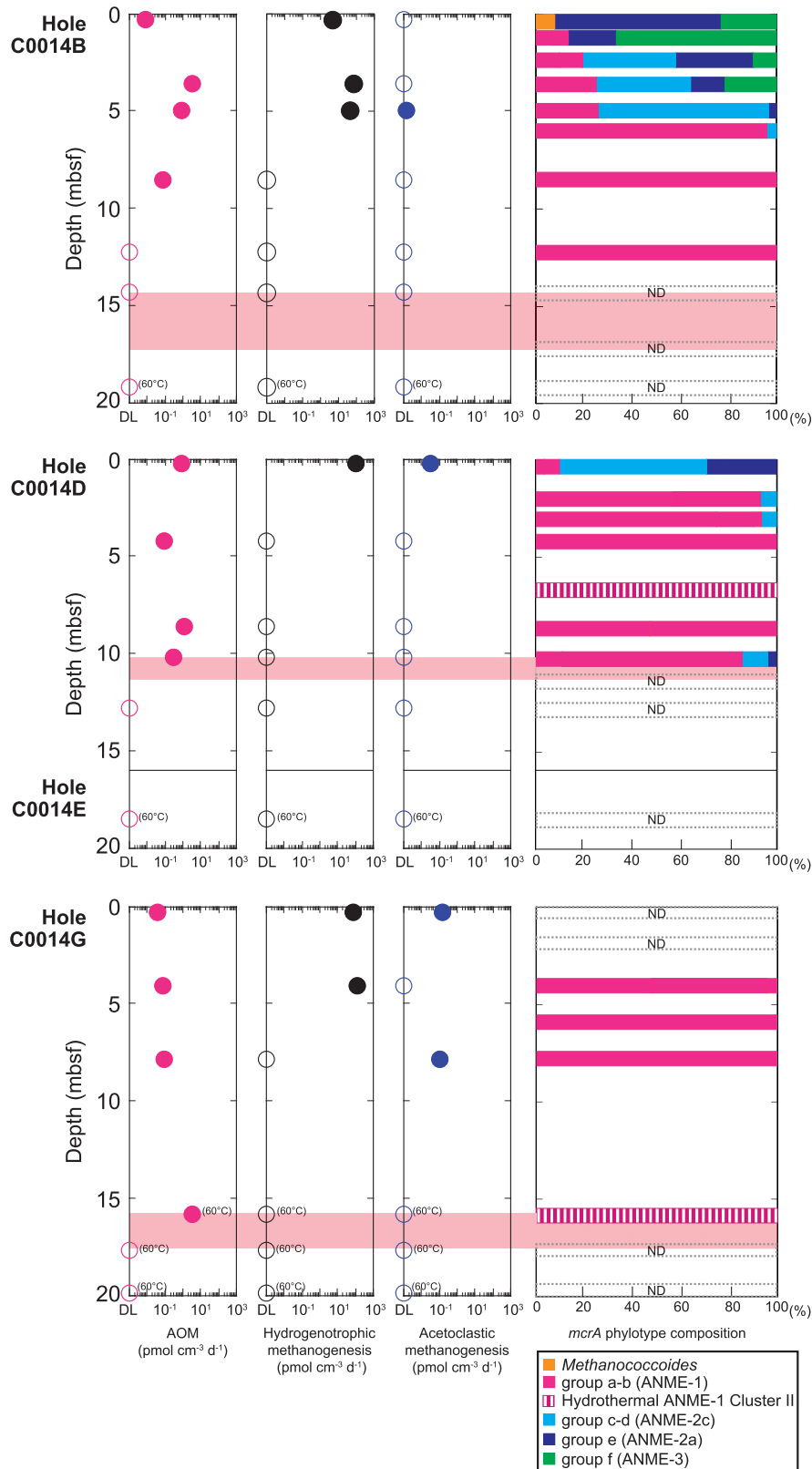
The metabolic activities of hydrogenotrophic methanogenesis, acetoclastic methanogenesis and AOM were traced using radioisotope-labeled substrates at *in situ* temperatures. Hydrogenotrophic and acetoclastic methanogenic activities were detected at certain depths in the relatively shallow zones at Site C0014, whereas AOM activity occurred widely in the seafloor environment, from which positive signals of the existence of microbial communities were obtained (Figure 4). Although the hydrogenotrophic methanogenesis activity outcompeted the AOM activity in the shallower zone and the AOM activity dominated in the deeper zone, the opposing microbial processes of methane production and consumption occurred at the same depths (0.3, 3.6 and 5.0 mbsf in Hole C0014B, 0.2 mbsf in Hole C0014D and 0.3, 4.1 and 7.8 mbsf in Hole C0014G). In addition, relatively high AOM activities and extremely low pore-water sulfate concentrations were

observed at the approximate depths of 3.6, 8.6 and 15.8 mbsf in Holes C0014B, C0014D and C0014G, respectively (Figure 1a).

Phylogenetic diversity and the abundance of functional genes related to methanogenesis and AOM were also characterized via both Q-PCR for genes associated with methyl coenzyme M reductase (*mcrA*), dissimilatory sulfite reductase (*dsrA*) and adenosine 5'-phosphosulfate reductase (*aprA*) and clone analysis for *mcrA* (Figures 1d and 4). The Q-PCR data indicated that the *mcrA*, *dsrA* and *aprA* genes were widely distributed in most of the shallow sediments that yielded many positive signals for the existence of microbial communities, and the abundances decreased with increasing depth in the sediment. This pattern also indicated that the complex biogeochemical processes associated with the microbial methane- and sulfate-related metabolisms co-occur in the sub-vent biosphere at Site C0014. The abundances of these functional genes were one to two orders of magnitude lower than those of the prokaryotic 16S rRNA genes in the same samples.

On the basis of the sequences of the *mcrA* genes obtained from the clone analysis, the *mcrA* genes were conventionally classified into the types derived from potential methanogenic and methanotrophic populations. The methanogenic type of *mcrA* genes were obtained only from the uppermost sediment at 0.3 mbsf in Hole C0014B (Figure 4) and were phylogenetically related to the *mcrA* genes of *Methanococcoides* (Supplementary Figure S5). The *mcrA* group *a-b* genes, which were derived from the ANME-1 (Knittel and Boetius, 2009), were most frequently obtained throughout the entire depth of the potential microbial habitable zone at Site C0014. In contrast, most of the *mcrA* gene sequences of groups *c-d*, *e* and *f*, hosted by ANME-2a, -2c and -3 (Knittel and Boetius, 2009), respectively, were found in the shallower depths of sediments (Figure 4). Molecular ecological studies have indicated that the ANME-1 populations occur in deeper, more reductive and more sulfate-depleted habitats than the ANME-2 populations (Knittel *et al.*, 2005; Krüger *et al.*, 2008; Nunoura *et al.*, 2008; Rossel *et al.*, 2011; Yanagawa *et al.*, 2011). Furthermore, the potentially thermophilic ANME-1 group has been recognized as a key component in certain hydrothermal ecosystems, such as Guaymas Basin and Juan de Fuca Ridge (Biddle *et al.*, 2012; Lever *et al.*, 2013; Merkel *et al.*, 2013). This *mcrA* group of thermophilic ANME-1 is defined as Hydrothermal ANME-1 Cluster II (Lever *et al.*, 2013) (alternatively classified as *mcrA*-Guaymas (Biddle *et al.*, 2012) or ANME-1GBa (Merkel *et al.*, 2013)). The optimal growth temperatures of the thermophilic ANME-1 in the Guaymas site have been estimated to be above 70 °C (Merkel *et al.*, 2013). We detected the same group of *mcrA* gene sequences at 6.7 mbsf in Hole C0014D and 15.8 mbsf in Hole C0014G (Figure 4; Supplementary Figure S5). The bacterial 16S rRNA gene sequences of potential thermophiles (*Thermotoga* and OP1)





**Figure 4** Site C0014 depth profiles of the potential activity of AOM, hydrogenotrophic methanogenesis and acetoclastic methanogenesis (from left to right). The phylogenetic affiliation of *mcrA* genes is shown in the rightmost column. Open circles on the y-axes denote analyses below the detection limit. The red-shaded layers represent the possible depth limit of active subsurface microbes, as determined in Figure 1.

were also detected at these sediment depths (Supplementary Data S1). Thus, the possible host archaeal populations of the Hydrothermal ANME-1 Cluster II *mcrA* genes may be thermophilic. Indeed, the radioisotope-tracer AOM activity measurements showed relatively high activity,  $3.1 \text{ pmol cm}^{-3} \text{ d}^{-1}$ , at  $60^\circ\text{C}$  at 15.8 mbsf in Hole C0014G (Figure 4).

### Synthesis and interpretation

*Development of methane-consuming microbial communities.* Previous studies of microbial communities associated with the hydrothermal vent fluids and chimney deposits in the Iheya North hydrothermal field have hypothesized the existence of active sub-vent microbial communities that are potentially dominated by hyperthermophilic *Thermococcales* and chemolithoautotrophs with  $\text{H}_2$ - and/or sulfur-compound metabolisms. These metabolisms were extrapolated from the variability in the microbial communities in the most interior and/or reductive seafloor habitats that are highly affected by the hydrothermal fluid input (Nakagawa *et al.*, 2005; Takai *et al.*, 2006). However, several thermodynamic estimates of chemolithotrophic microbial community development in hydrothermal-mixing zones have suggested that the populations that couple anaerobic methanotrophy with sulfate reduction would energetically dominate the microbial communities in the sediment-hosted seafloor and subseafloor habitats that are highly affected by hydrothermal fluid inputs (Takai and Nakamura, 2011; Nakamura and Takai, 2014; Takai *et al.*, 2014). In this study, all of the pore-water geochemical analyses, metabolic activity measurements and cellular and molecular microbial community analyses indicated the occurrence of functionally active microbial communities dominated by AOM populations in the relatively shallow subseafloor habitats down to 15.8 mbsf in Hole C0014G. Several studies have examined the abundance, phylogenetic diversity and function of AOM populations associated with seafloor hydrothermal activity in the Guaymas Basin and Yonaguni Knoll IV fields (Teske *et al.*, 2002; Nunoura *et al.*, 2010; Yanagawa *et al.*, 2013a; Dowell *et al.*, 2016). Because these investigations have focused on the shallow sediments just beneath the seafloor, where the diffusive mixing of hydrothermal fluids and seawater likely characterizes the geochemical environments, there remains a lack of knowledge on the subseafloor AOM communities associated with hydrogeologically controlled advection and the partitioning and mixing processes of hydrothermal fluids and infiltrated seawater near deep-sea vents.

The vertical profiles of pore-water methane and sulfate concentrations (Figure 1a) and stable isotopic values of  $\delta^{13}\text{C}_{\text{CH}_4}$  and  $\delta\text{D}_{\text{CH}_4}$  (Figure 2) indicated the abundant occurrence of potentially sulfate-reducing AOM functions in several specific horizons within

the microbially habitable subseafloor environment (for example, depths of 0–11.7 mbsf in Hole C0014B and 0–10.2 and 0–15.8 mbsf in Hole C0014G). In addition, previous study of significant  $^{34}\text{S}$  enrichment in pore-water sulfate strongly suggested the presence of microbial sulfate reduction at several depths within the microbially habitable terrain, that is,  $\sim 5$  mbsf in Hole C0014B and 16.1 mbsf in Hole C0014G (Aoyama *et al.*, 2014). These compositional and isotopic profiles of pore-water methane and sulfate are largely dependent on the mass balance of hydrothermal fluid and seawater inputs and *in situ* microbial consumption. Thus, the geochemically identified peaks and valleys in the potential microbial methane oxidation and sulfate reduction are not necessarily equivalent to the abundance of *in situ* microbial functions. Indeed, the potential *in situ* microbial activity profile of AOM, as determined via radioisotope-tracer experiments, did not match the geochemically identified peaks and valleys of microbial methane oxidation (Figure 4), and the *mcrA* and *dsrA* gene abundances gradually decreased with increasing depth (Figure 1d). However, overall, the pore-water geochemistry, the *in situ* metabolic activities and the 16S rRNA and functional gene distributions indicated a significant association between subseafloor AOM activity and sulfate reduction. On the other hand, previous studies have suggested that microbial AOM activity might be coupled to the reduction of iron (hydr)oxide minerals but not sulfate reduction in metalliferous hydrothermal sediments (Wankel *et al.*, 2012). The physical and chemical variations in the subseafloor environment resulting from advective hydrothermal fluid and seawater flows related to the lithostratigraphic and hydrogeologic conditions would create diverse biogeochemical processes.

*Constraints on microbial community development in the sub-vent biosphere.* All of the direct microscopic observations, PCR-based molecular analyses, cultivation tests and metabolic activity measurements indicated that functionally active, metabolically diverse microbial communities developed in the shallow zones of subseafloor sediments associated with hydrothermal fluid flows. The positive signals of the existence of microbial communities were obtained from the sediments down to 14.3, 10.2 and 15.8 mbsf in Holes C0014B, C0014D and C0014G, respectively. Microbial populations were not detected in the deeper, high-temperature hydrothermal fluid regimes, due to the limit of microbial habitability and/or the methodological detection limit in this study. The Q-PCR and clone library analyses for 16S rRNA genes showed that the abundance of archaeal 16S rRNA gene phylotypes increased with depth, whereas bacterial phylotype populations dominated the microbial communities at shallower depths (Supplementary Figure S3). However, neither the culture-dependent nor the culture-independent analyses detected the existence

of certain previously cultivated hyperthermophilic populations, such as *Thermococcales*, *Methanococcales*, *Archaeoglobales* and members of *Crenarchaeota*, even though such hyperthermophilic archaeal populations are known to dominate the microbial communities in other high-temperature hydrothermal fluid and chimney habitats (Nakagawa *et al.*, 2005; Takai *et al.*, 2006). One conceivable explanation for this may relate to unstable temperature conditions for their growth due to fluctuating hydrothermal fluids, as described below. In the 16S rRNA gene clone libraries obtained from the deepest sediments of the microbially habitable terrain in the subseafloor environment, putative thermophilic phylotypes, such as members of HWCGIV, thermophilic ANME-1, OP1 and *Thermotogae*, were found (Figure 3; Supplementary Data S1). According to the molecular thermometer calculation based on the GC content of the 16S rRNA gene sequences (Kimura *et al.*, 2010), the potential growth temperature range was estimated to be the highest (50–76 °C) for members of HWCGIV among the putative thermophilic populations.

The relationship between microbial habitability and the *in situ* temperature of the subseafloor biosphere has rarely been investigated. Recently, an active subseafloor microbial community has been discovered in deeply buried terrigenous sediments with a temperature of <60 °C at 2458 mbsf (Inagaki *et al.*, 2015). The low abundance of this community was attributed to the increase in energy used for the repair of essential biomolecules such as amino acid and DNA. The energetic costs of amino acid racemization and DNA depurination increase exponentially with temperature (Lever *et al.*, 2015). However, the physical and chemical conditions that form the boundary between habitable and uninhabitable terrains have not been directly explored in subseafloor environments (Takai *et al.*, 2014). Sub-vent microbial communities are believed to live near the upper temperature limits for life on this planet. However, based on the predicted thermal gradient from the temperatures measured during the drilling operation (Takai *et al.*, 2011), the temperature near the detection limit of microbial populations, activities and molecules in Hole C0014B (14.3 mbsf) was estimated to be ca. 45 °C. In contrast, the geochemical thermometer estimate based on the oxygen isotopic compositions of hydrothermally altered clay minerals from 12.8–15.1 mbsf in Hole C0014B suggested that the boundary habitat experienced higher temperatures (>106 °C) (Supplementary Table S1; Supplementary Figure S1). The former temperature (ca. 45 °C) and latter (>106 °C) temperature are far below and similar to the upper temperature limit for microbial growth, respectively (Takai *et al.*, 2008). Additionally, the molecular thermometer estimate based on the potentially most thermophilic 16S rRNA gene phylotype (HWCGIV) population at the depth limit yields a growth temperature range of 50–76 °C. It remains uncertain,

which of the estimated temperatures most accurately represents a realistic *in situ* temperature range because the bottom temperature of present microbial habitable zone was not directly measured during the drilling operation, and the geochemical and molecular thermometer estimates do not necessarily reflect the current *in situ* temperature range. One plausible interpretation is that the deepest microbial habitat in Hole C0014B has been exposed to considerably high temperatures (>106 °C) in the past and presently experiences fluctuating temperatures that are induced by ever-varying degrees of mixing between the subseafloor high-temperature hydrothermal fluids, diffusive and/or advective seawater flows and conductive cooling. Similarly, the hydrothermally altered sediments that were observed in the shallow sediments at Site C0013 and the oxygen isotopic values of the clay minerals indicate formation temperatures of >138 °C (Miyoshi, 2013; Supplementary Table S1). The excess levels of pore-water sulfate concentrations (relative to the sulfate concentrations of seawater) in these sediments were attributed to anhydrite dissolution related to cooling during core recovery and sampling (Supplementary Figure S2). Because anhydrite is stable only under high temperatures (>150 °C) (Gieskes *et al.*, 2002; Takai *et al.*, 2011), the subseafloor sediments at Site C0013 that contain excess pore-water sulfate concentrations (relative to seawater) are likely exposed to high temperatures that exceed the microbially habitable range at the present. The microbial habitability in the sub-vent biosphere is probably highly constrained by the latest temperature history and/or the present temperature conditions induced by spatiotemporally variable high-temperature hydrothermal fluid input.

Furthermore, the physical properties of the sediments are another important factor constraining the microbially habitable terrain in the sub-vent environment. The possible impermeable layers, which are predicted from the discontinuities in the pore-water chemical compositions, serve as not only shields limiting vertical flow and diffusion of fluids but also as barriers limiting vertical migration of microbial cells. Interestingly, the potential limit of microbial community development was always located above or near the possible impermeable layers at Site C0014 (Figure 1). Fluctuations in temperature in the boundary habitat probably occasionally sterilize the microbial community via exposure to high-temperature hydrothermal fluids, and this is followed by a gradual return to microbially habitable temperatures. However, the existence of impermeable layers may contribute to the slow recolonization of the active microbial community after sterilization because the impermeable layer limits migration of viable populations from refugia. In addition, the impermeable layers may also serve as the possible microbial refugia from the lethal temperature fluctuations associated with the occasional exposure to high-temperature hydrothermal fluids. The slow but

successful recolonization of the active microbial community after a temporary sterilization may be initiated from viable microbial cells disseminated throughout the safe interior of impermeable layers. Although the permeable and impermeable inter-layer sequences of cores are often difficult to recover in IODP-like drilling operations, these hypotheses of the limit and recolonization of the sub-vent biosphere should be clarified in future research.

## Conflict of Interest

The authors declare no conflict of interest.

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