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ORIGINAL PAPER

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# Geochemical and microbiological evidence for a hydrogen-based, hyperthermophilic subsurface lithoautotrophic microbial ecosystem (HyperSLiME) beneath an active deep-sea hydrothermal field

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**Abstract** Subsurface microbial communities supported by geologically and abiologically derived hydrogen and carbon dioxide from the Earth's interior are of great interest, not only with regard to the nature of primitive life on Earth, but as potential analogs for extraterrestrial life. Here, for the first time, we present geochemical and microbiological evidence pointing to the existence of hyperthermophilic subsurface lithoautotrophic microbial ecosystem (HyperSLiME) dominated by hyperthermophilic methanogens beneath an active deep-sea hydrothermal field in the Central Indian Ridge. Geochemical and isotopic analyses of gaseous components in the hydrothermal fluids revealed heterogeneity of both concentration and carbon isotopic compositions of methane between the main hydrothermal vent (0.08 mM and −13.8‰ PDB, respectively) and the adjacent divergent vent site (0.2 mM and −18.5‰ PDB, respectively), representing potential subsurface microbial methanogenesis, at least in the divergent vent emitting more $^{13}C$-depleted methane. Extremely high abundance of magmatic energy sources such as hydrogen (2.5 mM) in the fluids also encourages a hydrogen-based, lithoautotrophic microbial activity. Both cultivation and cultivation-independent molecular analyses suggested the predominance of *Methanococcales* members in the superheated hydrothermal emissions and chimney interiors along with the other major microbial components of *Thermococcales* members. These results imply that a HyperSLiME, consisting of methanogens and fermenters, occurs in this tectonically active subsurface zone, strongly supporting the existence of hydrogen-driven subsurface microbial communities.

**Keywords** Central Indian Ridge · Deep-sea hydrothermal vent · Hydrogen-oxidizing · Hyperthermophilic · Methanogen · Subsurface

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## Introduction

Recently, there has been increasing interest in the subsurface biosphere on Earth as a potential analogous habitat for extraterrestrial life in our own solar system, where liquid water and hydrolithologically provided energy and carbon sources may be more likely present in the subsurface (Stevens and McKinley 1995; Fisk and Giovannoni 1999; McCollom 1999). Based on terrestrial biology, this is not an unreasonable expectation; on Earth, chemolithoautotrophic, even methanotrophic and carboxydotrophic microorganisms, can gain energy from a variety of reduced inorganic compounds coupling with electron acceptors such as molecular oxygen, nitrate, metal oxides, sulfate, sulfur, carbon dioxide, or water. On modern Earth, these energy sources are readily generated by biological processes, and many of them can also be supplied from the Earth's interior directly associated with magmatism in volcanic and hydrothermal fields in tectonic margins and hot spots. In contrast to electron donors, the production of significant quantities of many electron-acceptor species such as nitrate, metal oxides, and sulfate ultimately requires the involvement of molecular oxygen, and is thus intimately

linked to past or present photosynthesis on Earth. Thus, the finding of microbial ecosystems based on chemolithoautotrophic primary producers utilizing photosynthesis-independent energy generation and carbon sources would have implications, not only for understanding early Earth ecosystems prior to emerging photosynthetic life, but as models or potential analogs for geochemically active planets or moons.

Such a system was first reported by Stevens and McKinley (1995) in the deep crystalline rock aquifers of the Columbia River Basalt (CRB) Group. The authors proposed that this system was driven by hydrogen generated from the interaction of basaltic rocks with anaerobic water (Stevens and McKinley 1995). However, Anderson et al. (1998) argued against the hydrogen-based microbial ecosystems [subsurface lithoautotrophic microbial ecosystem (SLiME) hypothesis] in the deep CRB aquifers proposed, citing a lack of evidence supporting the production of hydrogen by the experiments of the basalt-groundwater interactions as proposed by Stevens and McKinley (1995). Subsequently, Chapelle et al. (2002) reported the detection of *Archaea*-dominating microbial communities in the groundwater system beneath the Lidy Hot Springs in Idaho by means of a combination of molecular techniques and concluded that the microbial communities were derived from a hydrogen-based SLiME dominated by methanogens. While it was demonstrated that archaeal rRNA genes (rDNAs) were indeed present, there was no evidence presented that the predominant types were methane producers. However, there was no cultivation or molecular probing to show abundance of such organisms; there was no isotopic analysis of the methane to suggest a biological origin; and there was no discussion of how a system with nanomolar levels of $H_2$ could be producing millimolar amounts of $CH_4$ (Chapelle et al. 2002).

Perhaps a more promising site for a hydrogen-driven SLiME might be located beneath the active deep-sea hydrothermal seafloor in the Mid-Oceanic Ridge (MOR) spreading centers. These environments are located in sediment-poor areas, have very little input of organic carbon, and have the opportunity for production of abundant molecular hydrogen and carbon dioxide provided directly from degassing of magma or reaction between water and superheated rock (Von Damm 1995). Possible occurrence of microbial communities beneath active deep-sea hydrothermal vent systems (subvent biosphere) has been proposed on the basis of observations of microbial expulsion of hyperthermophiles immediately after submarine volcanic eruptions (Delaney et al. 1998; Summit and Baross 1998) and of the distribution profile of biomolecules in the deep-sea hydrothermal vent environment (Straube et al. 1990; Deming and Baross 1993). Direct measurements of the indigenous microbial communities in the superheated hydrothermal emissions and inside the sulfide chimneys (Baross et al. 1982; Takai and Horikoshi 1999; Takai et al. 2001a; Takai and Fujiwara 2002; Schrenk et al. 2003) and the potential subsurface microbial communities in the core samples penetrating beneath the hydrothermal seafloor (Kimura et al. 2003) have also supported the presence of a subvent biosphere. Most of the microbial components determined so far are hyperthermophilic chemolithoorganotrophs, obtaining energy from organic compounds probably provided from chemosynthetic microbial and animal communities utilizing photosynthesis-derived electron acceptors (molecular oxygen, nitrate, and sulfate) in the relatively surface zone of habitats. Superheated hydrothermal emissions, with minimal dilution by seawater, probably entrain the microbial components indigenous to the subsurface hydrothermal water–rock interface in a temperature range permitting microbial growth (up to around 120°C). In such systems, the input of photosynthetically derived energy and carbon sources will be negligible, and molecular hydrogen and carbon dioxide from the Earth's interior should be primary energy and carbon sources. However, previous studies of undiluted, superheated hydrothermal emissions have revealed limited information about the subvent biosphere because of the paucity of microbial cells and microbial products (Takai and Fujiwara 2002; Takai et al. 2003a), almost certainly due to the very high temperature ($>300$°C) inhibiting growth and survival of any entrained microbes.

In this study, we sought to investigate the microbial components entrained by the superheated hydrothermal emissions minimally mixing with seawater from indigenous subvent microbial ecosystem, by means of the fabrication of a microbial habitat called an in situ colonization system (ISCS) (Takai et al. 2003a). In a deep-sea hydrothermal field in the Central Indian Ridge (CIR), we analyzed the geochemical and isotopic properties of the hydrothermal fluids, and deployed the ISCS at several sites. The microbial components trapped in the ISCS devices and present in microbial habitats, such as sulfide chimney structures and hydrothermal fluids, were characterized by quantitative cultivation and culture-independent molecular techniques. The geochemical and microbiological results suggested the possible existence of a hydrogen-based, hyperthermophilic subvent chemolithoautotrophic ecosystem (HyperSLiME) beneath the deep-sea hydrothermal system.

## Materials and methods

### Sampling of hydrothermal emissions and chimneys and deployment of ISCS

The target deep-sea hydrothermal system in this study was the Kairei hydrothermal field in the CIR. The Kairei hydrothermal field (25°19.23′S, 70°02.42′E, water depth 2,415–2,460 m) was the first deep-sea hydrothermal vent system discovered in the Indian Ocean (Hashimoto et al. 2001), and the discovery of the second one, the Edmond field (23°52.68′S, 69°35.80′E, water depth 3,290–3,320 m), followed (Van Dover et al. 2001). Hydrothermal fluids and chimney

structures were obtained by means of the manned submersible *Shinkai 6500* in the cruise by "R/V Yokosuka" held in January–March 2002 (JAMSTEC YK01-15) and were obtained from two different chimney sites named the "Kali chimney" (KC) (25°19.2194′S, 70°02.3850′E, 2,451 m) and the "Fugen chimney" (FC) (25°19.2165′S, 70°02.4445′E, 2,422 m). The in situ temperature of the effluent black smoker emissions was measured by a self-recording thermometer (Table 1), and the vent emissions were collected by using a gas-tight fluid sampler water hydrothermal-fluid *Atsuryoku* tight sampler (WHATS) (Tsunogai et al. 2002a, b). After the recovery of the WHATS onboard, the collected fluids in gas-tight bottles (150 ml) were immediately opened to a vacuum line (ca. 1,500 ml) at the onboard laboratory to recover any gaseous components. For the degassing, we added $HOSO_2NH_2$ and $HgCl_2$ to the fluid in the vacuum line so as to decrease the pH of the fluid to promote degassing of $CO_2$ and precipitate HgS to prevent degassing of $H_2S$ from fluid. After the degassing, approximately 50 $cm^3$ of the gas phase and all of the filtered liquid phase were subsampled for the chemical and isotopic measurements at onshore laboratory.

For microbiological experiments, approximately 300 ml of the vent emission from the KC by using WHATS was filtered onboard using a 0.22-μm pore size, 25-mm-diameter cellulose acetate filters (Millipore) and the filter was stored at −80°C prior to DNA extraction. Another 25 ml of the Kali black smoker was fixed by formaldehyde at a final concentration of 3% (w/v), and the other 25 ml was anaerobically stored in the vial under 100% $N_2$ (200 kPa), sealed with butyl rubber stoppers for cultivation test. The chimney structures were successfully collected only from the FC site. The chimney was immediately divided onboard into two sections (surface layer of the chimney and vent orifice surface) as described by Takai et al. (2001a). Each of the subsamples was stored for the subsequent nucleic acid extraction (−80°C), microscope observation [in 30 ml MJ synthetic seawater (Sako et al. 1996) containing 0.05% (w/v) sodium sulfide and 3% (w/v) formaldehyde at 4°C] and cultivation of microorganisms [in 30 ml MJ synthetic seawater containing 0.05% (w/v) sodium sulfide at 4°C].

The characteristics of the ISCS device were described previously (Takai et al. 2003a). An ISCS was deployed in the hydrothermal conduit of a 365°C vent emission in the KC for 7 days and three ISCSs were settled at the vent passage of an approximately 250°C black smoker (3 days) at a site just adjacent to the smoker (3 days) and at a 2-m distance from the vent orifice (11 days) at the FC site. Unfortunately, the temperature data of the ISCSs during the deployment were not successfully retrieved because of instrumental failure. However, the short-term temperature shift (for 10–30 min) and the mean temperature were determined by another self-recording thermometer. The ISCS was retrieved from the deployed site and brought back to the ship in the cassette open to the seawater. This implies that the ISCS was exposed to oxygen for at least a couple of hours during the returning of the submersible to the surface. In addition, the seawater in the cassette was mixed with surface seawater during the submersible recovery to the ship. An ISCS incubated with surface seawater for 2 days was used as a control for the potential contaminated ISCS. Immediately after the recovery of the ISCS from the submersible, the substratum of the ISCS was stored in the same manner as the chimney subsamples. The frozen portions of the chimneys and the altered substratum of the ISCS were briefly characterized by using scanning electron microcopy and energy-dispersive X-ray spectroscopy (SEM-EDS).

## Geochemical analyses

Concentration and stable carbon isotopic composition of $CO_2$, CO, and $CH_4$ in degassed gaseous phases sample were determined by using isotope-ratio monitoring GC/MS as previously described (Tsunogai et al. 2002a, b). Concentrations of hydrogen in gaseous phase samples were determined using a gas chromatograph with a TRD detector (Seiler et al. 1980). The major chemical compositions in liquid phase samples were determined by inductively coupled plasma (ICP) emission spectrophotometry.

## Microbiological characterization

Using formalin-fixed subsamples, total microbial cell density and total autofluorescent cell density (F420-dependent autofluorescent methanogens) were determined with or without staining with 4′,6-diamidino-2-phenylindole (DAPI) as described previously (Takai and Horikoshi 1999, 2000a, b; Takai et al. 2001a). The microbial rDNA community structure and the proportion of the archaeal rDNA population in the whole microbial DNA assemblages obtained from the frozen subsamples were determined. Microbial DNA was directly extracted using a Soil DNA Kit Mega Prep (MO BIO Laboratories, Solana Beach, Calif., USA), following the manufacturer's suggested protocol. A blank tube (with no sample added) was processed as a negative control (Tanner et al. 1998). Quantification of the archaeal rDNA population in the whole microbial DNA assemblages was performed by a quantitative fluorescent PCR method as previously described (Takai and Horikoshi 2000a, b). Amplification of archaeal and bacterial rDNA, determination of the sequences, and sequence-similarity analysis were conducted in the same manner as described previously (Takai et al. 2001b, 2003c).

An MPN cultivation test was performed twice onboard and later onshore in the laboratory. The MPN cultivation test and subsequent cultivation and isolation of the microorganisms were conducted using the media and under the following conditions: for hyperthermophilic fermentative sulfur-reducing heterotrophs, MJYPS medium (Takai et al. 2000) was used, and

**Table 1** Description and microbiological characteristics of each of the samples. *FISH* Fluorescence in situ hybridization

| Sample ID | Description | Mean temperature (°C) | Microbial community density (cells $g^{-1}$ or $ml^{-1}$) | Strains obtained from MPN cultivation test in the medium | | | Proportion of rDNA for | | UV-excited autofluorescent cell counts (cells $g^{-1}$) | Proportion of *Methanococcales* by FISH analysis (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | MJYPS | MMJ | MMJHS | *Bacteria* (%) | *Archaea* (%) | | |
| K365-VE | 365°C of vent emission at the KC[a] site | 365 ± 0.1 | $2.3 \pm 0.6 \times 10^3$ | No strain | No strain | No strain | No rDNA | No rDNA | $< 10^{-1}$ | < 0.01 |
| K365-ISCS | Substratum of ISCS[b] deployed in vent emission at the KC site for 7 days | 365 ± 0.1 | $8.1 \pm 1.7 \times 10^6$ | Tc-365-95<br>Tc-365-85<br>Tc-365-70 | Mc-365-85<br>Mc-365-70 | Aq-365-85-$NO_3$<br>Aq-365-85-1%$O_2$<br>Aq-365-70-1%$O_2$<br>Ep-365-55-1%$O_2$ | 0.8 | 99.2 | $5.0 \pm 1.2 \times 10^6$ | 76.5 ± 9.5 |
| F1-ISCS | Substratum of ISCS deployed in vent emission at the FC[c] site for 3 days | 250 ± 34.2 | $1.5 \pm 0.4 \times 10^7$ | Tc-1-95<br>Tc-1-85<br>Tc-1-70 | Mc-1-85<br>Mc-1-70 | Aq-1-85-$NO_3$<br>Aq-1-85-1%$O_2$<br>Aq-1-70-1%$O_2$<br>Ep-1-55-1%$O_2$<br>Ep-1-37-1%$O_2$<br>Ep-1-37-10%$O_2$ | 44.3 | 55.7 | $1.0 \pm 0.4 \times 10^7$ | 72.1 ± 8.7 |
| F2-ISCS | Substratum of ISCS deployed adjacent to vent emission at the FC site for 3 days | 38.2 ± 28.8 | $6.1 \pm 1.8 \times 10^6$ | Tc-2-95<br>Tc-2-85<br>Tc-2-70 | Mc-2-85<br>Mc-2-70 | Ep-2-37-1%$O_2$<br>Ep-2-37-10%$O_2$ | 99.5 | 0.55 | $1.5 \pm 0.6 \times 10^4$ | 2.8 ± 0.9 |
| F4-ISCS | Substratum of ISCS deployed at 2 m away from vent emission at the FC site for 11 days | 2.1 ± 0.1 | $1.0 \pm 0.3 \times 10^6$ | Tc-4-85<br>Tc-4-70 | No strain | No strain | 98.7 | 1.3 | $1.2 \pm 0.4 \times 10^3$ | 0.7 ± 0.2 |
| FC-I | Subsample obtained from the interface part of the FC structure with vent emission | ND[d] | $1.0 \pm 0.2 \times 10^5$ | Tc-I-85<br>Tc-I-70 | Mc-I-85<br>Mc-I-70 | Aq-I-70-1%$O_2$<br>Ep-I-55-1%$O_2$ | 0.04 | 99.9 | ND | ND |
| FC-S | Subsample obtained from the surface layer (1–3 mm) of the FC structure | ND | $8.9 \pm 3.1 \times 10^7$ | Tc-S-85<br>Tc-S-70 | Mc-S-85<br>Mc-S-70 | Aq-S-70-1%$O_2$<br>Ep-S-37-1%$O_2$ | 92.2 | 7.8 | $4.6 \pm 1.3 \times 10^6$ | 3.3 ± 0.8 |
| SSW-ISCS | Substratum of ISCS deployed in surface seawater for 2 days | ND | $3.0 \pm 0.5 \times 10^4$ | No strain | No strain | No strain | 93.8 | 6.2 | $< 10^{-1}$ | ND |

[a]Kali chimney
[b]In situ colonization system
[c]Fugen chimney
[d]Not determined

cultivation was performed at 70, 85, and 95°C; for mesophilic to hyperthermophilic methanogens, MMJ medium (Takai et al. 2002) was used, and cultivation was performed at 25, 37, 55, 70, 85, and 95°C; for thermophilic to hyperthermophilic sulfate-reducers, MJYP medium (Takai et al. 1999) supplemented with 2 mM of acetate, lactate, pyruvate and tartrate was used, and cultivation was performed at 55, 70, 85, and 95°C; for mesophilic to hyperthermophilic hydrogen or sulfur oxidizers, MMJHS medium (Takai et al. 2003a) was used, and cultivation was performed at 25, 37, 55, 70, 85, and 95°C. The three-tube MPN method was employed to assess the viable population size of the targeted members of the microorganisms. The microorganism present in the most diluted series of the medium at each of temperatures was isolated by the subsequent extinction-dilution method. The partial sequences of the 16S rDNA (approximately 900 bp) were determined and subjected to sequence-similarity analysis.

Ribosomal RNA-targeted oligonucleotide probes specifically binding to 16S rRNA of *Methanococcales* were designed based on the multiple alignment of archaeal rDNA sequences representing various archaeal phylotypes. The probe sequences were analyzed using the CHECK-PROBE analysis function from the Ribosomal Data Project II (RDP-II) and the gapped-BLAST search algorithm to confirm the specificity of the probe to the rDNA sequences of the new isolate. Finally, the MC1188 probe (5′-ATGCGGACCTRTCGTTGC-3′), which corresponded to positions 1188–1205 in *Escherichia coli* 16S rDNA, was chosen. Based on in silico analysis, the MC1188 probe was found to bind specifically to the rRNA sequences of *Methanococcales* members and to have at least three bases of mismatch with any other archaeal rDNA sequences. In addition, dot-hybridization analysis (Takai and Horikoshi 2000a, b) was carried out with representative archaeal rDNA clones obtained in this study. These experiments supported the specificity of the MC1188 probe to *Methanococcales* rDNA sequences.

For the whole-cell hybridization experiment, the fixed microbial particles were concentrated by filtration or centrifugation and were immobilized on 3-aminopropyltriepoxysilane-coated slides. Hybridization was performed at 45°C for 5 h in hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.4], 0.1 wt% SDS, Denhardt's solution) containing 30% formamide and the FITC-labeled MC1188 probe (5 μg $ml^{-1}$). After hybridization, the slides were washed at 44°C for 20 min with hybridization buffer containing 50% formamide. The fluorescence signal from the MC1188 probe was intensified using an Alexa Fluor 488 signal-amplification kit for fluorescein-conjugated probes (Molecular Probes) following the manufacturer's instructions. Finally, the slides were stained with DDW containing DAPI (10 μg $ml^{-1}$) at 4°C for 20 min and were examined under epifluorescence using the Olympus BX51 microscope with the SPOT RT Slider CCD camera system (Diagnostic Instruments).

Two types of negative control experiments were employed using identical cell preparations and hybridization conditions. One was conducted in which the unlabeled MC1188 probe was added in 50-fold excess (250 μg $ml^{-1}$) of the FITC-labeled MC1188 probe added at the standard concentration (5 μg $ml^{-1}$). The other experiment was performed using an FITC-labeled negative (non-sense) probe having the identical length and base composition as the probe. No microbial cell with clear Alexa Fluor 488-derived fluorescence signal was obtained, and the standard for manually identifying the positive and negative hybridizations was established from both negative control experiments. Three slides from each sample were prepared, and microbial cells in at least $5\times10^{-7}$ $m^2$ on each slide were manually counted. Finally, an average of the ratio of probe-hybridized cells to the DAPI-stained cells was determined from three slides.

### Phylogenetic analyses

Single-strand sequences of approximately 400 nucleotides in length were analyzed from each of the clones in the archaeal or bacterial rDNA clone libraries from various subsamples. The rDNA sequences having ≥97% similarity by FASTA were assigned to the same clone type. A representative sequence of each clone type was subjected to sequence-similarity analysis against the prokaryotic SSU rRNA database and the non-redundant nucleotide sequence databases of GenBank, EMBL, and DDBJ using the gapped-BLAST. Approximately 0.9 kb of sequence of each representative rDNA clone was determined from both strand. The sequences were manually aligned to prokaryotic SSU rDNA data from the RDP-II (Maidak et al. 2000) and other databases based on primary and secondary structure considerations. Phylogenetic analyses were restricted to nucleotide positions that could be unambiguously aligned. Evolutionary distance matrix analysis (using the Kimura two-parameter method, the least square distance method, and transition/transversion rate of 2.0) and neighbor-joining analysis were performed using the PHYLIP package (version 3.5; obtained from J. Felsenstein, University of Washington, Seattle). Bootstrap analysis was used to provide confidence estimates for phylogenetic tree topologies.

## Results and discussion

### Environmental settings

The Kairei hydrothermal field in the CIR was the first deep-sea hydrothermal system discovered in the Indian Ocean (Hashimoto et al. 2001). In the light of the tectonic and geological settings, the CIR is a intermediate-rate spreading MOR (DeMets et al. 1988) and the hydrothermal fields discovered in the CIR are expected to have intermediate features between those in the fast-spreading and slowly spreading MOR like the East Pacific Rise (EPR) and the Mid-Atlantic Ridge (MAR), respectively.

**Table 2** Chemical and isotopic compositions of hydrothermal fluids

| | Mg (mM) | $CO_2$ (mM) | $\delta^{13}C(CO_2)$ (‰ PDB) | $H_2$ (mM) | $CH_4$ (μM) | $\delta^{13}C(CH_4)$ (‰ PDB) | CO (μM) | $\delta^{13}C(CO)$ (‰ PDB) |
|---|---|---|---|---|---|---|---|---|
| K365-VE | 0.2 | 7.8 | −6.1 | 2.5 | 84 | −13.8 | 0.5 | −41.6 |
| F250-VE | 0.5 | 8.0 | −6.0 | 2.5 | 199 | −18.5 | 0.7 | −38.2 |
| Ambient seawater | 53 | 2.3 | −1.0 | ND[a] | 0.001 | −35 | ND | ND |
| Analytical error (±) | 1 | 0.01 | 0.1 | 0.01 | 0.01 | 0.4 | 0.01 | 1 |

[a]Not detected

These CIR hydrothermal systems have been considered to be members of a new ecosystem with macrofaunal components that are biogeographically specific to these systems (Hashimoto et al. 2001; Van Dover et al. 2001), and microbial fauna that had been not fully determined (Van Dover et al. 2001; Takai et al. 2003a). Our studies focused on two sites within the Kairei field: a vigorous black smoker called the KC site, spouting from large hole (a diameter of approximately 50–100 cm) with a temperature maximum of 365°C, and a slow black smoker called the FC site, with a temperature maximum of approximately 250°C (Table 1). Chimney structures and vent emissions were collected by means of the manned submersible *Shinkai 6500*. In addition, ISCS devices were deployed into the hydrothermal conduit of the KC for 7 days, the vent passage of the FC (3 days), at the site adjacent to the FC (3 days), and at a 2-m distance from the FC (11 days). The short-term temperature shifts (for 10–30 min) at the ISCS deployment sites were determined by a self-recording thermometer, and the temperatures of ISCS devices deployed in the hydrothermal emissions were found to be stable (Table 1).

## Geochemical analyses

The chemical and isotopic compositions in the vent emissions collected from the KC and FC by means of a gas-tight fluid sampler WHATS (Tsunogai et al. 2002a, b) were determined and summarized in Table 2. As demonstrated by the Mg concentrations (0.2 and 0.5 mM in the K365-VE and F250-VE, respectively), the hydrothermal fluids from both the KC and FC were practically pure hydrothermal fluids with minimal contamination of the ambient seawater during the sampling (theoretically, none of Mg is present in the pure hydrothermal fluids). Major chemical compositions of the hydrothermal fluids including $\delta^{13}C$ compositions of dissolved $CO_2$ were almost the identical between two chimney vent emissions (K365-VE from KC and F250-VE from FC site), and the values corresponded to those previously described (Gamo et al. 2001) (Table 2). Dissolved $CH_4$, on the other hand, showed different concentrations (0.085 and 0.199 mM in K365-VE and F250-VE, respectively) and different $\delta^{13}C(CH_4)$ values (−13.8 and −18.5‰ PDB in K365-VE and F250-VE, respectively). The disparity was inconsistent with the conclusion that the two different chimney sites have the same origin of hydrothermal fluid based on characterization of major chemical composition in the previous (Gamo et al. 2001) and this study (data not shown).

The disparity of the concentration and isotopic compositions of methane could not be explained by any of the following processes: (1) incorporation of $^{13}C$-depleted methane in seawater into F250-VE due to subsurface mixing of seawater (in such a case, methane concentration should be lower in F250-VE than in K365-VE), (2) subsurface mixing of seawater into K365-VE and subsequent $^{13}C$-enrichment by kinetic isotope effect of microbial methane oxidizing activity (in this case, the Mg concentration in K365-VE should be much higher than in F250-VE), and (3) abiotic reaction between $CO_2$ and $H_2$ in a hydrothermal fluid (Horita and Berndt 1999) (this reaction should result in much higher concentrations of intermediate products such as CO).

Another possibility is that the differences in dissolved $CH_4$ in two hydrothermal emissions might be explained by contributions from subsurface microbial methanogenesis. House et al. (2003) reported carbon isotopic fractionation of approximately 17–30‰ between $CO_2$ and $CH_4$ during the growth with $H_2$ and $CO_2$ as the sole energy and carbon sources for most of the hyperthermophilic methanogens within *Methanopyrales* and *Methanococcales*. According to the experimental carbon isotopic fractionation from $CO_2$ to $CH_4$ by hydrogen-oxidizing hyperthermophilic methanogens, we can anticipate $\delta^{13}C(CH_4) = -22$ to −36‰ PDB for biogenic $CH_4$ in F250-VE from $CO_2$ having $\delta^{13}C(CO_2) = -6$‰ PDB (Table 2). If we assume that the amount of $CH_4$ (0.115 mM) added in F250-VE from K365-VE is contributed by hyperthermophilic methanogens and that $\delta^{13}C(CH_4)$ of the whole methane (0.199 mM) in F250-VE finally represents −18.5‰ PDB, the carbon isotopic fractionation of the potential subsurface microbial methane beneath the FC site is calculated to be ca. −22‰ PDB, which is consistent with the $\delta^{13}C$ values of the biogenic methane produced from $CO_2$ having $\delta^{13}C(CO_2) = -6$‰ PDB. In addition, extremely high abundance of molecular hydrogen in the superheated vent emissions may serve as the primary energy source for the microbial methanogenesis coupling with carbon dioxide in the emissions (Table 2). These geochemical properties are most easily explained via the input of biogenic methane from hyperthermophilic methanogens in the subsurface environment of the FC.

Even for the KC emitting relatively $^{13}C$-enriched methane, subsurface contribution of microbial methanogenesis seems likely. Prior to this study, by using same sampling and analytical methods, we determined $\delta^{13}C(CH_4)$ value in >300°C of black smoker hydrothermal emissions at the Suiyo Seamount hydrothermal field. All the hydrothermal emissions had homogeneous

$\delta^{13}C(CH_4)$ values to be −6‰ PDB, while all the $\delta^{13}C(CO_2)$ were 0‰ PDB (Tsunogai et al. 1994, 2000). In the deep-sea hydrothermal vent system at the Suiyo Seamount field, several investigations indicated that microbial communities might be limited in the surface habitats of the chimney structures, and that they might be deficient in the number of detectable methanogens due to relatively higher partial $O_2$ pressure in the hydrothermal fluids (Nakagawa et al. 2003, 2004; Takai et al. 2003b, d). The $\delta^{13}C(CH_4)$ values of the hydrothermal emissions obtained from the Suiyo Seamount field appear to completely exclude the potential contribution of the subsurface microbial methanogenesis, and thus, to represent the fractionation value of pure magmatic methane (Tsunogai et al. 1994, 2000). Thus, it may be possible that the $\delta^{13}C(CH_4)$ value of the hydrothermal emission from the KC, and even the values previously reported from other sediment-starved deep-sea hydrothermal systems in the EPR (Shanks 2001) and from the MAR other than the TAG site (Charlou et al. 2002), are accepted to be highly $^{13}C$-depleted as compared to the values from the Suiyo Seamount field.

## Microbiological characterization

The microbial population densities for each of the ISCS samplers were determined, indicating that both successfully gathered the microbial populations in the superheated vent emissions. The microbial population density in the 365°C vent emission of the KC was $2.3 \pm 0.6 \times 10^3$ cells $ml^{-1}$, while the ISCS in the vent emission had $8.1 \pm 1.7 \times 10^6$ cells $g^{-1}$ wet weight (Table 1). Although the population density of the Fugen black smoker was not determined because of the lack of sufficient sample, the microbial cell density was expected to be similar to or slightly higher ($10^3$–$10^4$ cells $ml^{-1}$) than that in the Kali smoker due to a slightly lower temperature of the vent emission. In comparison, the ISCS in the FC vent orifice had a high microbial population density (Table 1). The microbial population densities in the ISCSs deployed at different microhabitats in the Fugen chimney site were shifted from $1.5 \pm 0.4 \times 10^7$ to $1.0 \pm 0.3 \times 10^6$ cells $g^{-1}$ wet weight with increasing distance from the vent emission site (or with decreasing temperature of the habitats) as shown in Table 1. The interface zone between vent fluids and chimney (FC-I) contained $1.0 \pm 0.2 \times 10^5$ cells $g^{-1}$ wet weight, while the chimney surface layer (FC-S) had a higher population density (Table 1). This distribution profile was quite similar with that previously observed in the black smoker chimney structure obtained from the Manus Basin (Takai et al. 2001a), clearly indicating that the considerable high microbial cell counts in the ISCSs deployed in the superheated vent emissions were not contaminants from the inside structures of the chimneys (Table 1).

As a supplement to the cultivation-independent population analysis, we attempted to obtain an estimate of the population density by means of the MPN cultivation technique. On the basis of the results from recent culture-dependent and -independent microbiological surveys (Takai and Horikoshi 1999, 2000a, b; Takai et al. 1999, 2000, 2001a, 2002, 2003a, b, d; Nakagawa et al. 2003, 2004), we designed the media and the conditions culturing hyperthermophilic fermentative sulfur-reducing heterotrophs (members of the orders *Thermococcales* and *Thermotogales*), mesophilic to hyperthermophilic methanogens, thermophilic to hyperthermophilic sulfate-reducers, and mesophilic to hyperthermophilic hydrogen- or sulfur-oxidizers. The microorganisms growing in the most diluted series of various media under defined conditions were subsequently isolated by the extinction-to-dilution method (Takai and Horikoshi 2000a, b), and then identified by partial rDNA sequence analysis (Takai et al. 2001a). From the 365°C vent emission in the KC, no viable microorganisms were cultivated with any of the media under various conditions (Fig. 1a). In contrast, several different microorganisms were cultivated from the ISCSs. Members of the *Thermococcales* were the most frequently cultivated from all the ISCSs and the chimney samples, and their population size represented 4.0–7.5% of the total cell counts in the superheated ISCSs of K365-ISCS and F1-ISCS (Fig. 1a). The second most frequently cultivated group (one to two orders of magnitude lower abundance) was thermophilic to hyperthermophilic *Methanococcales* members in K365-ISCS, F1-ISCS, F2-ISCS, FC-I and FC-S. The proportion of these *Archaea* in the total population was reduced with increasing distance from the vent emission and decreasing temperature of the habitats. The viable population sizes of *Thermococcales* and *Methanococcales* in FC-I were two or three orders magnitude lower than those in the superheated ISCSs, further excluding the potential contamination from the habitats in the chimney structures (Fig. 1a). Both *Aquificales* and *ε-Proteobacteria* members were also cultivated in low numbers, while no sulfate-reducers were obtained from the MPN cultivation test.

All in all, the cultivation MPN methods yield results similar to those obtained in other environments; only a few percent of the population was sampled via cultivation. However, the most remarkable fact is that any organisms at all are found surviving in the ISCS samples from these high temperatures. Since the ISCS is exposed to ambient seawater and ambient oxygen on the return to the surface at least for a few hours, the possibility of some organisms, specifically oxygen-sensitive methanogens, being killed by the oxic conditions could be likely.

In contrast to the cultivation-dependent methods, cultivation-independent molecular methods revealed many details concerning the populations in vent fluid and the ISCSs. From 300 ml of 365°C vent emission of the KC, no evidence of archaeal or bacterial rDNA was obtained. In contrast, abundant archaeal signatures were retrieved from the ISCS deployed in the Kali hydrothermal conduit and the FC interior. The phylogenetic analysis indicated that all of the archaeal rDNA clones obtained from the Kali vent ISCS and the most interior

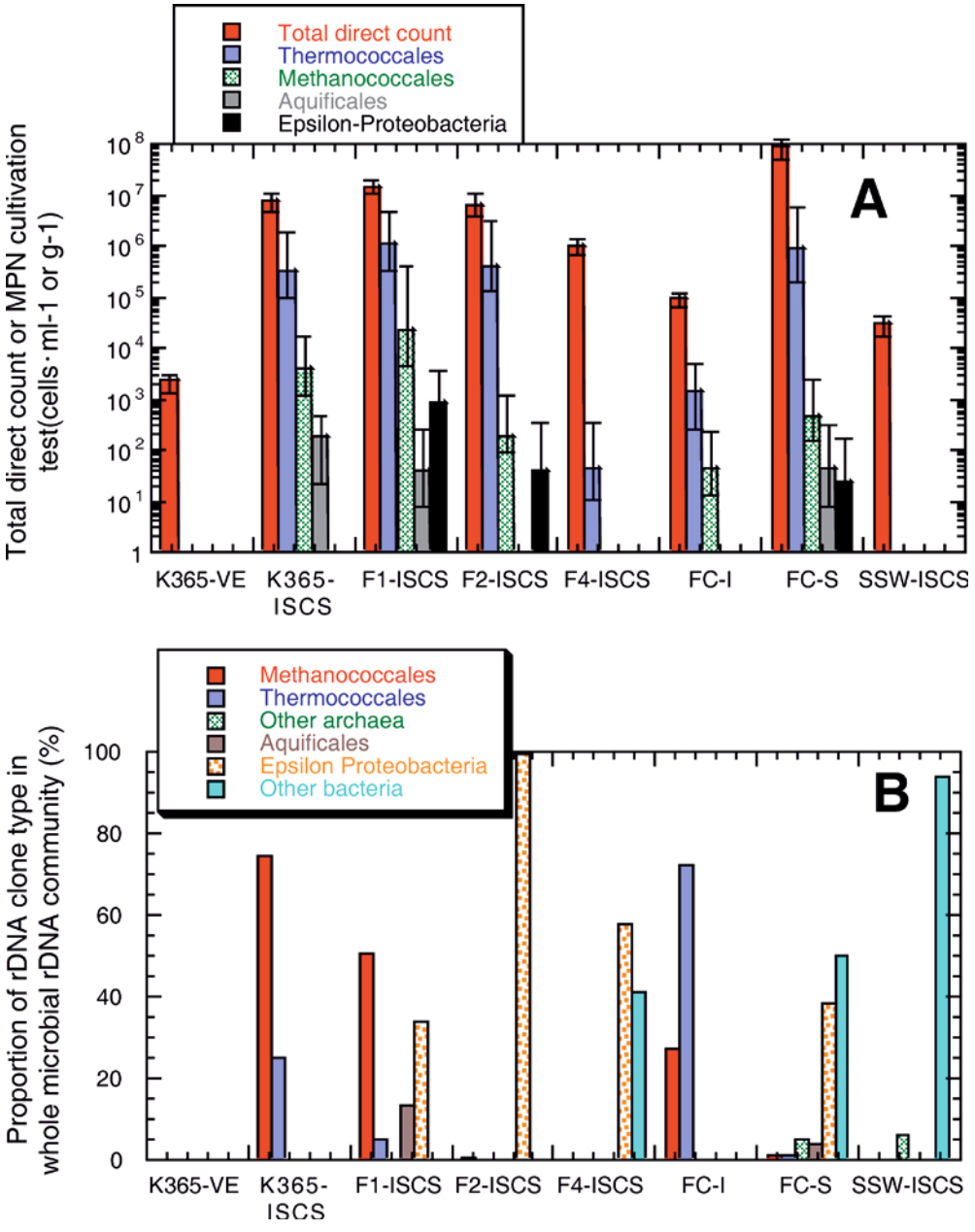



**Fig. 1a, b** Microbial population density determined by 4′,6-diamidino-2-phenylindole-staining direct cell count and the three-tube MPN test (**a**) and proportion of rDNA clone type in whole microbial rDNA community (**b**). The viable population size of *Thermococcales*, *Methanococcales*, *Aquificales*, or *ϵ-Proteobacteria* was represented by value obtained from the three-tube MPN cultivation at a temperatures of 70, 70, 70 or 37°C. The proportion of the rDNA clone type in the whole microbial rDNA community was calculated as follows: (1) the proportion of rDNA clones phylogenetically associated with *Thermococcales*, *Methanococcales*, or other archaeal phylotypes was determined in the archaeal rDNA clone library from each sample shown in Table 1; (2) similarly, the proportion of rDNA clones affiliated within *Aquificales*, *ϵ-Proteobacteria*, or other bacterial phylotypes was determined for each sample; and (3) the proportion of the rDNA clone type in the archaeal or bacterial rDNA clone library was multiplied with the proportion of archaeal or bacterial rDNA determined by quantitative PCR shown in Table 3. *Bar* indicates a range giving 95% confidence

part of the FC were closely related with *Methanococcales* and *Thermococcales* (Fig. 2; Table 3). On the basis of rDNA clone analysis and quantitative PCR calculations (Takai and Horikoshi 2000a, b; Tables 1, 3), it was estimated that almost 70% of the clones from K365-ISCS were phylogenetically associated with hyperthermophilic *Methanococcales* members, while 70% of the rDNA clones from FC-I were related to *Thermococcales* (Fig. 1b). In contrast, from the ISCSs deployed at different sites of the FC, both bacterial and archaeal rDNAs were successfully recovered. The proportion of archaeal rDNA in the whole microbial rDNA community apparently decreased with increasing distance from the vent emission or with decreasing temperature of the habitat (Fig. 1b). The predominant phylotypes in the rDNA community structures of the ISCSs deployed at different sites of the FC also shifted from anaerobic hyperthermophilic archaeal phylotypes to mixed communities of thermophilic to mesophilic, anaerobic to aerobic *Archaea* and *Bacteria* potentially having a variety of metabolic features (Fig. 2). From the surface layer of the FC, a diversity of bacterial and archaeal rDNA phylotypes was detected (Fig. 2). The structures of both bacterial and archaeal rDNA communities were significantly different from those in the superheated ISCSs (K365-ISCS and F1-ISCS) and the ISCSs deployed in the ambient habitats of the vent emission (F2-ISCS and F4-ISCS). These results indicated that the ISCSs deployed in the superheated vent emissions were able to gather *Methanococcales*-dominated microbial communities that

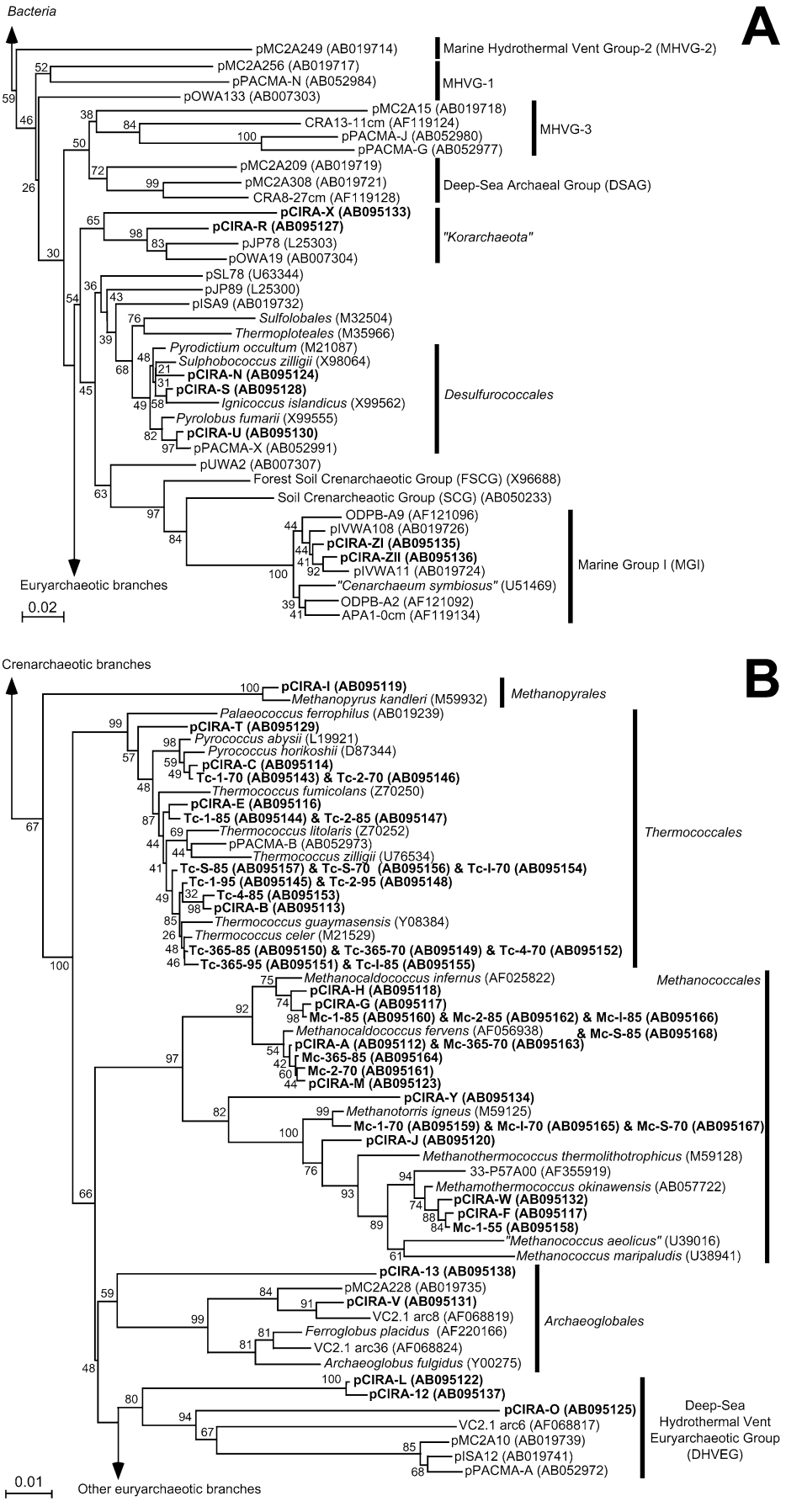
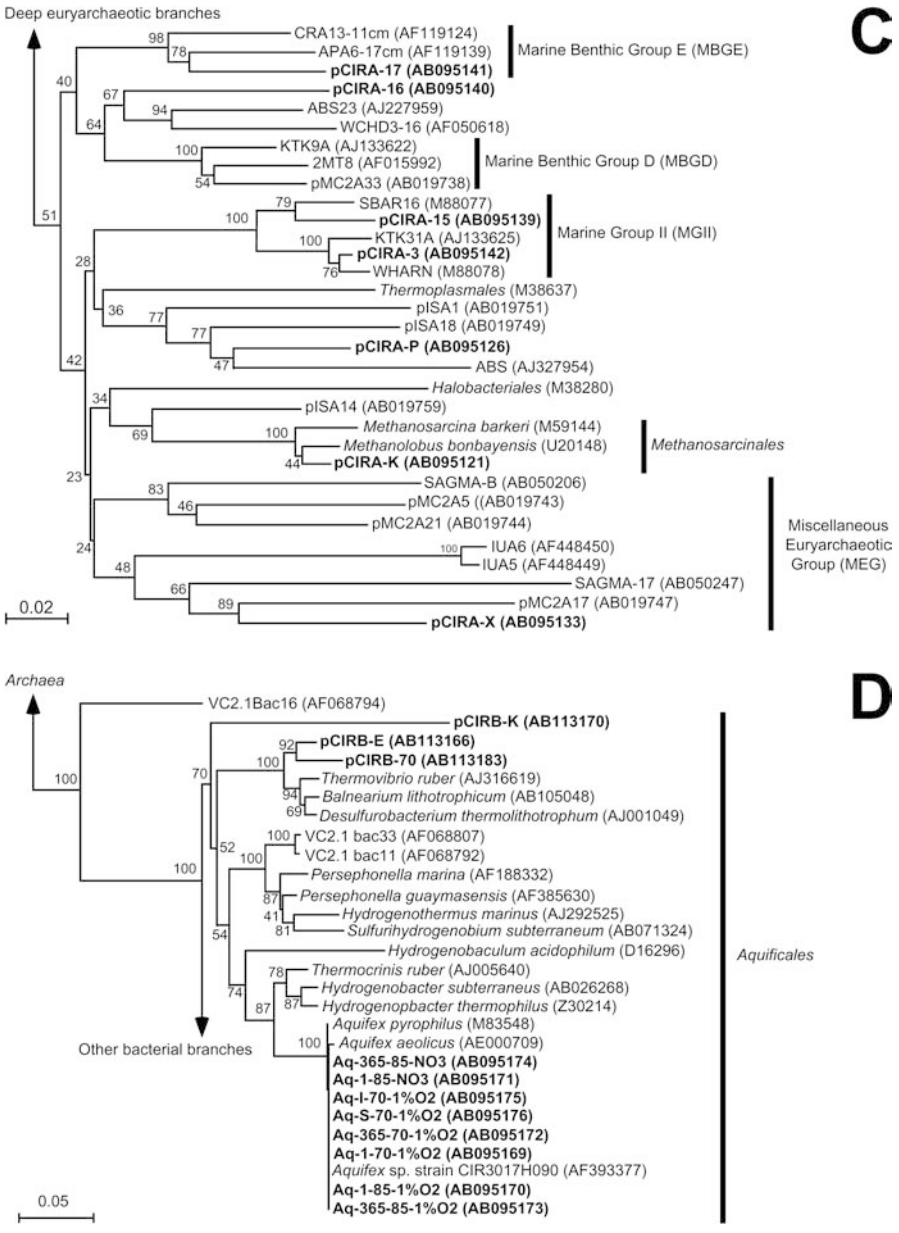



**Fig. 2a–e** Phylogenetic tree of representative 16S rRNA gene sequences of the strains and rDNA clones (*pCIR*) within the deep branches of *Archaea* and crenarchaeotic phylotypes (**a**); the deep branches of euryarchaeotic phylotypes such as *Methanopyrales*, *Thermococcales*, *Methanococcales*, and *Archaeoglobales* (**b**); the other euryarchaeotic phylotypes (**c**); the *Aquificales* (**d**); and the $\epsilon$-*Proteobacteria* (**e**). Neighbor-joining analysis was done using PHYLIP package (version 3.5). Bootstrap analysis was performed with 100 resampled data sets. *Boldface type* indicates the cultivated strains and the rDNA clones obtained in this study. Classification of the phylogenetic group of *Archaea* shown in **a**, **b**, and **c** are according to previous studies (Takai and Horikoshi 1999; Takai et al. 2001b), and the phylogenetic group and the undescribed strains of $\epsilon$-*Proteobacteria* tree (**e**) were described previously (Takai et al. 2003a)

**Fig. 2** (Continued)




were distinct from other microhabitats occurring in the deep-sea hydrothermal environment.

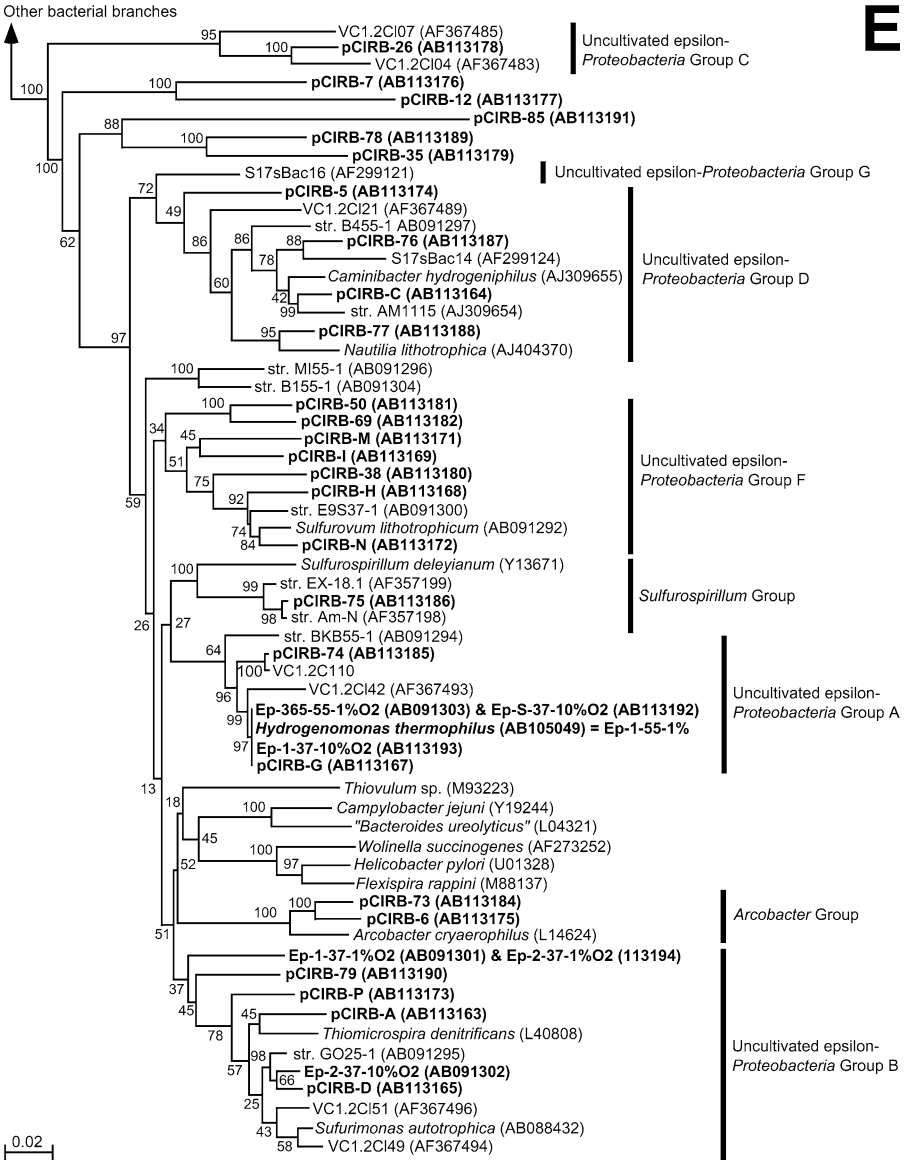
The predominance of hyperthermophilic methanogens in the microbial community of the superheated ISCSs was verified by microscope observation, using whole cell fluorescence in situ hybridization analysis (Table 1). The proportion of the cells displaying UV-excited autofluorescence and the proportion of the cells having fluorescence signals derived from a *Methanococcales*-specific oligonucleotide probe in the total cell count by using DAPI were similar (Table 1) and were also equivalent to the proportion of *Methanococcales* rDNA in the microbial rDNA community (Fig. 1b). These results based on culture-independent molecular analyses and microscope observation clearly indicated the presence of a *Methanococcales*-dominating microbial community in the hydrothermal fluids and the superheated ISCSs deployed.

## Environmental constraints of HyperSLiME

We have presented both geochemical and cultivation-independent microbiological evidence that the ISCSs acted as collectors for the microbial components probably entrained by hydrothermal fluids from a hydrogen-based HyperSLiME beneath the FC site in the Kairei field. While the cultivation-dependent methods are much less convincing, they are consistent with the conclusion reached using cultivation-independent methods. More data at more sites will be needed to make the same claim for the deep subsurface beneath the Kairei deep-sea hydrothermal field.

The Kairei field is located in the intermediate-rate-spreading CIR and has intermediate features with respect to the tectonic and geological settings between those in the fast-spreading and slowly spreading MOR like the

**Fig. 2** (Continued)




EPR and MAR, respectively (DeMets et al. 1988). The geochemical characteristics of the hydrothermal fluids also appeared to be for the most part common in other sediment-starved MOR deep-sea hydrothermal systems (Gamo et al. 2001; Hashimoto et al. 2001; Van Dover et al. 2001). However, in geochemistry of gas components in the hydrothermal fluids, the Kairei field resembles the Logatchev and Rainbow sites in the MAR. While the hydrogen concentration in the hydrothermal fluids of many MOR deep-sea hydrothermal systems is usually less than 0.5 mM (Von Damm 1995; Kelly et al. 2001), the highly enriched hydrogen in the Kairei field (2.5 mM) was the third highest value ever detected following the Rainbow (16 mM) and Logatchev (12 mM) sites (Charlou et al. 2002). In addition, the shift in carbon isotopic ratio from the $\delta^{13}C(CH_4)$ to $\delta^{13}C(CO_2)$ of the hydrothermal fluid was similar among the Kairei field ($-7.7$ – $-12.5‰$), the Logatchev ($-9.3‰$), and Rainbow ($-12.7‰$) sites (Charlou et al. 2002). Both the Logatchev and Rainbow sites are novel, deep-sea hydrothermal systems founded in the ultramafic environments hosted by the mantle rocks, and the extraordinary abundance of hydrogen might be derived from the hydrothermal serpentinization of the ultramafic rocks (Charlou et al. 2002). Although the geological setting of the CIR Kairei field should be further addressed, the highly enriched hydrogen in the hydrothermal fluids of the Kairei field may result from the contribution of ultramafic rocks containing olivine and orthopyroxene minerals in the deep subsurface hydrothermal reaction. Thus, the tectonic and geological settings potentially resulting in the abundant supply of hydrothermally produced hydrogen may be a basis of the occurrence of HyperSLiME. Further comparative characterization of deep-sea hydrothermal systems based on different geographical locations, tectonic and geological settings, geohydrological structures, geochemical features, and microbial communities will lead to the identification of the

**Table 3** Distribution of representative bacterial and archaeal rDNA clone types among the samples

| Representative bacterial and archaeal rDNA clone types | K365-VE | K365-ISCS | F1-ISCS | F2-ISCS | F4-ISCS | FC-I | FC-S | SSW-ISCS |
|---|---|---|---|---|---|---|---|---|
| ***Archaea*** | **ND**[a] | **40** | **42** | **40** | **40** | **38** | **41** | **37** |
| *Methanopyrales* | | | | | | | | |
| pCIRA-I | | | 1 | | | | | |
| *Methanococcales* | | | | | | | | |
| pCIRA-A | | 30 | | 2 | | | | |
| pCIRA-F | | | 9 | 29 | | | 3 | |
| pCIRA-G | | | 26 | 5 | 3 | 1 | 2 | |
| pCIRA-H | | | 2 | | | | | |
| pCIRA-J | | | | 1 | | | | |
| pCIRA-W | | | | | | | 2 | |
| pCIRA-Y | | | | | | | 2 | |
| *Thermococcales* | | | | | | | | |
| pCIRA-B | | 6 | | 1 | 17 | 35 | 2 | |
| pCIRA-C | | 4 | | | | 2 | | |
| pCIRA-E | | | 4 | | | | 4 | |
| pCIRA-T | | | | | | | 1 | |
| *Archaeglobales* | | | | | | | | |
| pCIRA-V | | | | | | | 3 | |
| pCIRA-13 | | | | | 1 | | | |
| Deep-sea hydrothermal vent *Euryarchaeota* | | | | | | | | |
| pCIRA-L | | | | 1 | | | | |
| pCIRA-O | | | | | | | 10 | |
| pCIRA-12 | | | | | 2 | | | |
| Other *Euryarchaeota* | | | | | | | | |
| pCIRA-K | | | | 1 | | | | |
| pCIRA-P | | | | | | | 1 | |
| pCIRA-X | | | | | | | 1 | |
| pCIRA-3 | | | | | | | | 11 |
| pCIRA-15 | | | | | 2 | | | |
| pCIRA-16 | | | | | 1 | | | |
| pCIRA-17 | | | | | 5 | | | |
| "*Korarchaeota*" | | | | | | | | |
| pCIRA-R | | | | | | | 6 | |
| pCIRA-X | | | | | | | 1 | |
| *Desulfurococcales* | | | | | | | | |
| pCIRA-N | | | | | | | 1 | |
| pCIRA-S | | | | | | | 1 | |
| pCIRA-U | | | | | | | 1 | |
| Marine Group I | | | | | | | | |
| pCIRA-ZI | | | | | 9 | | | 25 |
| pCIRA-ZII | | | | | | | | 1 |
| ***Bacteria*** | **ND** | **ND** | **46** | **46** | **41** | **ND** | **46** | **42** |
| *Aquificales* | | | | | | | | |
| pCIRB-E | | | 14 | | | | 1 | |
| pCIRB-K | | | 1 | | | | | |
| pCIRB-70 | | | 1 | | | | 1 | |
| *ϵ-Proteobacteria* | | | | | | | | |
| pCIRB-A | | | | 2 | 13 | | | |
| pCIRB-C | | | 4 | | | | | |
| pCIRB-D | | | 5 | 8 | | | | |
| pCIRB-G | | | 3 | | | | | |
| pCIRB-H | | | 3 | 2 | | | | |
| pCIRB-I | | | 3 | | | | 6 | |
| pCIRB-M | | | 1 | | | | | |
| pCIRB-N | | | 5 | 7 | 7 | | 6 | |
| pCIRB-P | | | | 2 | | | | |
| pCIRB-5 | | | | 1 | | | | |
| pCIRB-6 | | | 1 | 16 | | | | |
| pCIRB-7 | | | | 2 | | | | |
| pCIRB-12 | | | | | 3 | | | |
| pCIRB-26 | | | | | 1 | | | |
| pCIRB-35 | | | | | | | 2 | |
| pCIRB-38 | | | | | 1 | | 3 | |
| pCIRB-50 | | | | | | | 1 | |
| pCIRB-69 | | | | 3 | | | | |
| pCIRB-73 | | | 1 | | | | | |
| pCIRB-74 | | | 1 | | | | | |
| pCIRB-75 | | | 1 | | | | | |
| pCIRB-76 | | | 1 | | | | | |
| pCIRB-77 | | | 1 | | | | | |
| pCIRB-78 | | | | 2 | | | | |
| pCIRB-79 | | | | 1 | | | | |
| pCIRB-85 | | | | | | | 1 | |
| *δ-Proteobacteria* (data not shown) | | | | | 1 | | 12 | |
| *γ-Proteobacteria* (data not shown) | | | | | 12 | | 3 | 3 |
| *α-Proteobacteria* (data not shown) | | | | | 3 | | 1 | 9 |
| Other bacterial phyla (data not shown) | | | | | | | 9 | 30 |

[a]Not detected

fundamental conditions determining the formation of a hydrogen-based HyperSLiME.

On a final note, we have reported here the successful recovery of viable hyperthermophilic *Archaea*, the primary microbial components in the HyperSLiME, from at least >250°C of the superheated ISCSs. This does not imply that the HyperSLiME would occur in the temperature range exceeding the generally accepted temperature limit of growth for hyperthermophilic *Archaea* up to 120°C (Blöchl et al. 1997). Considering the geohydrological structure and the water circulation beneath the active hydrothermal seafloor, the subsurface microhabitats for hyperthermophilic *Archaea* (presumably along with the passage of hydrothermal liquid) might be formed within the temperature gradient, which is controlled with increasing distance from the heat source and by physical equilibration between the hydrothermal fluid and the infiltrated subseafloor water. Thus, we assume that the material we obtained for enrichments was being constantly supplied as entrained cells in the hot hydrothermal fluid and being captured and concentrated on the ISCS. To our knowledge, however, the most thermal-resistant survival of life is the reproducible growth after exposure at 121°C for 1 h demonstrated by the hyperthermophilic microorganism *Pyrolobus fumarii*, growing at the highest temperature among all the life (Blöchl et al. 1997). None of the *Thermococcales* strains is known to be able to reproduce itself for more than 5 min at 120°C (Jannasch et al. 1992). In fact, we tested the thermal sensitivity at 121 and 180°C of the representative strains of *Thermococcales* (strain Tc-365-95) and *Methanococcales* (strain Mc-365-85) isolated from the superheated ISCS deployed in the Kali vent orifice. Approximately $10^9$ cells $ml^{-1}$ of the exponentially growing cells of both strains demonstrated survival maximally for up to 10 min at both temperatures, but no recovery was obtained from the longer incubation (data not shown). After the ISCS recovery from the Kali and Fugen vent orifices, it was observed that the surfaces of the steel pipes were covered with sulfide and anhydrite minerals and that the pumice, the substratum of the ISCS, had been altered to clay-like minerals. To clarify the mechanism of survival for hyperthermophilic microorganisms with minerals in the superheated emission and to determine how long they are able to survive under the extraordinary conditions, simulating the naturally occurring hydrothermal emission are the foci in our future work.

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