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Fumio Inagaki · Ken Takai · Hisako Hirayama
Yu Yamato · Kenneth H. Nealson · Koki Horikoshi

Distribution and phylogenetic diversity of the subsurface microbial community in a Japanese epithermal gold mine

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Abstract Distribution and phylogenetic diversity of microbial communities in hot, deep underground environments in the Hishikari epithermal gold mine, southern part of Kyushu, Japan, were evaluated using molecular phylogenetic analyses. Samples included drilled cores such as andesitic volcanic rock (0.95–1.78 Ma) and the oceanic sedimentary basement rock of Shimanto-Supergroup (100 Ma), as well as geothermal hot aquifer waters directly collected from two different sites: AW-site (71.5°C, pH 6.19) and XW-site (85.0°C, pH 6.80) at a depth of 350 mbls (meters below land surface). Based on PCR-amplified 16S rRNA gene clone analysis, the microbial communities in the drilled cores and the hot aquifer water from the XW-site consisted largely of the 16S rRNA gene sequences, closely related to the sequences often found in marine environments, while the aquifer water from the AW-site contained 16S rRNA gene sequences representing members of *Aquificales*, thermophilic methanotrophs within the γ-subdivision of the *Proteobacteria* and uncultivated strains within the β-subdivision of *Proteobacteria*. The cultivable microbial community detected by enrichment cultivation analysis largely matched that detected by the culture-independent molecular analysis.

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F. Inagaki (✉) · K. Takai · H. Hirayama · K. H. Nealson
K. Horikoshi
Subground Animalcule Retrieval (SUGAR) Project,
Frontier Research System for Extremophiles,
Japan Marine Science and Technology Center (JAMSTEC),
Yokosuka 237-0061, Japan
E-mail: inagaki@jamstec.go.jp
Tel.: +81-46-8679687
Fax: +81-46-8679715

Y. Yamato
Sumitomo Metal Mining Company,
Kagoshima, Japan

K. H. Nealson
Department of Earth Sciences,
University of Southern California,
Los Angeles, CA, USA

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Introduction

In the past few years, our perspective on the Earth's biosphere has expanded from just the terrestrial and oceanic realms to include deep subterranean and subseafloor environments. Results of calculations suggest that the subsurface biosphere is one of the largest reservoirs of biomass in this planet (Whitman et al. 1998). Advances in cultivation-independent molecular genetic surveys now enable the analysis of the diversity of naturally occurring microbial habitats (Giovannoni et al. 1990; DeLong 1992; Pace 1997; Hugenholtz et al. 1998; Takai and Horikoshi 1999a). Because of these new approaches, it is now recognized that the numerous newly detected but as-yet uncultivated microorganisms represent dominant components of natural microbial assemblages. While the occurrence of indigenous and endolithic microbial populations has been demonstrated in the deposits and rocks by a number of investigations (Parkes et al. 1994; Stevens and McKinley 1995; Krumholz et al. 1997; McKinley et al. 2000; Warthmann et al. 2000; Inagaki et al. 2001, 2002a,2002b; Takai et al. 2001c), little is still known of the diversity and lifestyle of deep subterranean and subseafloor microbial communities, and also of the relationship between their distribution and the geological and geochemical background.

Microbial communities of subterranean aquifers have been more intensively studied than those in subseafloor environments because of the better accessibility. The existence of microorganisms in deep ground waters was evidenced by several studies within the Columbia River Basalt Group (CRB) in south-central Washington State (Stevens and McKinley 1995; Fry et al. 1997) and in deep granitic environments at Äspö Hard Rock Laboratory (HRL) in Sweden (Pedersen 1997). Reports have been also focused on subsurface contaminated aquifers

(Ludvigen et al. 1999; Cho and Kim 2000). In addition to these mesophilic subsurface aquifer systems, active thermophilic subsurface microbial populations have been detected in geothermal hot aquifer systems such as non-volcanically heated groundwater in the Great Artesian Basin of Australia (Anderews and Partel 1996), deep subsurface geothermal water in the Hachoubaru geothermal electric power plant (Takai and Horikoshi 1999a; Takai et al. 2001a), the subterranean hot springs in Iceland (Marteinsson et al. 2001) and, recently, the non-volcanically heated fissure water in the deep South African gold mines (Takai et al. 2001b). Most of these studies have focused on subsurface microbial communities composed of currently active microorganisms. Recently, the presence of microbial (genetic) remnants possibly buried in the habitats during the sedimentation processes was also detected from deep-sea rock and subseafloor sediments (Inagaki et al. 2001, 2002a).

The Hishikari subterranean environment is an epithermal gold-silver deposit located in northeastern Kagoshima Prefecture, Kyushu, Japan (Izawa et al. 1990); it contains one of the most productive gold deposits in the western Pacific, and the Sumitomo Metal Mining Company owns the mineral-production rights. Gold is found primarily in auriferous quartz veins that were formed approximately 1 million years ago, whose content is 45–50 g per ton, about 10 times higher than the world average. Around these veins are aquifer zones with geothermal hot water at a temperature of 65–150°C, with gases composed of hydrogen, methane and carbon dioxide (Izawa et al. 1990). In a previous study, using a combination of culture-dependent and culture-independent techniques, we characterized the indigenous, active subsurface microbial community in a geothermal hot aquifer sample obtained from a gold-bearing vein (AW-site) in the Hishikari gold mine (Takai et al. 2002a). These studies concluded that these samples consisted primarily of thermophilic, hydrogen- or sulfur-oxidizing chemolithoautotrophic bacteria. Here we report the expansion of this work to include drilled core samples and geothermal hot aquifer waters from a variety of habitats of the gold mine, and the distribution and phylogenetic diversity of genetic microbial communities in the gold mine as determined by PCR-mediated molecular phylogenetic surveys. Furthermore, preliminary enrichment and cultivation were conducted to evaluate the occurrence of microbial components revealed by 16S rRNA gene signatures and their physiological characteristics. The unique microbial diversity and distribution depending on the tectonics and geochemical characteristics of the geothermal deep underground are also discussed.

Materials and methods

Geological setting of the Hishikari gold mine

The Hishikari gold mine is located in the northeastern Kagoshima Prefecture, Kyushu, Japan (32°00′N, 130°41′E). The deposit is about 40 km from the nearest coast. The Hishikari mine is immediately west of Kagoshima Graben where the active Kirishima and Sakurajima volcanoes are located. We studied two distinct deposit zones, the Main deposit site (AW) and the Sanjin deposit site (XW) (Fig. 1).

The subsurface lithological structure of the Hishikari gold mine is mainly composed of three different strata; basement shale of the Cretaceous Shimanto-Supergroup, the Quaternary andesitic volcanic rocks, and the Pleistocene auriferous quartz veins as shown Fig. 1 (Izawa et al. 1990). The quartz veins are intrusive, occurring vertically along the fractures in the Shimanto basement rocks. The subground geothermal hot water in the vein system is the circulated meteoric water, on the basis of tritium analysis (Izawa et al. 1990)

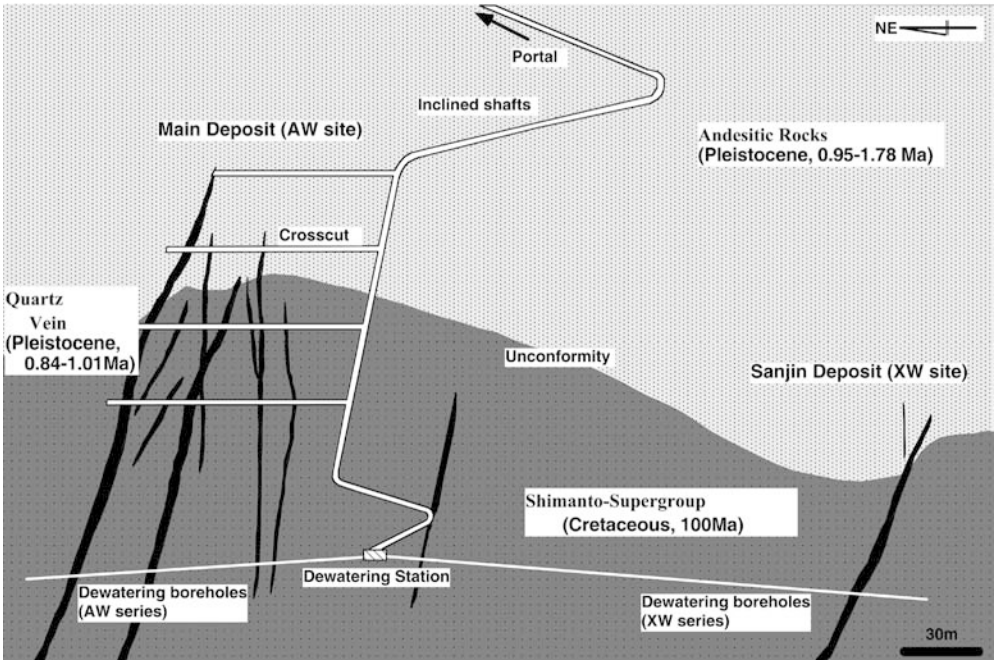

Fig. 1 Schematic cross section and the subsurface geologic structure in the Hishikari gold mine

and deuterium-oxygen isotope (Matsushima and Aoki 1994). The super-heated meteoric water changes to gas-fluid phases in the deepest zone near the magma. As the steam and fluid ascend through the basement up to the unconformity zone, the auriferous quartz vein is formed, accompanied by siliceous mineralization with cooling temperatures.

Sample collection

The rock cores of the andesite and Shimanto-basement were kindly provided by Sumitomo Metal Mining. The drilled rock cores were immediately put into the sample bag and stored at 4°C prior to analysis in the laboratory. The surface of the core was burned with ethanol by gas burner on a clean bench to remove contaminants, and then crushed by a rock trimmer. Inner parts of the crushed core were picked, and then ground into a powder sample with a stainless steel mortar.

Subground geothermal hot waters were collected from the dewatering system located at the deepest pit of the mine, at a depth of 350 m below the surface (Fig. 1). Sampling procedures for determination of microbial-community densities, nucleic acid extractions, and chemical analyses were followed as previously described (Takai et al. 2002a).

Chemical characterizations of geothermal aquifer waters and gases

Physical properties of aquifer water samples such as temperature, pH, conductivity, concentration of dissolved oxygen (DO), salinity, total concentration of dissolved saline (TDS), and oxidation-reduction potential (ORP), were measured by a multi-parameter water-quality-monitoring system MULTI-PROBE U-20 (Horiba, Japan) according to the manufacturer's recommendations.

Gas composition was measured by gas chromatograph Micro GC CP2002 (GL Sciences, Tokyo, Japan). Cation (Na, K, Mg and Ca) concentrations were obtained from Sumitomo Metal Mining. Anion samples were analyzed by ion chromatography using a Shim-pack IC column (Shimadzu, Kyoto, Japan). The concentrations of silica, nitrate, nitrite, ammonia, iron, sulfide, arsenate, arsenite, phosphate, and thiosulfate in aquifer waters were determined as previously described (Takai et al. 2002a).

Microscopic observation

Total microbial mass was estimated by direct count using epifluorescence microscopy with 4′, 6-diamidino-2-phenylindole (DAPI)-stained cells (Porter and Feig 1980). The hot aquifer water samples were filtered through 0.22-μm-pore-size, 13-mm-diameter polycarbonate filters (Advantec, Tokyo. Japan) and the filters were fixed for 20 min in 3.7% formaldehyde. The filter was rinsed twice in deionized, distilled water (DDW), and then stained by treatment with DDW containing DAPI at a final concentration of 10 μg ml^{-1} at room temperature for 20 min. The crushed rock samples were suspended in ''mj'' 10% synthetic sea water (Takai et al. 2001a) containing 3.7% formaldehyde for 10 min. DAPI was added to the mixture at a final concentration of 10 μg/ml. The suspension was incubated for 20 min on ice and briefly centrifuged. The supernatant was filtered through the same polycarbonate filters (Advantec). The filter was briefly rinsed in DDW and examined under epifluorescence using the Olympus BX51 microscope with the Olympus Camedia C3030 digital camera system.

DNA extraction and PCR amplification of 16S rRNA gene

Bulk DNA was extracted from 10 g of the powdery rock samples and the filter-concentrated aquifer-water samples using the Ultra Clean Large Scale Soil DNA kit (MO Bio Laboratory, Solana Beach, Calif.) following the manufacturer's instructions. The bacterial 16S rRNA gene was amplified by PCR using LA Taq polymerase with GC buffer I (TaKaRa, Tokyo, Japan) using Bac27F and Uni1492R primers (DeLong 1992). Archaeal 16S rRNA gene was amplified using the Arch21F and Arch958R primers for rock core samples and the Arch21F and Uni 1492R for the aquifer hot-water samples (DeLong 1992). Thermal cycling was performed using the GeneAmp PCR system 9600 (Perkin-Elmer, Foster City, Calif.) and the conditions were as follows: rock-core samples; denaturation at 96°C for 20 s, annealing at 52°C for 45 s, and extension at 72°C for 120 s for the bacterial 16S rRNA gene amplification of 36 cycles and the archaeal 16S rRNA gene of 40 cycles; aquifer hot-water samples; denaturation at 96°C for 25 s, annealing at 50°C for 45 s, and extension at 72°C for 120 s for the bacterial 16S rRNA gene amplification of 30 cycles and the archaeal 16S rRNA gene of 40 cycles.

Cloning, sequencing, and phylogenetic analyses of the 16S rRNA gene

Amplified 16 rRNA genes were gel-purified as previously described (Inagaki et al. 2001). The purified gene fragments were cloned in the vector pCR2.1 using the original TA cloning kit (Invitrogen, Carlsbad, Calif.), and then the 16S rRNA gene clone libraries were constructed from the rock-core samples and the aquifer hot waters. Bac27F and Arch21F primers were used in sequencing reactions to determine the phylogenetic clone type (phylotype) of bacteria and Archaea, respectively. The partial 16S rRNA gene sequences (approximately 400 bp) were determined using a model 3100 automated sequencer [Applied Biosystems Instruments (ABI), USA]. The sequence similarity was analyzed by the FASTA component program of DNASIS (Hitachi software, Tokyo, Japan). The sequences having ≥97% similarity by FASTA were tentatively assigned to the phylogenetically same phylotype as a species-level relationship in Bac27F and Arch21F primer-dependent sequences (Stackebrandt and Goebel 1994). 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 (Altschul et al. 1997; Benson et al. 1998).

The representative 16S rRNA gene sequences of about 900 bp were determined for both strands. Sequence alignment was performed by the DDBJ CLUSTAL-X ver. 1.81 System (Thompson et al. 1997). Phylogenetic analyses were manually restricted to nucleotide positions that were unambiguously alignable in all sequences. Least-squares distance matrix analysis, based on evolutionary distances, was performed using the correction of Kimura (Kimura 1980). Neighbor-joining analysis was accomplished using the DDBJ CLUSTAL-X ver. 1.81 System. Bootstrap analysis was used to provide confidence estimates for phylogenetic tree topologies.

Enrichment and isolation

To test the culturability of the potential microbial components represented by 16S rRNA gene signatures in clone libraries, enrichment was undertaken at a wide range of temperature (45–80°C) under a variety of chemotrophic conditions (Table 1). The media used contained different electron donors, electron acceptors, and carbon sources and were designed for the growth of microorganisms with metabolic activities predicted by the physical and geochemical features in the subsurface hot aquifer.

Inoculation of the hot aquifer water samples was performed at the site of the dewatering station. A 1-ml syringe was used to sample the hot aquifer waters (AW and XW) immediately after vigorous emission from the valve of the boreholes, and was inoculated into 3 ml of media. The inoculated tubes were incubated at the designated temperatures in the laboratory within 7 h. Inoculation of the rock-core samples was performed in the laboratory.

Table 1 Enrichment and isolation of the potential microbial components from various samples

Targeted microorganisms	Medium	Positive enrichment	Strain obtained from dilution-to-extinction method	Most closely related rDNA clone and organisms	Similarity (%)
Anoxic hydrogen-oxidizers with nitrate-reduction	mjANHOX-NO_3	AW water	HGM-K1 at 60, 65, 70°C	Environmental clone pHAuB-D	100.0
				Persephonella guaymasensis	91.2
Anoxic hydrogen-oxidizers with arsenate-reduction	mjANHOX-As	AW water	HGM-K2 at 60, 65, 70°C	Environmental clone pHAuB-D	100.0
				Strain HGM-K1	100.0
Microaerobic thiosulfate- or sulfur-oxidizers	mjAESOX	AW water	HGM-K3 at 60, 65, 70°C	Environmental clone pHAuB-D	99.6
				Strain HGM-K1	99.6
			HGM-C55 at 55°C	*Hydrogenophilus thermoluteolus*	96.2
				Hydrogenophilus hirschii	95.9
Microaerobic ammonia-oxidizers	mjAEAMOX	AW water	HGM-H1 at 50°C	Environmental clone OPB37	93.0
				Azoarcus sp. strain EbN1	93.0
Microaerobic nitrite-oxidizers	mjAENIOX	AW water	HGM-H2 at 60°C	Environmental clone OPB37	85.0
				Azoarcus sp. strain EbN1	90.8
Aerobic methane-oxidizers	mjAEMEX	AW water	HGM-G1 at 55, 60, 65, 70, 75°C	Environmental clone pHAuB-P	100.0
				Thermophilic methanotroph strain HB	93.4
Thermococcales	MJYPS	None	None	None	None
Methanococcales	MMJ	None	None	None	None

Approximately 20 cc crushed rock samples was suspended in 40 ml of "mj" 10% synthetic seawater containing 0.05% (w/v) Na_2S $9H_2O$ in 100-ml glass bottles with butyl rubber stoppers under 100% N_2. The suspension was inoculated into the designated media and incubated. The test media were mjANHOX-NO_3, mjANHOX-As, and mjAESOX, whose compositions have been previously described (Takai et al. 2002a), and were mjAEAMOX, mjAENIOX and mjAEMEX. The media contained 25 mM $NaHCO_3$, 1 mM NH_4Cl, 5 mM Na_2SiO_3, and 1 ml vitamin mixture (Balch et al. 1979) per liter of the "mj" diluted synthetic seawater. Additionally, the mjAEAMOX and mjAENIOX media contained 10 mM NH_4Cl and 5 mM $NaNO_2$, respectively, and were supplied with a gas mixture of N_2/O_2 (95/5; 100 kPa). For the mjAEMEX medium, 0.01% (w/v) yeast extract and 1 mM $CuSO_4$ were added, and a gas mixture of $N_2/CH_4/O_2$ (40/50/10; 100 kPa) was used. To prepare the media, 5 mM of Na_2SiO_3 and 1 mM of NH_4Cl were dissolved in the "mj" 10% synthetic seawater, and the pH of the media was adjusted to around 7.5 with HCl before autoclaving. After autoclaving, a concentrated solution of vitamins (Balch et al. 1979), $NaHCO_3$, and other inorganic or organic substrates were added to the media. These solutions were sterilized separately by autoclaving except for the vitamins, which were filter-sterilized. The pH of the medium was adjusted at 7.5 with H_2SO_4 or NaOH under an anaerobic chamber under 95% N_2 and 5% H_2. The medium was dispensed at 20% of the total bottle or tube volume, and it was tightly sealed with a butyl rubber stopper under the designed gas phase. For enrichment of members of *Thermococcales* and *Methanococcales*, MJYPS medium (Takai et al. 2001a) and MMJ medium (Takai et al. 2002b) were used.

Positive enrichment cultures were partially purified by the dilution-to-extinction technique (Takai and Horikoshi 1999b). The partial sequence of the 16S rRNA gene of the strain was determined and applied to sequence similarity analysis.

Nucleotide sequence accession numbers

The sequences from this study are available though DDBJ under accession numbers as follows: pHAuB-D; AB071326, pHAuB-J; AB071327, other bacterial 16S rRNA gene sequences of pHAuB; AB072705–AB072722; archaeal 16S rRNA gene sequences of pHAuA; AB072723–AB072728.

Results

Geochemical characteristics of deep hot aquifers

Physical properties and geochemical characteristics of the geothermal hot aquifer waters were measured in situ (Table 2). The temperature of the AW hot water was 71.5°C, while the XW hot water was 85.0°C. pH values of both aquifer waters were neutral or slightly acidic. Since the DO and ORP indicated low values and both aquifer waters contained high concentrations of ferrous iron, the hot aquifer waters in the AW and XW deposit sites were under extremely reductive conditions.

Chemical properties of hot aquifer water samples from both deposit sites indicated different geochemical features of the aquifers between the AW and XW sites (Table 2). The concentration of silica in the XW water was 4.3 times higher than that of AW water. The concentration of ammonium ion was relatively high in both hot aquifer waters (Table 2). Nitrate ion (26.9 μM) was detected in the AW hot aquifer water, while the nitrite ion was below detection limit in both waters (Table 2). In addition, trace amounts of arsenate were detected at 9–10 μM; however, arsenite was not detected in either of the hot-water samples (Table 2). Gas phases of hot aquifers in both deposit sites were mainly composed of carbon dioxide and methane (Table 2). The concentration of methane in the XW gas phase was about 8 times higher than that in the AW, indicating that more active thermo-degradation of organic compounds in the subducted Shimanto basement deposit occurred in the XW site than in the AW site. Hydrogen sulfide was not detected in either of the hot aquifers (Table 2). A higher temperature, a more reduced state, and higher concen-

Table 2 Geochemical characteristics of geothermal hot aquifer waters collected from AW and XW sites (*n.d.*not detected)

	AW	XW
Gas phase		
H_2 (ppm)	150.3	11.6
CH_4 (ppm)	3002	23300
CO_2 (ppm)	74000	62900
H_2S (ppm)	n.d.	n.d.
Physical property		
Temperature (°C)	71.5	85
pH	6.19	6.80
Conductivity (S m^{-1})	0.18	0.34
DO (mg l^{-1})	0.15	0.00
Salinity (%)	0.1	0.2
TDS (g l^{-1})	1.2	2.1
ORP (mV)	−110	−212
Chemical property		
Na^+ (mg l^{-1})	520	670
K^+ (mg l^{-1})	25	37
Mg^{2+} (mg l^{-1})	17	5
Ca^{2+} (mg l^{-1})	80	47
Cl^- (mg l^{-1})	310	460
HCO_3^- (mM)	16.1	16.4
SiO_2 (mM)	1.7	7.4
S^{2-}/HS^- (μM)	102	254
NH_4^+ (μM)	96.2	140
NO_3^- (μM)	26.9	n.d
NO_2^- (μM)	n.d.	n.d
Fe (III) (μM)	74	9.7
Fe (II) (μM)	72.7	2.2
As (V) (μM)	9.9	9.4
As (III) (μM)	n.d.	n.d.
PO_4^{3-} (μM)	5.3	5.7
$S_2O_3^{2-}$ (μM)	4.1	12.1

trations of silica and methane found in XW water may be caused by a deeper and hotter origin of the XW aquifer water than that of the AW aquifer water.

Direct cell count

Microbial population density in all examined aquifer-water samples was estimated by the direct epifluorescence microscopic count of DAPI-stained cells. The geothermal hot aquifer water samples from AW and XW sites contained 1.2×10^4 and 1.0×10^4 cells per liter, respectively. The DAPI-stained cells observed in the AW aquifer water were composed of rods and coccoids with bright fluorescence signals, but in the XW water they were rods only with weak signals.

Phylogenetic analyses of bacterial 16S rRNA gene clone libraries

A total of 230 bacterial 16S rRNA gene clones from rocks and aquifer hot waters were characterized by partial sequencing and similarity analysis (Table 3). The representative clone type of pHAuB-1 was dominant in these clone libraries: 17 of 54 clones (31.5%) from the Shimanto-Supergroup and 19 of 49 clones (38.7%) from andesite. The sequence of pHAuB-1 was very similar to the 16S rRNA gene of the α-proteobacterium *Caulobacter crescentus* (99.3%) (Brun 2001). The phylotypes

Table 3 Phylotype analysis of bacterial 16S rRNA gene clone sequences recovered from the drilled rock cores and aquifer hot waters from AW and XW deposit sites (*CFB group Cytophaga-Flexibacter-Bacterioides* group)

Representative clone	Phylogenetic affiliation	Number of relative clones				Similar rDNA sequence	Similarity (%)
		Shimanto-Super Group	Andesite	AW	XW		
pHAuB-D	*Aquificales*			38		*Aquificales* strain SRI-40	97.5
pHAuB-1	α-*Proteobacteria*	17	19	2	3	*Caulobacter crescentus*	99.3
pHAuB-15	α-*Proteobacteria*	2	1			*Methylobacterium extorquens*	95.9
pHAuB-20	α-*Proteobacteria*		1			*Brevundimonas vesicularis*	99.7
pHAuB-27	α-*Proteobacteria*		1			*Bradyrhizobium japonicum*	98.4
pHAuB-7	α-*Proteobacteria*	1	2			*Sphingomonas paucimobilis*	96.7
pHAuB-J	β-*Proteobacteria*	1		29		OPB37	96.1
pHAuB-Q5	β-*Proteobacteria*	1				*Comamonas denitrificans*	99.7
pHAuB-18	β-*Proteobacteria*	4	3			*Pseudomonas saccharophila*	98.6
pHAuB-34	β-*Proteobacteria*	5	2		1	Denitrifying Fe(II)-oxidizing bacteria BrG2	99.1
pHAuB-3	β-*Proteobacteria*	1				*Stenotrophomonas maltophila*	99.3
pHAuB-A	γ-*Proteobacteria*	2	8		35	*Marinobacter hydrocarbonoclasticus*	99.6
pHAuB-P	γ-*Proteobacteria*	3		7		Thermophilic methanotroph HB	93.4
pHAuB-3	γ-*Proteobacteria*	1				*Stenotrohomanas maltophila*	99.3
pHAuB-24	γ-*Proteobacteria*	3		2		*Psychromonas arctica*	94.8
pHAuB-Q27	γ-*Proteobacteria*	4	2			*Halomonas meridiana*	97.8
pHAuB-33	γ-*Proteobacteria*	2				*Moritella yayanosii*	98.2
pHAuB-45	γ-*Proteobacteria*		1	3		Psychrophilic marine bacterium PS39	98.8
pHAuB-5	δ-*Proteobacteria*	8	9	1	2	*Desulfocapsa sulfoexigens*	92.8
pHAuB-L	CFB group*			3		OPB56	90.2
pHAuB-N	Low G+C Gram Positives			1		*Thermoanaerobacter ethanolicus*	78.5
Total number of rDNA clones		54	49	86	41		

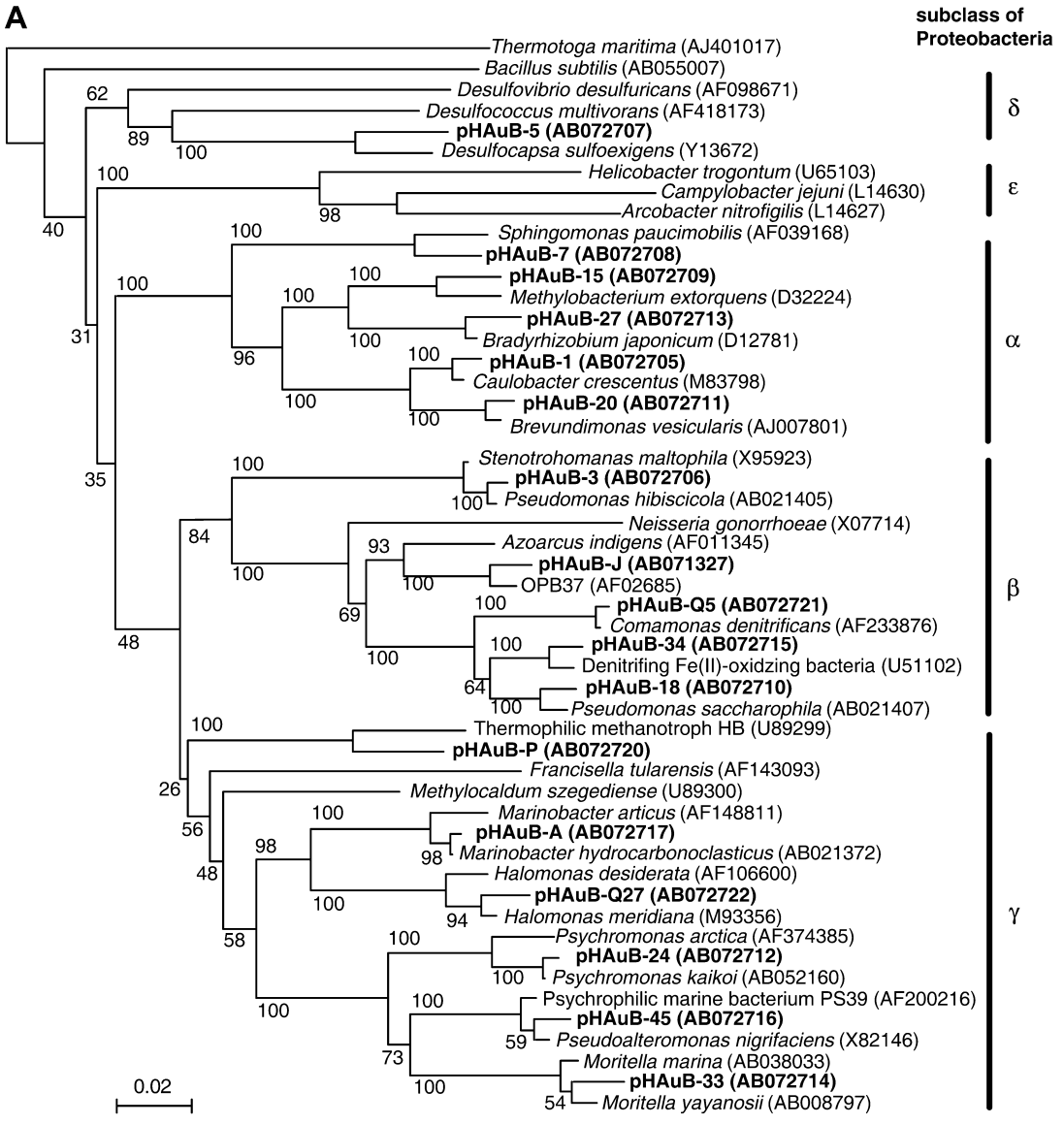

Fig. 2A, B Phylogenetic relationship between the representative 16S rRNA gene clones from the Hishikari gold mine and 16S rRNA gene sequences of related pure cultures and environmental clones among the class *Proteobacteria* in domain Bacteria (**A**) and among the kingdom Crenarchaeota in domain Archaea (**B**). The trees were inferred by the neighbor-joining analysis based on approximately 600 homologous positions of all 16S rRNA gene sequences. The values of 100 bootstrap trial replications are given for nodes in trees. The *scale bar* represents 0.02 nucleotide substitutions per sequence position. Representative bacterial and archaeal 16S rRNA gene sequences obtained from the Hishikari gold mine (pHAuB- and pHAuA-, respectively) are indicated by **bold letters**. The accession number of the sequence is given in *parentheses*

of pHAuB-7 and pHAuB-15 indicated 96.7% and 95.9% similarity with *Sphingomonas paucimobilis* and *Methylobacterium extorquens*, respectively. These phylotypes were detected from both rock-core samples but represented a low proportion in the bacterial 16S rRNA gene clone libraries (Table 3).

Within β-Proteobacteria, the 16S rRNA gene sequences closely related to *Pseudomonas saccharophila* and the denitrifing Fe (II)-oxidizing bacterium BrG2 were abundantly detected from the drilled rock samples. *P. saccharophila* was known as an aromatic hydrocarbon-degrading bacterium, which was typically detected from a variety of terrestrial surface environments such as agricultural soil and activated sludge (Puhakka et al. 1995; Nohynek et al. 1996). Strain BrG2 was an anaerobic ferrous iron-oxidizing, nitrate-reducing autotrophic bacterium which was isolated from terrestrial freshwater sediments (Buchholz-Cleven et al. 1997).

A variety of γ-proteobacterial sequences related to marine bacteria was obtained from the rock-core samples. Twenty-six of 103 clones (25%) from rock samples were affiliated within the γ-subclass of *Proteobacteria*. From the bacterial clone libraries constructed from Shimanto-Supergroup basement rock, the 16S rRNA gene sequences phylogenetically associated with the sequences of the genera *Marinobacter*, *Psychromonas*, *Halomonas* and *Moritella* were detected, which have

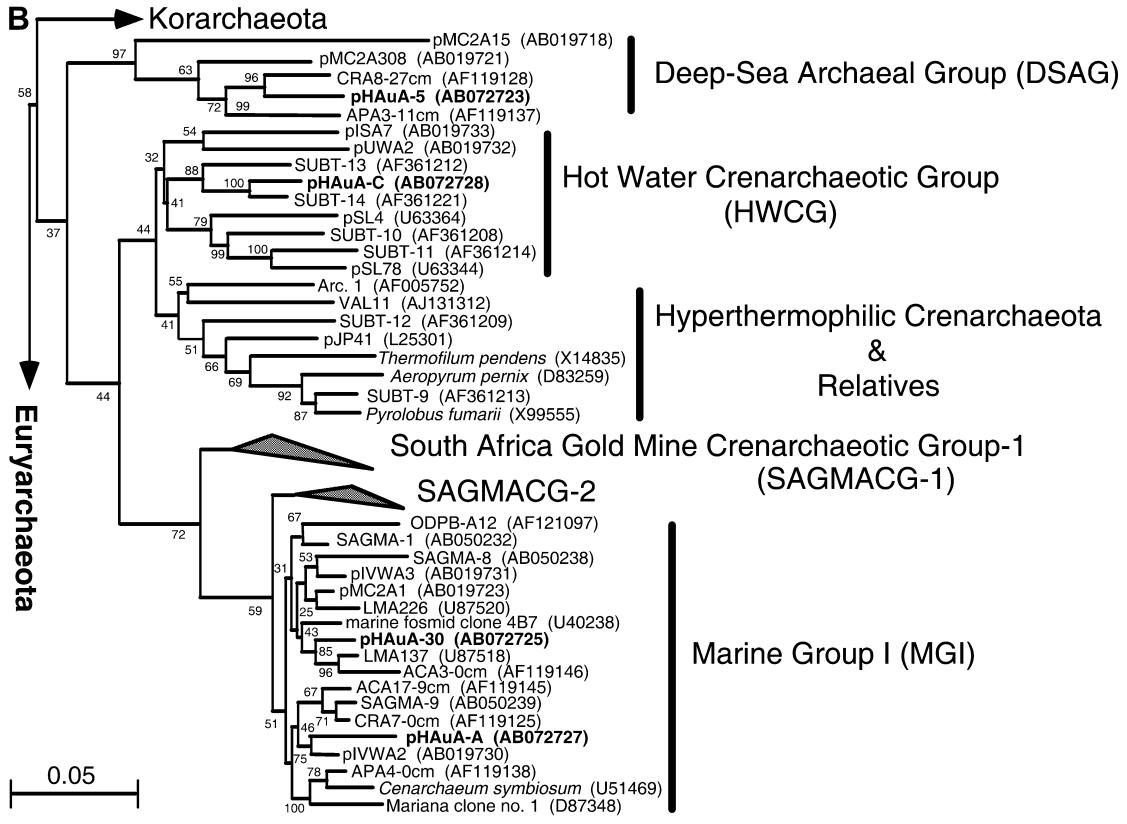

Fig. 2 (Continued)

been typically found in cold deep-sea sediments. Eight clones of pHAuB-A closely related to *Marinobacter hydrocarbonoclasticus* were detected from the andesite core sample. One sequence of 16S rRNA gene was found to be closely related to the psychrophilic marine bacterium PS39 and *Pseudoalteromanas nigrifaciens* (Table 2, Fig. 2A). In addition to the bacterial components typically found in marine environments, three related clones of pHAuB-P were obtained from Shimanto-Supergroup basement rock. These clones had 93.4% similarity with thermophilic methanotroph HB isolated from an underground hot spring in Hungary (Table 3) (Bodrossy et al. 1999). The phylogenetic analysis suggested that the thermophilic methanotroph HB and pHAuB-P were closely related to each other, with phylogenetic positions deeply located within the γ-subclass of *Proteobacteria* (Fig. 2A).

The phylotype of pHAuB-5 was one of the dominant components among bacterial clones retrieved from the rock-core samples. The sequence of pHAuB-5 was 92.8% similar to that of *Desulfocapsa sulfoexigens* within the δ-*Proteobacteria* (Table 3, Fig. 2A). *D. sulfoexigens* is a freshwater sulfur- and sulfate-reducing bacterium (Finster et al. 1998) whose relatives have been predominantly detected at the reductive cold-seep environments (Inagaki et al. 2002b).

In marked contrast, 16S rRNA gene sequences recovered from hot aquifer water samples of AW and XW deposit sites were less diverse than those from rock-core samples. The phylotypes of pHAuB-D, pHAuB-J, and pHAuB-P were predominantly obtained from the clone assemblages from the AW aquifer water sample (Table 3). In agreement with previously published results (Takai et al. 2002a), the AW water sample was dominated by the phylotype of pHAuB-D, which contributed 38 of 86 clones (44.2%) of the bacterial 16S rRNA gene clone library (Table 3). This sequence was very similar to the 16S rRNA gene of facultatively anaerobic, hydrogen- or sulfur/thiosulfate-oxidizing, thermophilic chemolithoautotrophs within the order *Aquificales* using molecular oxygen, nitrate, ferric iron, arsenate, selenate, and selenite as electron acceptors (Takai et al. 2002a). The phylotype of pHAuB-J was the second-most abundant sequence (33.7%) in the bacterial clone library from the AW aquifer hot water (Table 3). This sequence showed 96.1% similarity to that of OPB37 recovered from Yellowstone National Park hot-spring sediments (Hugenholtz et al. 1998); thus, it is affiliated with the β-subclass of the *Proteobacteria* and phylogenetically located at the downstream of *Azoarcus indigens* (Fig. 2A). Seven 16S rRNA genes represented by pHAuB-P (found also in the Shimanto-Supergroup basement as described above) were detected from the AW-water bacterial clone library (Table 3). In addition, a few sequences related to 16S rRNA genes of putative psychrophilic or mesophilic bacteria such as *Psychromonas*, *Pseudoalteromonas*, and *Desulfocapsa* were detected from the AW aquifer hot-water sample.

Table 4 Phylotype analysis of archaeal 16S rRNA gene clone sequences constructed from the drilled core of the Shimanto-Super Group and geothermal hot aquifer water samples from AW and XW sites (*DSAG* Deep-Sea Archaeotic Group, *HWCG* Hot Water Crenarchaeotic Group)

Representative clone	Phylogenetic affiliation	Number of relative clones			Similar rDNA sequence	Similarity (%)
		Shimanto-Super Group	AW	XW		
pHAuA-A	Marine Group I	3	85	45	PVA-OTU-4	94.3
pHAuA-30	Marine Group I	2			APA3–0 cm	96.7
pHAuA-5	DSAG	4			CRA8–27 cm	96.9
pHAuA-C	HWCG	2	3		SUBT-14	94.8
pHAuA-9	*Thermococcales*	20			*Thermococcus siculi*	98.6
pHAuA-43	*Methanococcales*	3			*Methanothermococcus okinawensis*	98.7
Total		34	88	45		

In contrast to the AW water sample, a very different 16S rRNA gene community structure was seen in the XW aquifer sample (Table 3). Most of the examined clone sequences were extremely similar to the phylotype of pHAuB-A that closely related to *Marinobacter hydrocarbonoclasticus* (Table 3). 16S rRNA genes related to *Caulobacter* and *Desulfocapsa* were also detected. Every 16S rRNA gene sequence seen in the XW hot aquifer sample was also found in both rock-core samples.

Phylogenetic analyses of archaeal 16S rRNA gene clone libraries

Three archaeal 16S rRNA gene libraries were constructed from the successful PCR amplicons from the Shimanto-Supergroup rock core and the geothermal hot aquifer water samples from the AW and XW deposit sites. The most dominant archaeal 16S rRNA gene component recovered from the archaeal clone library of the Shimanto-Supergroup basement was pHAuA-9 (58.8%), which was closely related to the sequence of the hyperthermophilic archaeon *Thermococcus siculi* (98.6%) isolated from the hydrothermal vent at the Okinawa Trough in Japan (Table 4) (Grote et al. 1999). The phylotype of pHAuA-43 was also closely related to the isolate from the same hydrothermal vent field, *Methanothermococcus okinawensis* (Table 4) (Takai et al. 2002b). Other phylotypes of pHAuA-5 (4 of 34 clones) and pHAuA-C (2 of 34 clones) were obtained from the Shimanto-Supergroup rock core (Table 4). Phylogenetic analysis revealed that the representative sequences of pHAuA-5 and pHAuA-C were affiliated to the Deep-Sea Archaeal Group (DSAG) and the Hot Water Crenarchaeotic Group (HWCG), respectively (Fig. 2B). The representative 16S rRNA gene sequence of pHAuA-5 indicated 96.9% similarity to CRA8-27 cm within DSAG (Table 4, Fig. 2B). The 16S rRNA genes within DSAG were recovered from miscellaneous deep-sea environments; pMC was detected from a hydrothermal vent in Japan (Takai and Horikoshi 1999a), CRA and ACA were obtained from deep-sea sediments in the Atlantic Ocean (Vetriani et al. 1999) while SUBT-14 was recovered from the borehole of a subterranean geothermal field in Iceland (Marteinsson et al. 2001). The phylogenetic cluster of HWCG contains 16S rRNA gene sequences recovered from terrestrial and subterranean geothermal hot waters and deep-sea hydrothermal vent environments (Fig. 2B). In addition, five clones within Marine Group I (MGI), which represents the most abundant archaeal phylotypes detected from global oceanic environments, were obtained from the basement rock-core sample (Table 4, Fig. 2B).

The compositions of the archaeal clone libraries from the AW and XW hot aquifer samples were less diverse than those from the Shimanto-Supergroup basement rock. Almost all clones were similar to the phylotype of pHAuA-A in both libraries (Table 4). The representative sequence of pHAuA-A indicated 94.3% similarity with PVA-OTU4 within MGI detected from a hydrothermal vent environment (published only in database: U46680) (Table 4). In addition, three clones within HWCG were also detected from the AW aquifer hot-water sample.

Enrichment analyses

The culturability of the potential microbial components represented by 16S rRNA gene signatures in the libraries prepared from enrichment cultures was tested at a wide range of temperature (45–80°C) using various media (Table 1). Table 1 shows the results of enrichment cultures. The microorganisms that grew in the most diluted series of cultures in mjANHOX-NO_3, mjANHOX-As, and mjAESOX media at 60, 65 or 70°C containing the inocula from the AW hot aquifer were found to be facultatively anaerobic, hydrogen- or sulfur/thiosulfate-oxidizing, thermophilic chemolithoautotrophs within the order *Aquificales* as described previously (Takai et al. 2002a). However, a strain grown in the mjAESOX medium at 55°C from the AW aquifer water had the partial 16S rRNA gene sequence related to *Hydrogenophilus thermoluteolus* strain TH-1 (96.2%) (Hayashi et al. 1999) within the *β-Proteobacteria*. The partial 16S rRNA gene sequences of the microorganisms that grew in mjAEAMOX at 50°C and mjAENIOX at 60°C with

the inocula from the AW hot aquifer were similar to the sequences of OPB37 (93.0% and 85.0%, respectively) and pHAuB-J (92.5% and 83.3%, respectively) within the *β-Proteobacteria*. In addition, the microorganisms obtained from the mjAEMEX medium at 55, 60, 65, 70 and 75°C from AW water had the identical partial 16S rRNA gene sequence, which was also identical to the sequence of pHAuB-P (100%). None of these microorganisms was obtained from any of the test media containing the XW hot aquifer water samples or any of the rock-core samples. Based on the clone analysis, from the Shimanto-Supergroup basement rock, the 16S rRNA gene signatures of thermophilic Archaea *Thermococcales* and *Methanococcales* were detected. Given that these anaerobic thermophilic Archaea are preserved as microbial components in the reductive, endolithic environments buried in the oceanic sedimentary rock, it may be possible to recover the cultures of these Archaea from the populations. However, no positive enrichment of *Thermococcales* and *Methanococcales* members was retrieved from the Shimanto-Supergroup basement-rock sample and even any other core samples and hot aquifer samples (Table 1).

Discussion

The subsurface biosphere in an epithermal type of gold deposit, the Hishikari gold mine, was evaluated by molecular ecological surveys. Most sequences recovered from the drilled rock cores and the geothermal aquifer hot waters were closely related to sequences of microorganisms typically obtained from habitats occurring in various marine environments. Phylotype analysis of the rock-core samples, however, revealed 16S rRNA gene signatures related to psychrophilic or mesophilic marine bacteria such as genera *Moritella* and *Psychromonas* (Table 3). 16S rRNA gene sequences related to a sulfate-reducing marine bacterium within the *δ-Proteobacteria*, *D. sulfoexigens*, were also found from rock samples of andesite and the Shimanto-Supergroup. Since in-situ temperatures were too high for any mesophilic bacterial growth, and the geochemical data suggested that the aquifer was originally derived from super-heated meteoric water (Izawa et al. 1990), molecular signals for various oceanic mesophiles such as *Marinobacter* and *Psychrobacter* might reflect on inactive cells possibly transferred from the cold aquifer zone by subterranean hydrological process.

Oceanic bacterial components were also detected from the andesite samples (Table 3). The Shimanto-Supergroup basement uplifted in the early Cretaceous, and the andesitic rock was deposited on it by the activity of the Kirishima volcano (Izawa et al. 1990). Vertical geothermal flow may carry and disperse the microbial communities compacted in the Shimanto-Supergroup shale to the overlain andesite. The presence of phylotype pHAuB-1 related to the genus *Caulobacter* might also be associated with such a geohydrological flow in the Hishikari subterranean environments.

The composition of PCR-amplified archaeal 16S rRNA genes from the oceanic sedimentary rock, Shimanto-Supergroup, also indicated the presence of oceanic archaeal components such as MGI, *Thermococcus*, and *Methanococcus* (Table 4). In addition, thermococcal and methanococcal 16S rRNA gene sequences indicated high similarity values with previously isolated Archaea from the geographically associated hydrothermal field. The phylotypes of pHAuA-9 and pHAuA-43 were the most similar to the sequences of *T. sicui* and *Methanothermococcus okinawensis*, respectively, which were both isolated from the mid-Okinawa Trough (Table 4) (Grote et al. 1999; Takai et al. 2002b). To this end, our recent investigations have revealed the presence of archaeal 16S rRNA gene signatures, such as *Thermococcus*, *Haloarcula*, and *Sulfolobus*, to be widely distributed in cold, marine sediment and sedimentary rock environments (Inagaki et al. 2001, 2002a). At present, we cannot define whether these extremophilic Archaea were present as active microbial populations or preserved as microbial components buried during the sedimentation and subduction process. However, preliminary enrichment and cultivation experiments suggested that the thermophilic archaeal populations were culture-resistant and might be the potential microbial relics.

However, a distinct bacterial 16S rRNA gene community structure was obtained from the geothermal aquifer water of the AW site. The phylotype of pHAuB-D was the most abundant in the bacterial 16S rRNA clone library, and phylogenetically belonged to the order *Aquificales* (Table 3). The phylotype represented the sequence of facultatively anaerobic, hydrogen- or sulfur/thiosulfate-oxidizing, thermophilic chemolithoautotrophs within the order *Aquificales* using molecular oxygen, nitrate, ferric iron, arsenate, selenate, and selenite as electron acceptors (Takai et al. 2002a). Furthermore, we obtained sequences of pHAuB-J and pHAuB-P as dominant 16S rRNA gene components in the AW aquifer water (Table 3). Although we have not yet succeeded in the cultivation of the microorganism whose 16S rRNA gene sequence matches pHAuB-J, several sulfur/thiosulfate- or ammonia/nitrite-oxidizing, thermophilic chemolithoautotrophs were successfully cultivated. These microorganisms were phylogenetically associated with the *β*-subclass of *Proteobacteria* and were most closely related to pHAuB-J and OPB37. It seems likely, therefore, that the previously uncultivated bacterium OPB37 and its relative pHAuB-J are chemolithoautotrophic thermophiles, potentially utilizing abundant geochemical resources such as hydrogen, hydrogen sulfide, sulfur, thiosulfate ammonia, and nitrite. In contrast, a methanotrophic, thermophilic bacterium represented by the phylotype pHAuB-P was cultivated from the AW hot aquifer water (Table 1). The predominant occurrence of a thermophilic, methanotrophic bacterial population in the AW aquifer was demonstrated by both culture-dependent and culture-

independent analyses, and may be strongly associated with the abundance of methane in the gas phase of the AW aquifer. The thermophilic methanotroph strain HB was an aerobic coccoid isolated from the underground hot spring in Hungary (Bodrossy et al. 1999). These cells probably use a small amount of oxygen dissolved in the AW aquifer as an electron acceptor (Table 2). The enrichment and cultivation experiments suggest that the 16S rRNA gene community structure obtained from the AW hot aquifer water represents the presently active, indigenous thermophilic microbial community, primarily consisting of chemolithoautotrophs associated with the geochemical conditions.

In contrast to the bacterial population in the AW aquifer water, the XW aquifer water yielded a different community structure (Table 3). Thirty-five of 41 (~85%) randomly sequenced clones from the XW aquifer water were phylotype pHAuB-A, which were closely related to *Marinobacter hydrocarbonoclasticus*, isolated from Mediterranean seawater near a petroleum refinery (Gauthier et al. 1992) (Table 3). The direct count of the DAPI-stained cells indicated that approximately 10^4 rod-shaped cells l/ml were present in the XW water; however, the fluorescence signals were weak in comparison with the cells in the AW water. We previously reported that the presence of *Aquificales* cells was detected from the AW hot aquifer water by in-situ hybridization (FISH) analysis (Takai et al. 2002a); however, there was no fluorescence signal and positive culture for *Aquificales* in the XW hot water. In addition, since the temperature of the XW aquifer water (85.0°C) was too high for the growth of *Marinobacter*, the retrieved genetic signatures are likely derived from the dead or inactive microbial components preserved in or transferred from the cold subducted marine sediment, the Shimanto-Supergroup basement aquifers. Higher temperature, a more reductive state and higher concentrations of silica and methane found in XW water may be indicative of a deeper and hotter origin of the XW aquifer water than the AW aquifer, conditions that may prevent the occurrence of presently active, thermophilic microbial communities in the XW aquifer water.

Based on several criteria, including geological setting, geochemical characteristics, and the distribution, abundance, and phylogenetic diversity of microbial communities in the Hishikari gold mine, we hypothesize that the geothermal subsurface biosphere is characterized by co-existence of the presently active, thermophilic chemolithotrophic microbial communities in the geothermal hot aquifer environment and of the endolithic microbial relics (or dormant cells) buried over geological time scales. The microbial distribution was directed by the specific geological settings such as the subduction system of the Pacific Pate and the perpendicular geothermal hydrology. It is generally difficult to determine the distribution and function of the subsurface microbial communities since the local geohydrological features are often so complex and incompletely characterized. The first step in resolving such issues is the combined use of culturing and molecular surveys to partly resolve the distribution and function of the indigenous microbial communities, and the entrainment of the background microbial or genetic signatures, such as the microbial relics and the contaminated microbial components from surface or distinct environments. In the case of the Hishikari epithermal gold mine, on the basis of this hypothesis, the 16S rRNA genes of mesophilic bacteria typically obtained from deep-sea environments will likely be traces transferred from a subducted accretionary wedge, i.e., the Shimanto-Supergroup basement, by the deep-subsurface hydrothermal fluids. In order to verify this dynamic hypothesis, we will need to improve and enlarge our culture methods, sample at higher spatial resolution, and investigate new subsurface environments. These are the foci of our ongoing efforts.

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