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Bacterial community shift along a subsurface geothermal water stream in a Japanese gold mine

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Abstract Change of bacterial community occurring along a hot water stream in the Hishikari gold mine, Japan, was investigated by applying a combination of various culture-independent techniques. The stream, which is derived from a subsurface anaerobic aquifer containing plentiful CO_2, CH_4, H_2, and NH_4^+, emerges in a mine tunnel 320 m below the surface providing nutrients for a lush microbial community that extends to a distance of approximately 7 m in the absence of sunlight-irradiation. Over this distance, the temperature decreases from 69°C to 55°C, and the oxidation-reduction potential increases from −130 mV to +59 mV. In the hot upper reaches of the stream, the dominant phylotypes were: 1) a deeply branching lineage of thermophilic methane-oxidizing *γ-Proteobacteria*, and 2) a thermophilic hydrogen- and sulfur-oxidizing *Sulfurihydrogenibium* sp. In contrast, the prevailing phylotypes in the middle and lower parts of the stream were closely related to ammonia-oxidizing *Nitrosomonas* and nitrite-oxidizing *Nitrospira* spp.. Changes in the microbial metabolic potential estimated by competitive PCR analysis of genes encoding the enzymes, particulate methane monooxygenase (*pmoA*), ammonia monooxygenase (*amoA*), and putative nitrite oxidoreductase (*norB*), also substantiated the community shift indicated by 16S rRNA gene analysis. The diversity of putative *norB* lineages was assessed for the first time in the hot water environment. Estimation of dominant phylotypes by whole-cell fluorescent in situ hybridization and changes in inorganic nitrogen compounds such as decreasing ammonium and increasing nitrite and nitrate in the mat-interstitial water along the stream were consistent with the observed transition of the bacterial community structure in the stream.

Keywords Methane-oxidizer · Ammonia-oxidizer · Nitrite-oxidizer · *Aquificales* · Subsurface

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Introduction

Terrestrial hot spring environments support a diverse unique microbial community including thermophilic microorganisms with novel physiological properties and industrial potential (Bruins et al. 2001; Huber and Stetter 1998; Huber et al. 2000; Stetter 1999). The microbial community that occurs in the various habitats of hot spring environments is strongly influenced by environmental conditions such as temperature, pH, redox potential, sulfide concentration, sunlight-irradiation, etc. (Kurosawa et al. 1998; Ruff-Roberts et al. 1994). Of these environmental factors, the effects of temperature and sulfide concentration have been relatively well studied, and transitions in these factors have been observed to have a great impact on microbial community structure (Hiraishi et al.1999; Skirnisdottir et al. 2000). A predominant occurrence of members of the order *Aquificales* is observed in relatively high temperature habitats (approx. >70°C) such as hot water pools and upper reaches of hot water flows, whereas in habitats with lower temperatures and lower sulfide concentrations the prevailing phototrophic bacterial populations are the Green-Non-Sulfur (GNS) Group (*Chloroflexus*) and

Cyanobacteria (Ferris and Ward 1997; Hugenholtz et al 1998; Marteinsson et al. 2001; Reysenbach et al. 1994; Reysenbach et al. 2000; Takacs et al. 2001; Takai et al. 2002; Ward et al. 1998; Weller et al. 1992; Yamamoto et al. 1998). These observations imply a shift in predominant microbial energy metabolism from chemolithotrophy to phototrophy, which should be attributed to sufficient and stable energy supply by sunlight in surface environments. In contrast, little is known about the microbial communities that occur when subsurface geothermal aquifers emerge into oxic environments under dark conditions, such a subsurface environment is the subject of the current paper.

The Hishikari gold mine is located at the northwestern part of a volcanically active region near Mt.Kirishima in the Kagoshima prefecture, Japan. A subsurface volcanic hot aquifer (>72°C) exists under the mine (Izawa et al. 1990), containing phylotypes related to the order *Aquificales* (Inagaki et al. 2003; Takai et al. 2002). One of these phylotypes has been successfully cultivated from the vein water of this mine, and identified as a new genus of the *Aquificales*, *Sulfurihydrogenibium subterraneum* (Takai et al. 2003).

In the deepest part of the mine tunnel, a dense microbial community occurs at the point of emergence of a geothermal water stream on a tunnel surface. In this part of the tunnel, there is an electric light system used only during times of maintenance, so this stream offers an excellent opportunity to examine a transition in the microbial community structure along a hot water stream in the absence of sunlight. The input of plentiful energy and carbon sources such as methane, hydrogen, sulfide, carbon dioxide, and ammonium from the geothermal aquifer were considered as a likely determinant of the microbial community structure. In this study, we investigated the changes that occur in the microbial mat formed along the stream by applying a combination of 16S rRNA gene clone analysis, whole-cell fluorescent in situ hybridization (FISH) and competitive PCR (cPCR) for quantification of the key metabolic enzyme genes.

Materials and methods

Site description, sampling and measurement of physical properties

The location and geological setting of the Hishikari gold mine has been previously described (Izawa et al. 1990; Takai et al. 2002; Inagaki et al. 2003). Our study site within this mine was a dense microbial mat formation along a 7-m geothermal water stream located in the deepest level of the mine (320 m below the land surface). This stream begins as a natural discharge of a subsurface aquifer through the cracks of the basement rocks (Shimanto-Supergroup) onto the surface of a mine tunnel, and terminates by emptying into a waste pool. An abundant filamentous microbial mat system was observed along the entire length of the stream, being somewhat thicker in the middle and lower sections.

Properties of the stream water, such as temperature, pH, dissolved oxygen (DO) content, oxidation-reduction potential (ORP), and conductivity were measured during the sampling time in April 2001, November 2001 and December 2002, with a multisensor probe U-21DX system (Horiba, Kyoto, Japan). The measurements conducted in November 2001 are summarized in Table 1. Mat samples for DNA extraction for PCR-mediated analyses were collected at three points of the stream in April 2001; the mat at the origin or discharging point (designated "upper mat"), the mat in the middle stream, 2.5 m from the origin (designated "middle mat"), and the mat in the lower stream, 6 m from the origin (designated "lower mat") (Table 1). For analyses of whole-cell FISH and interstitial water chemistry, the mats sampled in November 2001 at the corresponding upper, middle and lower site of the stream were used.

Samples were collected in sterile polypropylene tubes, which were: 1) frozen at −80°C prior to DNA extraction in the laboratory; 2) fixed in situ with 3.7% formaldehyde for whole-cell FISH analysis and stored at 4°C in the laboratory; and, 3) squeezed to separate interstitial porewater, which was kept cool for later chemical analysis (see below). Based on the geological setting, the discharging geothermal water is derived from the aquifer vein called AW-S, the physical properties and chemical compositions of which have been previously described (Takai et al. 2002).

Chemical analyses in interstitial water inside mat structure

The interstitial water contained in each of the mat structures was squeezed through a polypropylene syringe with a 0.22 μm pore-sized filter at the sampling time. The collected interstitial water was cooled on ice, placed in a plastic bag under 100% nitrogen, and transferred to a laboratory within 5 h. Ammonia was measured by Nessler's reagent (Allen et al. 1974). Nitrite, nitrate, and sulfate concentrations were measured by ion chromatography with a Shim-pack IC-A3 column (Shimadzu, Kyoto, Japan) with a buffer system consisting of 50 mM boric acid, 8 mM *p*-hydroxybenzoic acid, and 3.2 mM bis (2-hydroxyethyl) imino-tris (hydroxymethyl) methane.

Extraction of DNA

The mat samples were centrifuged at 5000× *g* for 10 min for removal of interstitial water, and 1 g of each mat material was used for DNA extraction, using a Soil DNA Mega Prep kit (Mo Bio Laboratories, Inc., Solana Beach, Calif.) according to the manufacturer's instructions.

Table 1 Physical properties of stream water and chemical components in mat-interstitial water

Stream[a]	Physical property of the water						Chemical component in the mat-interstitial water[f]			
	Temp[b] (°C)	pH	DO[c] (mg/L)	ORP[d] (mV)	Salinity (%)	Cond[e] (S/m)	NH_4^+ (μM)	NO_2^- (μM)	NO_3^- (μM)	SO_4^{2-} (μM)
Upper										
(Origin)	69	5.1	0.3	−130	0.1	0.2	116 ± 3	n.d.	n.d.	620
(0.4 m)	69	5.0	0.4	−85	0.1	0.2				
(1.5 m)	63	4.9	0.4	−32	0.1	0.2				
Middle										
(2.5 m)	62	4.9	0.4	−16	0.1	0.2	69 ± 2	159	173	598
(3.5 m)	59	4.8	0.5	28	0.1	0.2				
Lower										
(6.0 m)	55	4.8	0.6	40	0.1	0.2	52 ± 2	304	224	617
(7.0 m)	50	4.8	0.8	59	0.1	0.2				

[a] The measuring points in the stream were shown by the distance (in parentheses) from the discharging point (origin) of the stream
[b] Temperature
[c] Dissolved oxygen
[d] Oxidation-reduction potential
[e] Conductivity
[f] Colorimetric determination of ammonium was presented as mean ± standard deviation of triplicate test tubes. Nitrite, nitrate, and sulfate were determined by HPLC. Analytical errors in HPLC analysis were estimated within 4% for each standard substances

Bacterial 16S rRNA gene clone analysis and phylogenetic characterization

Bacterial 16S rRNA genes were amplified from the extracted DNA by PCR using the oligonucleotide primer set of Bac27F and Uni1492R (Lane 1991). A 50-μl volume of PCR reaction mixture containing 1×GC buffer I (TaKaRa Bio, Shiga, Japan), 0.2 mM each deoxynucleoside triphosphate, 0.4 μM each primer, 2.5 U of *LA Taq* polymerase (TaKaRa Bio), and 50 ng of DNA, was prepared. The amplification was performed with a thermal cycler GeneAmp 9600 (PE Applied Biosystems) with the following program for a total of 30 cycles; 96°C for 25 s, 50°C for 45 s, and 72°C for 120 s. Five replicate amplifications were pooled and separated via agarose gel electrophoresis to cut target bands from the gel. The rRNA genes were recovered by using the GelSpin DNA purification kit (Mo Bio Laboratories), and then cloned into vector pCR2.1 with a TA cloning kit (Invitrogen, Carlsbad, Calif.). The insert rRNA genes were directly amplified with M13 primers and treated with exonuclease I and shrimp alkaline phosphatase (Amersham Pharmacia biotech). The preparations were directly sequenced using a dRhodamine terminator cycle sequencing kit (PE Applied Biosystems) and ABI PRISM 3100 sequencing analyzer (PE Applied Biosystems). The primer Bac27F was used for the initial single-strand sequencing of bacterial rRNA gene sequences.

Sequence similarity among all of the single-strand rRNA gene sequences approximately 0.5 kb long was analyzed by the FASTA-composing algorithm in a DNASIS software (Hitachi Software, Tokyo, Japan). The sequences showing ≥97% identity by DNASIS analysis were assigned to the same clone type (phylotype). Each phylotype was represented by the clone showing the highest-sequence-matching with other clones within the same phylotype. Each phylotype was further subjected to sequencing and 0.7–1.5 kb of sequence was determined from both strands. The phylotype sequences were subjected to similarity search against the DDBJ/EMBL/GenBank databases by FASTA3, BLAST, and Smith-Waterman Search programs (http://www.ddbj.nig.ac.jp/E-mail/homology-j.html). The sequences were automatically aligned with closely related rRNA gene sequence data by using CLUSTALX ver.1.81 program and revised by manual removal of ambiguously aligned regions. Phylogenetic trees were constructed by neighbor-joining algorithm using CLUSTALX ver.1.81 program. Bootstrap analyses for 1000 replicates were performed to assign confidence levels to tree topology.

Whole cell FISH analysis

Specific oligonucleotide probes targeting unique regions of 16S rRNA in the predominant bacterial phylotypes (HAuD-UB26, HAuD-MB27 and HAuD-LB38) (see Table 2) were designed. For detection of the phylotype HAuD-UB5, the previously designed probe AF175 was used. (Takai et al. 2002). The probe sequences, positions in *Escherichia coli*, detectable phylotypes and microorganisms which had fully complementary sequences with the probes are summarized in Table 3.

Specificity of the probes was checked before the FISH experiment. Firstly, the probe sequences were checked by both Probe Match analysis in the Ribosomal Database Project II (http://rdp.cme.msu.edu/html/, Cole et al. 2003) and BLAST search. Secondly, all of the probes were analyzed in silico for the specificity and mismatches with sequences of the closely related phylotypes and microorganisms. Probe HAuD-UB26 had a three bases mismatch with a sequence of the closest bacterium thermophilic methanotroph HB (Bodrossy et al. 1999). Probe AF175 had one to three bases mismatches with the closest members in the phylogenetic tree. Probe HAuD-MB27 had one to three bases mis-

Table 2 Distribution of bacterial 16S rDNA phylotypes identified in the discharging hot water stream

Phylotype	No. of clones			Phylogenetic relationship			
	Upper	Middle	Lower	Close affiliation	Closest database match	% identity	Accession no.
HAuD-UB26	22			*γ-Proteobacteria*	Thermophilic methanotroph HB	90	U89299
HAuD-UB5	14			*Aquificales*	*Sulfurihydrogenobium subterraneus*	99	AB071324
HAuD-UB44	1			*Aquificales*	*Hydrogenobacter subterraneus*	98	AB026268
HAuD-UB10	10			Green nonsulfur	Hot spring clone OPB65	97	AF027036
HAuD-UB1	9			*β-Proteobacteria*	*Hydrogenophilus thermoluteolus*	98	AB009828
HAuD-UB30	5	1		OP1	Hot spring clone OPB14	83	AF027045
HAuD-UB8	2			CFB[c]	Hot spring clone OPS2	90	AF018187
HAuD-UB19	2			OP10	Hot spring clone SM2G08	93	AF445740
HAuD-UB28	2			*Nitrospira*	*Nitrospira moscoviensis*	93	X82558
HAuD-UB38	1			*Nitrospira*	*Nitrospira moscoviensis*	87	X82558
HAuD-UB41	1			*Thermus*/*Deinococcus*	*Thermus* sp. SA-01	99	AF020205
HAuD-UB36	1			*Clostridiales*	*Eubacterium* sp. OS type K	91	L04711
HAuD-LB9			1	*Clostridiales*	*Eubacterium* sp. OS type K	91	L04711
HAuD-MB3		1		*Actinomycetales*	*Actinomyces* sp. TM36	86	X92694
HAuD-MB47		1		OP10	Sludge clone SBR1039	85	X84482
HAuD-MB19		1		Green nonsulfur	Sludge clone H8	84	AF234705
HAuD-MB4		1		Green nonsulfur	Sludge clone A11b	87	AF234682
HAuD-MB35		1		Green nonsulfur	Sludge clone A11b	95	AF234682
HAuD-LB29		2	4	Green nonsulfur	Sludge clone S16	94	AF234759
HAuD-MB2-35		1		*Planctomycetales*	*Candidatus* "Brocadia anammoxidans"	95	AF375994
HAuD-MB10		2	2	*Planctomycetales*	*Planctomyces* sp. strain 599	90	AJ231189
HAuD-MB15		5		*Planctomycetales*	Soil clone#0319-7F4	80	AF234144
HAuD-MB30		4		*Planctomycetales*	Bioreactor clone mle1-8	81	AF280847
HAuD-LB25		1	5	*Planctomycetales*	Bioreactor clone mle1-8	87	AF280847
HAuD-LB24			1	*Planctomycetales*	Bioreactor clone mle1-8	86	AF280847
HAuD-MB13		4		*γ-Proteobacteria*	*Methylococcus capsulatus*	97	X72770
HAuD-LB16			4	*γ-Proteobacteria*	*Methylococcus capsulatus*	93	X72770
HAuD-MB34		1		*β-Proteobacteria*	*Thiobacillus aquaesulis*	93	U58019
HAuD-MB40		1		*β-Proteobacteria*	arsenite-oxidizing bacterium NT-6	98	AY027499
HAuD-MB8		2	1	*β-Proteobacteria*	Hot spring clone MS8	94	AF232922
HAuD-MB2-1		1		*β-Proteobacteria*	*Thiobacillus aquaesulis*	95	U58019
HAuD-MB46		1		*β-Proteobacteria*	*Thiobacillus aquaesulis*	92	U58019
HAuD-MB38		1	1	*β-Proteobacteria*	*Thiobacillus denitrificans*	95	AJ243144
HAuD-LB2-3			2	*β-Proteobacteria*	*Thiobacillus aquaesulis*	92	U58019
HAuD-LB2-20			1	*β-Proteobacteria*	Ground water clone GOUTA12	95	AY050584
HAuD-LB2-36			1	*β-Proteobacteria*	Hot spring clone MS8	95	AF232922
HAuD-LB10			1	*β-Proteobacteria*	Hishikari gold mine clone pHAuB-J	99	AB071327
HAuD-MB26		2		*β-Proteobacteria*	*Nitrosomonas* sp. NM 107	90	AF272416
HAuD-MB27		14	3	*β-Proteobacteria*	*Nitrosomonas* sp. NM 107	98	AF272416
HAuD-MB7		1		*Nitrospira*	*Nitrospira* sp. clone b2	93	AJ224038
HAuD-MB37		6		*Nitrospira*	*Nitrospira* sp. clone g6	98	AJ224039
HAuD-LB38		16	28	Nitrospira	*Nitrospira moscoviensis*	98	X82558
HAuD-LB2-23			1	*Nitrospira*	*Nitrospira moscoviensis*	92	X82558
HAuD-LB13			1	*Nitrospira*	*Nitrospira moscoviensis*	88	X82558
HAuD-LB1			1	*Nitrospira*	*Nitrospira moscoviensis*	78	X82558
HAuD-LB2-47		1	8	*Nitrospira*	*Thermodesulfovibrio islandicus*	90	X96726
HAuD-LB48			1	*Nitrospira*	*Thermodesulfovibrio islandicus*	87	X96726
HAuD-LB4			4	CFB[a]/Green sulfur	Hot spring clone OPB 56	87	AF027009

[a]CFB, Cytophaga-Flexibacter-Bacterioides

matches with sequences from *Nitrosomonas* spp. and *Nitrosospira* spp. except for the species listed in Table 4. Probe HAuD-LB38 had four bases mismatch with a sequence of closely related phylotype HAuD-MB7. In addition, probe HAuD-LB38 had three bases mismatch with *Nirospira marina* and five or more bases mismatches with the relatively distant phylotypes HAuD-LB1, HAuD-LB48 and HAuD-LB/2-47. Thirdly, the specificity of the designed probes in the libraries was experimentally confirmed by dot blot hybridization analysis with amplified 16S rRNA gene fragments from dominant phylotypes, as previously described (Takai et al. 2002). Each probe had 4 to 14 bases mismatches with other predominant phylotype sequences from all three libraries.

The whole cell FISH experiment was performed as previously described (Takai et al. 2002). The dispersed mat structure that was fixed with 3.7% formaldehyde was immobilized on 3-aminopropyltriepoxysilane-coated slide and hybridized with fluorescein-labeled probes in a buffer containing 30% formamide at temperature optimized for each probe (Table 3). After hybridization, the slide was washed at a temperature 5°C lower than the hybridization temperature with 50%

Table 3 FISH probes and estimated population ratios of the detected cells in the mat

Probe designation	Probe sequence (5′ to 3′)	Positions in *E. coli*	Hybridization temperature (°C)	Detectable phylotypes & microorganisms	% of total cells		
					Upper	Middle	Lower
HAuD-UB26 probe	CCTTTAGGGTATTCGCCTAA	458-477	44	HAuD-UB26 pHAuB-P (Inagaki et al. 2003) (*γ-Proteobacteria*)	25	0.1	1
AF175 probe	GGGCTTTGGAGTCCCCTCTT	182-208	50	HAuD-UB5 pHAuB-D (Takai et al. 2002) *Sulfurihydrogenibium subterraneum* (*Aquificales*)	17	2	0.4
HAuD-MB27 probe	TTAAGGAYACGTTCCGATGYA	121-141	44	HAuD-MB26 HAuD-MB27 *Nitrosomonas europaea/ Nitrosococcus mobilis*-cluster spp. *Nitrosomonas ureae* *Nitrosospira multiformis* (*β-Proteobacteria*)	0.1	30	26
HAuD-LB38 probe	SAGGTACCGTCCGAACG	471-491	46	HAuD-UB28 HAuD-UB38 HAuD-MB37 HAuD-LB38 HAuD-LB13 HAuD-LB/2-23 *Nitrospira moscoviensis* (*Nitrospirae*)	0.1	52	66

Table 4 Primers used for detection and quantification of the key enzyme genes in this study

Primer	Sequence (5' to 3')	Target gene	Reference
A189F	GGNGACTGGGACTTCTGG	*pmoA*	Holmes et al. 1995
mb661R	CCGGMGCAACGTCYTTACC	*pmoA*	Costello & Lidstrom 1999
301F	GACTGGGACTTCTGGCTGGACTGGAA	*amoA*	Norton et al. 2002
302R	TTTGATCCCCTCTGGAAAGCCTTCTTC	*amoA*	Norton et al. 2002
norB-196F	TGCATCGGTTGCCACACCTGCAGCATC (positions 46-72)[a]	*norB*, *narH*, *narY*	This study
norB-870R	CCAGTTGAAGTAGGTCTTCTTGTACGGG (positions 693-720)[a]	*norB*, *narH*, *narY*	This study
Ns-norB-454F	TACTAYGARCCCTGGACTTACRA (positions 304-326)[a]	*norB*	This study
Ns-norB-710R	ATGCGYGGSAWRTAGAAGWAMAMSA (positions 536-560)[a]	*norB*	This study

[a]The positions of the sequences is in *norB* of *Nitrobacter hamburgensis*

formamide-containing buffer. The fluorescence signal from the probes was intensified as previously described (Takai et al. 2002). Finally, the slide was stained with 4',6-diamidino-2-phenylindole (DAPI) solution (10 μg ml^{-1}) and examined under an Olympus BX51 epifluorescence microscope with a Spot RT slider cooled digital camera system (Diagnostic Instruments, Inc., Sterling Heights, Mich.). Over 1000 DAPI-stained cells were counted to estimate the ratio of probe-hybridized cells to the total number of cells.

Amplification and phylogenetic analysis of *pmoA*, *amoA*, and putative *norB* genes

The genes encoding components of the enzymes, particulate methane monooxygenase (*pmoA*), ammonia monooxygenase (*amoA*), and putative nitrite oxidoreductase (*norB*), were amplified by PCR from genomic DNA extracted from the mat. The PCR reaction mixtures were prepared as described above for 16S rRNA gene amplification. Primers used for amplification are listed in Table 4. For amplification of *pmoA*, the reported primers A189F (Holmes et al. 1995) and mb661R (Costello and Lidstrom 1999) were used in the following PCR program for 30 cycles; 96°C for 25 s, 54°C for 45 s, and 72°C for 50 s. For amplification of *amoA*, the described primers 301F and 302R (Norton et al. 2002) were used in the following PCR program for 30 cycles; 96°C for 25 s, 58°C for 45 s, and 72°C for 1 min. For amplification of putative *norB*, the newly designed primers Ns-norB-454F and Ns-norB-710R were used in the following PCR program for 30 cycles; 96°C for 25 s, 53°C for 45 s, and 72°C for 25 s. The PCR products

were purified by electrophoresis, recovered, and cloned into vector pCR2.1 (Invitrogen). Forty to 46 clones from each library were randomly sequenced with M13 primers. All the procedures were performed as described above for 16S rRNA genes.

The clone sequences showing ≥97% DNA identity by DNASIS analysis were assigned to the same phylotype. Each phylotype was represented by the clone showing the highest-sequence-matching with other clones within the same phylotype. The DNA and deduced amino acid sequences of the phylotypes were compared with sequences available in the databases by using the BLAST Search program. Phylogenetic trees of the deduced amino acid sequences were constructed with procedures as described above for trees of 16S rRNA genes.

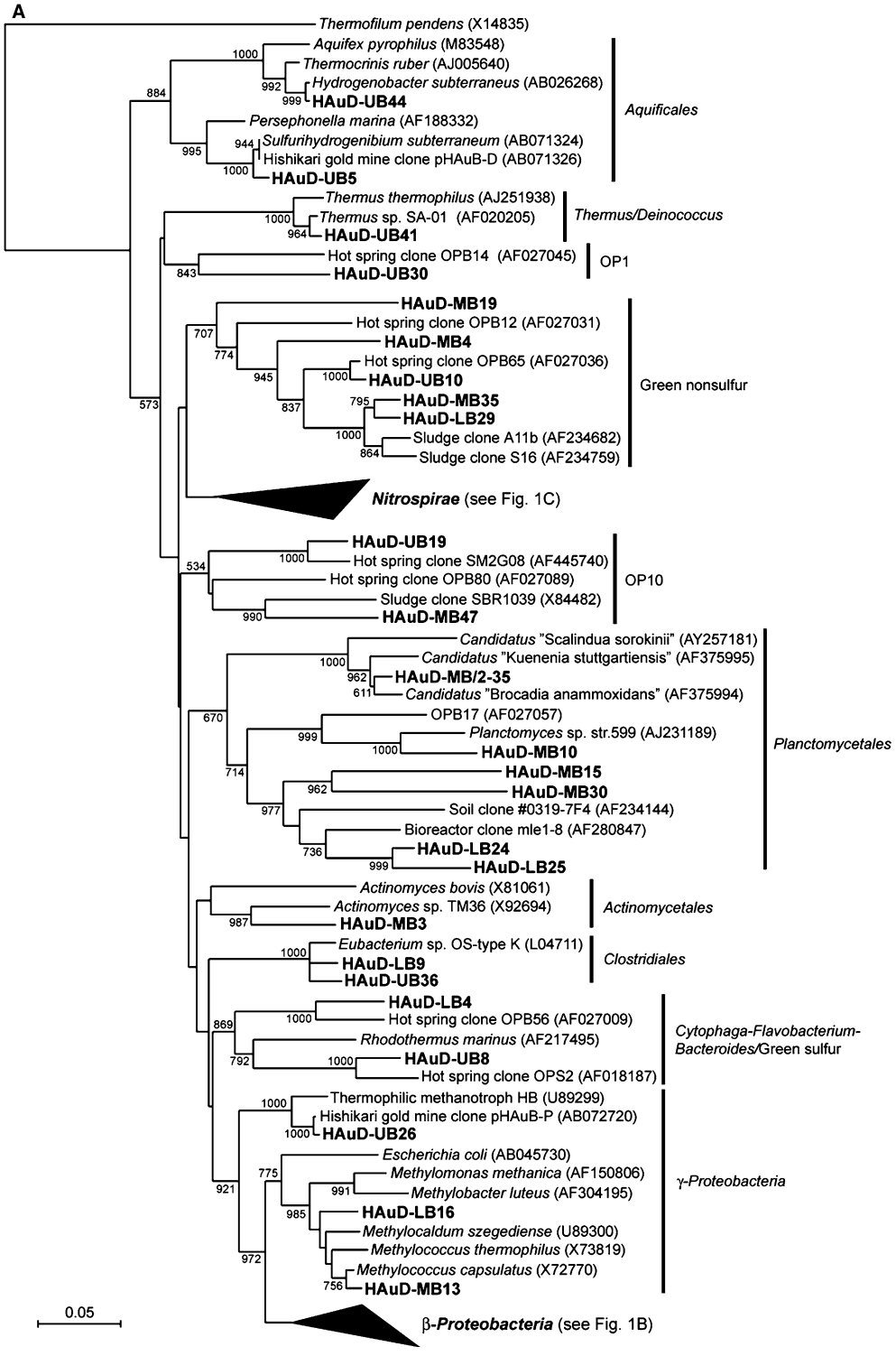
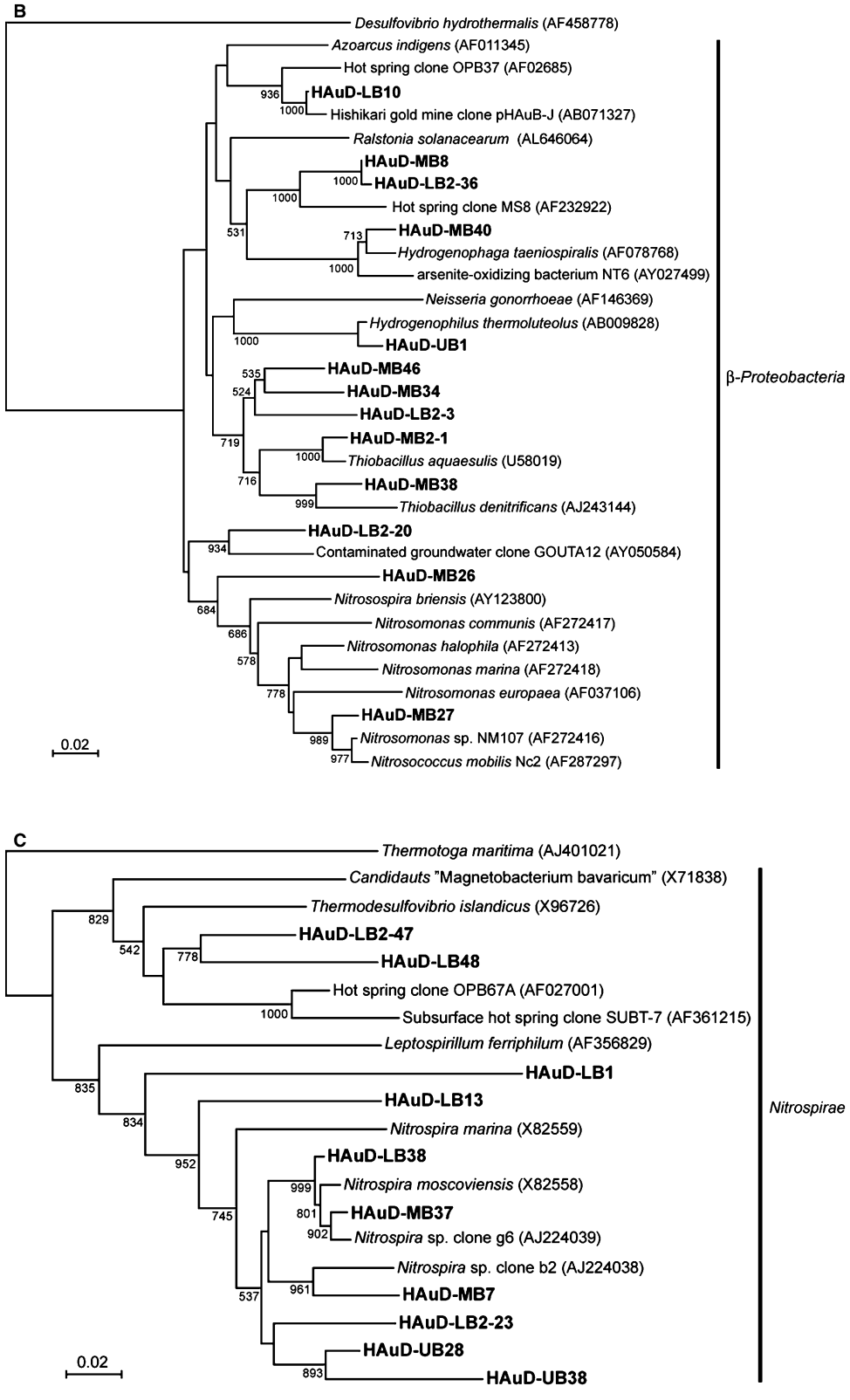


Fig. 1 **A** Phylogenetic tree of 16S rRNA gene sequences of representative bacterial strains and phylotypes based on neighbor-joining analysis with 520 homologous positions in sequences. The numbers at nodes are bootstrap confidence values based on 1000 replicates. Only values higher than 50% are shown. The bar indicates the expected number of changes per sequence position. Boldface type indicates rRNA gene clones obtained in this study. **B** Phylogenetic tree of 16S rRNA gene sequences of representative *β-proteobacteria* strains and phylotypes based on neighbor-joining analysis with 571 homologous positions in sequences. The numbers at nodes are bootstrap confidence values based on 1000 replicates. Only values higher than 50% are shown. The bar indicates the expected number of changes per sequence position. Boldface type indicates rRNA gene clones obtained in this study. **C** Phylogenetic tree of 16S rRNA gene sequences of representative *Nitrospirae* strains and phylotypes based on neighbor-joining analysis with 620 homologous positions in sequences. The numbers at nodes are bootstrap confidence values based on 1000 replicates. Only values higher than 50% are shown. The bar indicates the expected number of changes per sequence position. Boldface type indicates rRNA gene clones obtained in this study

Fig. 1 (Contd.)



Quantification of *pmoA*, *amoA*, and *norB* by competitive PCR

DNA competitors for competitive PCR (cPCR) analyses were constructed by PCR amplifications to quantify *pmoA*, *amoA*, and *norB* in the mat DNA assemblage. The 407 bp-competitor for *pmoA* was generated from 509 bp of *pmoA* sequence in pmoU1 clone, by using the A189F primer and a newly designed internal primer having the mb661 primer sequence at that end. The

Table 5 Clone analyses of PCR products generated with specific primer sets for paticulate methane monooxygenase gene *pmoA* in methane oxidizers (A), ammonia monooxygenase gene *amoA* in ammonia oxidizers (B), and nitrite oxidoreductase gene *norB* in the relatives of *Nitrospirae* members (C)

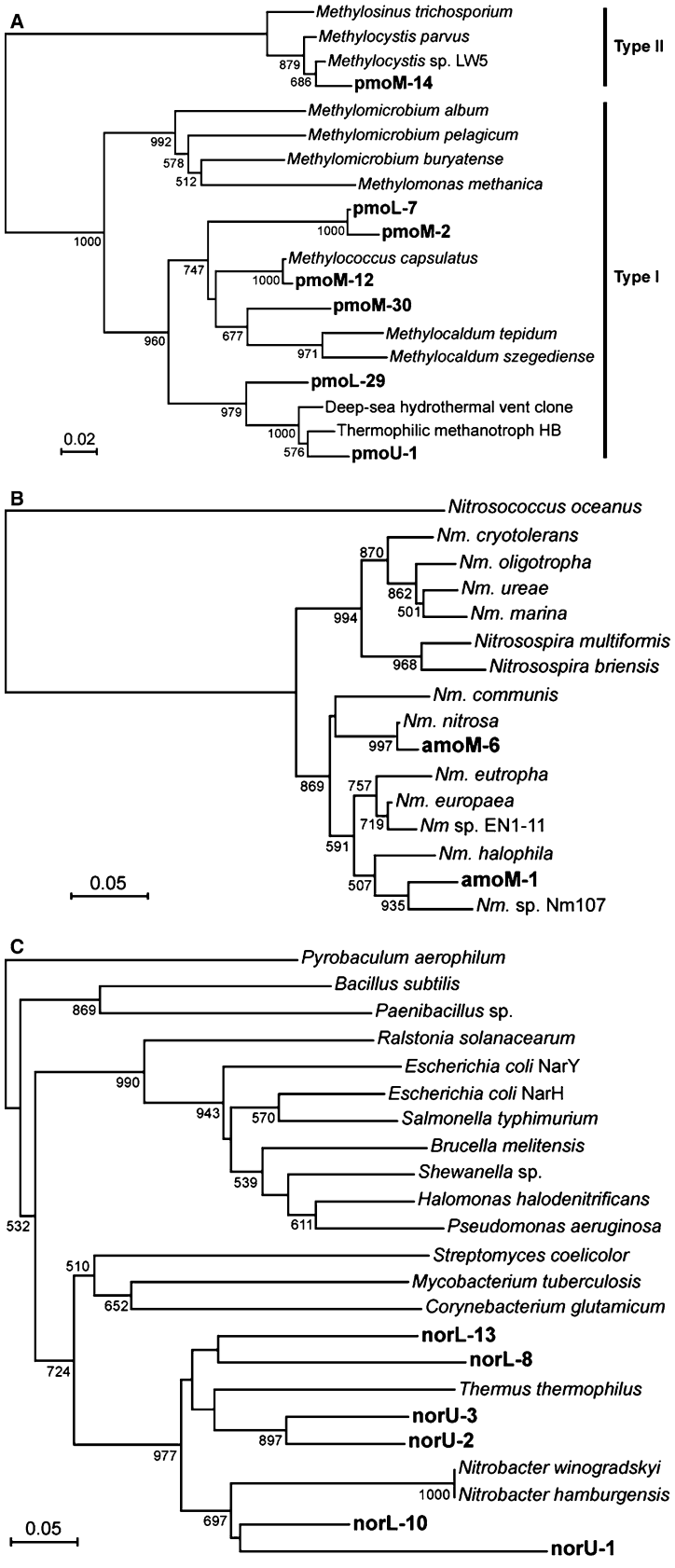
Phylotype	No. of clones			% identity in deduced amino acid	Closest database match	Accession no.
	Upper	Middle	Lower			
(A) Clone analysis of methane monooxygenase gene *pmoA*[a]						
pmoU-1	43	1	13	98% in 160 aa	*pmoA* in thermophilic methanotroph HB	U89302
pmoM-2		10	23	88% in 160 aa	*pmoA* in *Methylococcus capsulatus*	U94337
pmoM-12		17		99% in 169 aa	*pmoA* in *Methylococcus capsulatus*	U94337
pmoM-14		1		98% in 160 aa	*pmoA* in *Methylocystis* sp.	AF150791
pmoM-30		1		91% in 169 aa	*pmoA* in *Methylococcus capsulatus*	U94337
pmoL-7			1	88% in 169 aa	*pmoA* in *Methylococcus capsulatus*	U94337
pmoL-29			1	91% in 169 aa	*pmoA* in thermophilic methanotroph HB	U89302
Others		13	5			
(B) Clone analysis of ammonia monooxygenase gene *amoA*[b]						
amoM-1	–[c]	33	40	91% in 225 aa	*amoA* in *Nitrosomonas europaea*	AF058691
amoM-6	–[c]	7		99% in 151 aa	*amoA* in *Nitrosomonas nitrosa*	AF272404
(C) Clone analysis of nitrite oxidoreductase gene *norB*[d]						
norL-10	3	40	30	72% in 85 aa	*norB* in *Nitrobacter hamburgensis*	X66067
norU-3	6			58% in 85 aa	*norB* in *Nitrobacter hamburgensis*	X66067
norU-2	7			59% in 85 aa	*norB* in *Nitrobacter hamburgensis*	X66067
norU-1	1			68% in 68 aa	*norB* in *Nitrobacter hamburgensis*	X66067
norL-8			5	58% in 84 aa	*norB* in *Nitrobacter hamburgensis*	X66067
norL-13			7	58% in 85 aa	*norB* in *Nitrobacter hamburgensis*	X66067
Others	27	4	4			

[a]Each phylotype contains clones of sequences showing >98.5% identities
[b]Each phylotype contains clones of sequences showing >99% identities
[c]Clone was not obtained from the upper mat
[d]Each phylotype contains clones of sequences showing >97% identities

569 bp-competitor for *amoA* was generated from 675 bp of *amoA* sequence in amoM1 clone, by using the 301F primer and a newly designed internal primer having the 302R primer sequence at that end. The 202 bp-competitor for *norB* was generated from 257 bp of *norB* sequence in a norL10 clone, by using a newly designed internal primer having a Ns-norB-454F primer sequence at that end and a Ns-norB-710R primer. Each competitor fragment was cloned into vector pCR2.1 (Invitrogen) and amplified by PCR. The amplified competitor fragments were purified with a SUPREC-02 filter column (TaKaRa Bio) and quantified by measurement of OD_{260} with a DU7400 Spectrophotometer (Beckman Coulter, Inc., Fullerton, CA).

►

Fig. 2 **A** Phylogenetic tree of deduced PmoA sequences from methane-oxidizing α- and *γ-proteobacteria*, and *pmoA* clones obtained in this study, based on neighbor-joining analysis with 160 homologous positions in amino acid sequences. The numbers at nodes are bootstrap confidence values based on 1000 replicates. Only values higher than 50% are shown. The bar indicates the expected number of changes per sequence position. The accession numbers of the referred PmoA sequences are: Thermophilic methanotroph HB, U89302; *Methylocaldum.tepidum*, U89304; *Methylocaldum.szegediense* U89303; *Methylococcus. capsulatus*, U94337; *Methylomicrobium pelagicum*, U31652; *Methylomonas methanica*, U31653; *Methylomicrobium buryatense*, AF307139; *Methylomicrobium album*, U31654; *Methylocystis* sp. LW5, AF150791; *Methylocystis parvus*, AJ459042; *Methylosinus trichosporium*, AJ431388; Deep-sea vent clone, AY354042. **B** Phylogenetic tree of deduced AmoA sequences from ammonia-oxidizing *β-proteobacteria* and *amoA* clone obtained in this study, based on neighbor-joining analysis with 136 homologous positions in amino acid sequences. The numbers at nodes are bootstrap confidence values based on 1000 replicates. Only values higher than 50% are shown. The bar indicates the expected number of changes per sequence position. The accession numbers of the referred AmoA sequences are: *Nitrosomonas* sp. Nm107, AF272407; *Nitrosomonas halophila*, AF272398; *Nitrosomonas communis*, AF272399; *Nitrosomonas eutropha* U51630; *Nitrosomonas* sp. EN1-11, AB079055; *Nitrosomonas europaea*, AF058691; *Nitrosomonas nitrosa* AF272404; *Nitrosomonas cryotolerans*, AF272402; *Nitrosomonas oligotropha* AF272406; *Nitrosomonas ureae*, AF272403; *Nitrosomonas marina*, AF272405; *Nitrosospira briensis*, U76553; *Nitrosospira multiformis*, U91603; *Nitrosococcus oceanus*, U96611. **C** Phylogenetic tree of deduced NorB sequences from nitrite-oxidizing *Nitrobacter* spp. within *α-proteobacteria*, similar sequences encoding *β*-subunits of nitrate reductases in several microorganisms, and *norB* clones obtained in this study, based on neighbor-joining analysis with 82 homologous positions in amino acid sequences. The numbers at nodes are bootstrap confidence values based on 1000 replicates. Only values higher than 50% are shown. The bar indicates the expected number of changes per sequence position. The accession numbers of the referred sequences of NorB in nitrite oxidoreductase and *β*-subunits in nitrate reductases are: *Nitrobacter hamburgensis*, X66067; *Nitrobacter winogradskyi*, AY508477; *Thermus thermophilus*, Y10124; *Mycobacterium tuberculosis*, AE006997; *Corynebacterium glutamicum*, AP005277; *Streptomyces coelicolor*, AL109989; *Paenibacillus* sp., AJ288131; *Bacillus subtilis*, Z49884; *Pyrobaculum aerophilum* AE009941; *Halomonas halodenitrificans*, AB076402; *Pseudomonas aeruginosa*, Y15252; *Shewanella* sp. AJ288123; *Brucella melitensis*, AE009728; *Escherichia coli* NarH, X16181; *Salmonella typhimurium*, AE008778; *Escherichia coli* NarY, AE000243; *Ralstonia solanacearum*, AL646082


A
Methylosinus trichosporium
Methylocystis parvus
Methylocystis sp. LW5
pmoM-14
Type II
Methylomicrobium album
Methylomicrobium pelagicum
Methylomicrobium buryatense
Methylomonas methanica
pmoL-7
pmoM-2
Methylococcus capsulatus
pmoM-12
pmoM-30
Methylocaldum tepidum
Methylocaldum szegediense
pmoL-29
Deep-sea hydrothermal vent clone
Thermophilic methanotroph HB
pmoU-1
Type I
992
578
512
1000
1000
747
1000
677
971
960
979
1000
576
879
686
0.02
B
Nitrosococcus oceanus
Nm. cryotolerans
Nm. oligotropha
Nm. ureae
Nm. marina
Nitrosospira multiformis
Nitrosospira briensis
Nm. communis
Nm. nitrosa
amoM-6
Nm. eutropha
Nm. europaea
Nm sp. EN1-11
Nm. halophila
amoM-1
Nm. sp. Nm107
870
862
501
994
968
997
869
757
719
591
507
935
0.05
C
Pyrobaculum aerophilum
Bacillus subtilis
Paenibacillus sp.
Ralstonia solanacearum
Escherichia coli NarY
Escherichia coli NarH
Salmonella typhimurium
Brucella melitensis
Shewanella sp.
Halomonas halodenitrificans
Pseudomonas aeruginosa
Streptomyces coelicolor
Mycobacterium tuberculosis
Corynebacterium glutamicum
norL-13
norL-8
Thermus thermophilus
norU-3
norU-2
Nitrobacter winogradskyi
Nitrobacter hamburgensis
norL-10
norU-1
869
990
943
570
539
611
532
510
652
724
897
977
1000
697
0.05

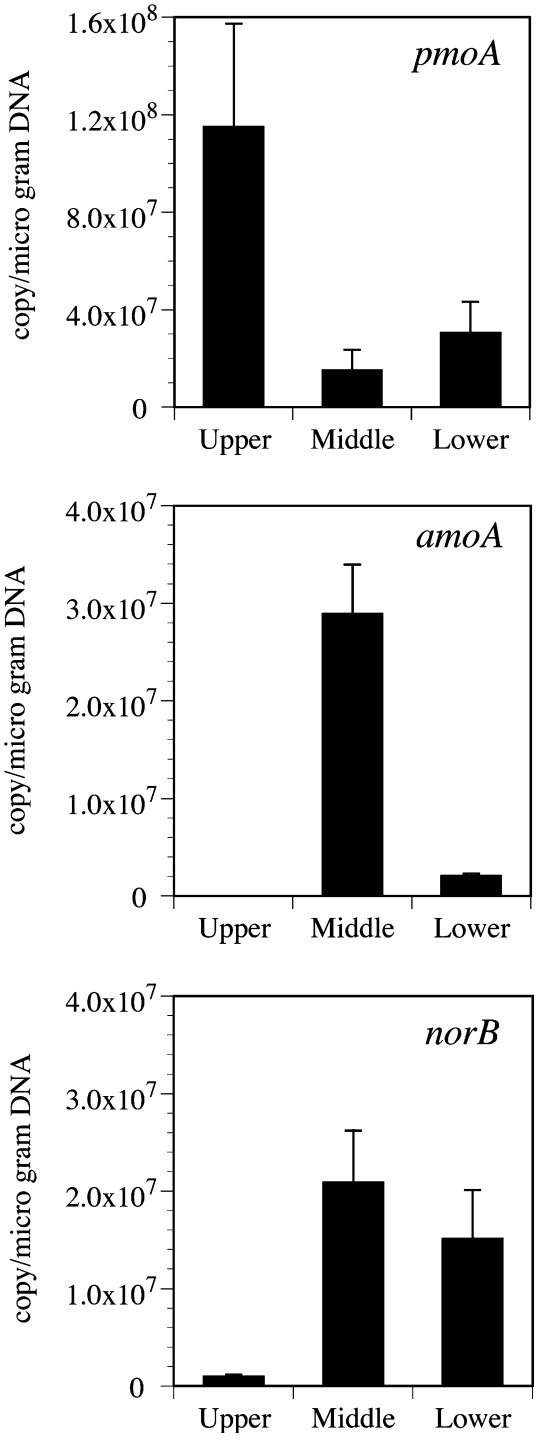


Fig. 3 The amounts of *pmoA*, *amoA*, and *norB* genes in microbial mat DNA assemblages from the upper, middle and lower stream. The content of the each gene was quantified by cPCR analysis and estimated to show as gene copy number per μg of microbial mat DNA. The averages of three independent trials are presented with the standard deviations

cPCR was performed to quantify *pmoA*, *amoA*, and *norB* genes in the mat DNA assemblages. The cPCR was carried out in 25 μl (total volume) mixture containing 1×GC buffer I (TaKaRa Bio), 0.2 mM each deoxynucleoside triphosphate, 0.4 μM each primer, 2.5 U of *LA Taq* polymerase (TaKaRa Bio), 100 ng of mat DNA, and a dilution of a DNA competitor. Seven-series of half dilutions of the competitor, with the range from 25000 to 1×10^8 copies, were employed for each calculation. The employed primers and thermal cycle programs were same as described for the library construction of enzyme genes. PCR products were separated by electrophoresis by using 2.5% (w/v) agarose for 50–800 bp fragment (Nacalai tesque, Inc., Kyoto, Japan) with 1×TBE buffer (90 mM Tris, 90 mM Boric acid, 2 mM Na_2-EDTA [pH 8.0]), and stained with SYBR Green I Nucleic Acid Gel Stain (Molecular Probes). The fluorescence intensities of the individual bands were measured with a Typhoon 8600 variable mode imager (Amersham Biosciences) and quantitatively analyzed with ImageQuant ver. 5.1 software (Amersham Biosciences). The calculation of competition equivalence point was performed as previously described (Dionisi et al., 2002).

To adjust differences in fluorescence intensities caused by size differences of the products from genomic DNA and the competitor, the fluorescence intensities of the products from genomic DNA were corrected with factors, 0.80 (407/509) for *pmoA*, 0.84 (569/675) for *amoA*, and 0.79 (202/257) for *norB*, before calculations of the competition equivalence points. Amplification efficiencies were assumed to be equal in the target and the competitor.

Nucleotide sequence accession number

The 16S rRNA gene, *pmoA*, *amoA*, and *norB* sequences determined in this study were deposited to DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers as follows: HAuD-UB, AB113582-AB113593; HAuD-MB, AB113594-AB113611, AB176695-176696; HAuD-LB, AB113612-AB113622, AB176697-176701; *pmoA*, AB113636-113639, AB176703-176705; *amoA*, AB113640, AB176702; *norB*, AB113641-AB113646.

Results

Physical and chemical properties of the stream water

The water with a high temperature and a low ORP value at the discharging point was consistent with the highly reductive state of the original hot aquifer water (Takai et al. 2002) (Table 1). In the middle to lower stream, the temperatures lowered and ORP values rose. A slight decrease in the pH value and increase in DO content were also observed in the lower stream. The low levels of salinity and conductivity, and the low sulfate in the original aquifer water (514 μM) (Takai et al. 2002) and mat-interstitial water (average 612 μM) indicated the geothermal water in this mine is like freshwater as previously reported (Izawa et al. 1990). Slightly bubbling gas was observed at the discharging point, which is de-

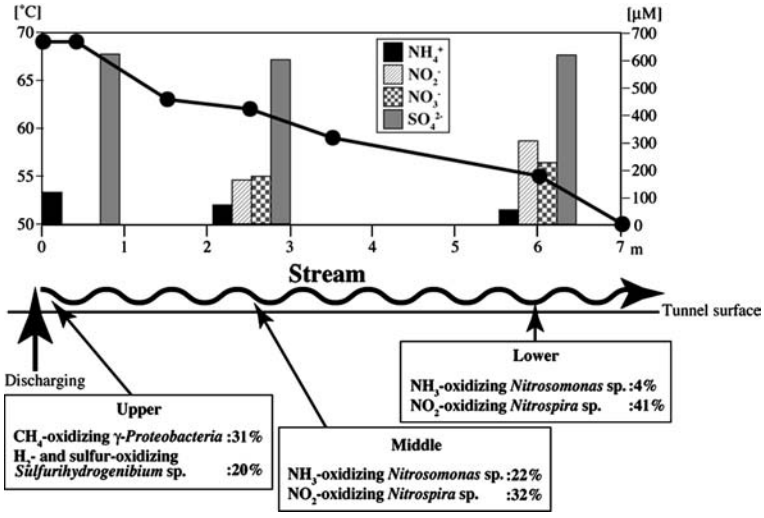


Fig. 4 The summary of changes in the predominat microorganisms, chemical components, and temperature of the stream. Solid circle shows the temperature (a left y-axis) at each point in the stream of 7-m long. The contents of NH_4^+, NO_2^-, NO_3^-, and SO_4^{2-} are expressed by bars (a right y-axis)

rived from the original aquifer vein as well as the discharging water (Inagaki et al. 2003; Takai et al. 2002). Physical properties of the water have been measured three times in one and half years, and the same patterns of the changing properties along the stream have been seen each time, which is likely to mean that a stable supply of subsurface aquifer water into the stream has been continuing for at least several years.

Phylogenetic diversity of bacteria in hot water stream microbial mats

The sequence similarity analysis of 70–72 bacterial clones per each microbial mat sample (total 213 clones) revealed a significant transition in bacterial community structure from the upper to the middle and lower parts of the mat (Table 2). About half of the clones from the upper mat area were accounted for by two phylotypes, HAuD-UB26 (22 of 70 clones) and HAuD-UB5 (14 of 70 clones), both of which had previously been identified from vein waters of this mine (Inagaki et al. 2003; Takai et al. 2002). The former is closely related to a thermophilic methanotroph strain HB within *γ-Proteobacteria* isolated from a hot spring in Hungary (Bodrossy et al. 1999) (Fig. 1A), while the latter is closely related to the phylotype pHAuB-D previously detected in the vein water (Takai et al. 2002) and a member of the genus *Sulfurihydrogenibium*, a representative of which was previously isolated from the vein water of this mine (Takai et al. 2003) (Fig. 1A). The remainder of the upper mat population contained a variety of phylotypes related to thermophilic microorganisms commonly detected in global terrestrial geothermal environments (Hayashi et al. 1999; Hugenholtz et al. 1998, Kieft et al. 1999, Takai et al. 2001). Minor but unique phylotypes moderately related to *Nitrospira moscoviensis*, known to be mesophilic nitrite-oxidizers (Ehrich et al. 1995), were also obtained from the upper mat.

In the middle mat, half of the 72 clones were closely related to nitrifying bacteria, including 16 clones closely or moderately related to ammonia-oxidizing *Nitrosomonas* sp. Nm107 (Purkhold et al., 2000) within the *β-Proteobacteria* (Fig. 1B) and 24 clones related to nitrite-oxidizing *Nitrospira moscoviensis* (Ehrich et al. 1995) and other *Nitrospira* spp. (Schramm et al. 1998) (Fig. 1C). The lower mat was similar in overall composition to the middle mat, but had a higher percentage of phylotypes related to nitrite-oxidizers (40 of 71 clones). Phylotypes in the *Planctomycetales*, including a close relative of members responsible for anaerobic ammonium oxidation (anammox), were also abundant in the middle and lower mats (Fig. 1A). In addition, phylotypes of the Green-Non-Sulfur Group (but distinct from HAuD-UB10 from the upper mat), *β-Proteobacteria* phylotypes apparently different from the *Nitrosomonas* lineage, and the relatives of type I methanotrophs within the *γ-Proteobacteria* were present (Fig. 1A, B).

The cells of predominant phylotypes in the mats were examined by whole-cell FISH. The ratios of the number of probe-positive cells to number of DAPI-stained cells are shown in Table 3. The estimated ratios were relatively consistent with the abundance of the phylotypes in 16S rRNA gene analyses.

Chemical shift in interstitial water of microbial mat structure

Since 16S rRNA gene clone analyses and FISH observations in the middle and lower mat revealed the predominance of phylotypes associated with ammonia- and nitrite-oxidizing bacteria, the mat community was expected to influence the amount of inorganic nitrogen compounds such as ammonia, nitrite, and nitrate in the mat structure.

Ammonium was detected at the highest concentration (116 μM) in the upper mat, and decreased to 69 μM in middle and 52 μM in lower mat-interstitial water

(Table 1). The nitrite concentration in the upper mat-interstitial water was undetectable, but rose to 159 μM in the middle mat, and 304 μM in the lower mat-interstitial water. Similarly, nitrate was undetectable in the upper mat zone, and easily detectable in the middle and lower mat-interstitial waters (173 μM and 224 μM, respectively). In contrast, sulfate remained constant throughout. The chemical properties of the mat interstitial water (at the discharge point) are similar to those of the vein aquifer water source previously characterized, with the exception that small amounts of nitrate (27 μM) were detected in the aquifer (Takai et al. 2002).

Phylogenetic characterization of *pmoA*, *amoA*, and putative *norB*

On the basis of the clone analyses, the FISH observations, and the chemical analysis of interstitial waters, we selected three genes for copy number analysis, with the goal of looking for patterns of specific metabolic gene abundance in the mat environment. These included *pmoA* for particulate methane monooxygenase, *amoA* for ammonia monooxygenase, and the putative *norB* for nitrite oxidoreductase. The obtained phylotypes of *pmoA*, *amoA*, and putative *norB*, and the results of their similarity analyses are summarized in Table 5A-C. Phylogenetic trees based on deduced amino acids of PmoA, AmoA, and putative NorB are shown in Fig.2A-C.

From the upper mat, a single phylotype of *pmoA* (pmoU-1) was detected and the deduced PmoU-1 clustered with PmoA in the thermophilic methanotroph strain HB (Bodrossy et al. 1999) within the *γ-Proteobacteria* (Table 5A, Fig. 2A). In the middle mat, two *pmoA* clone types pmoM-2 and pmoM-12 were predominant, and the deduced PmoM-2 and PmoM-12 clustered with PmoA in the type I methanotrophs *Methylococcus* and *Methylocaldum*. In the lower mat, pmoU-1 and pmoM-2 were most abundant phylotypes. These results were consistent with those of rRNA gene clone analyses, namely; the most abundant rRNA gene and *pmoA* clone types in the upper mat were derived from putative thermophilic methanotrophs similar to thermophilic methanotroph strain HB, and a portion of rRNA gene clones and the *pmoA* clones in the downstream mats were from mesophilic and moderately thermophilic methanotrophs similar to *Methylococcus* and *Methylocaldum* spp.

From the upper mat DNA, no *amoA* fragment was amplified by repeated PCR experiments. Two *amoA* phylotypes amoM-1 and amoM-6 were detected from the middle mat, and amoM-1 was also obtained from the lower mat as a single phylotype (Table 5B). The phylogenetic tree of deduced AmoA showed that the predominant clone type amoM-1 was in the same branch as *Nitrosomonas* sp. Nm107 (Purkhold et al. 2000) (Fig.2B). This presented a good agreement with the result of rRNA gene clone analysis that the most abundant clone type HAuD-MB27 was highly associated with *Nitrosomonas* sp. Nm107 which was phylogenetically affiliated to the *Nitrosomonas europaea*/*Nitrosococcus mobilis* cluster.

The cloning process of putative *norB* was a little complicated because there had been no available *norB* sequence data from *Nitrospira* spp. and only *norB* sequences from *Nitrobacter* spp. within the *α-Proteobacteria*. The amino acid sequence of NorB in *Nitrobacter hamburgensis* was known to have significant similarity with the *β*-subunits (NarH, NarY) of the two dissimilatory nitrate reductases (NRA, NRZ) in *E. coli* (Kirstein and Bock 1993). To obtain a putative *norB* from the microbial mat DNA, a primer set binding to high similarity regions existing commonly among *norB*, *narH*, and *narY* in several microorganisms was first designed. The designed primers were norB-196F and norB-870R (Table 4). The primer set norB-196F/norB-870R generated approximately 660 bp fragments from the mat DNA, and 32 clones of the fragments were obtained, sequenced, and subjected to similarity analysis. Most of the sequences obtained indicated much higher similarity with *norB* in *N. hamburgensis* than with any known *narH* and *narY* sequences. From a multiple alignment of the clone sequences, *norB* in *N. hamburgensis* and *N. winogradskyi*, and available sequences encoding *β*-subunits of nitrate reductases in several microorganisms, the regions specific to *norB* were screened and finally the primers Ns-norB-454F and Ns-norB-710R were designed.

Table 5C shows the summary of putative *norB* clone analysis using the primer set Ns-norB-454F/Ns-norB-710R. Almost all clone types indicated relatively high similarity with NorB in *N. hamburgensis*. The phylogenetic tree for NorB and the *β*-subunit of nitrate reductases represented a monophyletic lineage clustering NorB with *Nitrobacter* spp., the *β*-subunit of nitrate reductase in *Thermus thermophilus*, and all of the putative NorB sequences obtained from the microbial mats (Fig. 2C). Within this lineage, three groups were observed; one containing NorB in *Nitrobacter* spp.; another containing *β*-subunit of nitrate reductase in *T. thermophilus*; the other of NorB from clone types norL-8 and norL-13. The most abundant clone type norL-10 in the middle and lower mat was affiliated to the group containing NorB from *Nitrobacter* spp..

Quantification of *pmoA*, *amoA*, and putative *norB* by competitive PCR

A cPCR assay was conducted for the quantification of *pmoA*, *amoA*, and *norB* in the microbial mat DNA assemblages. In the estimation of competition equivalence points by the amount of cPCR products from both mat DNA and competitors, the regression lines to calculate copy numbers of the genes in the mat DNA indicated high correlation coefficients (r^2) as >0.97 ($n=7$), which indicates statistically significant linearity (data not shown).

In the upper mat, the abundant *pmoA* gene was detected, whereas *norB* was scarce (only 1% of the amount of *pmoA*) and *amoA* was below the detection limit (approximately $< 5\times10^4$ copies per μg DNA under the employed experimental conditions) (Fig.3). This result revealed the predominant occurrence of methane oxidation metabolism by *γ-Proteobacteria* and very little ammonia oxidation by *β-Proteobacteria* in the upper mat. The middle mat contained the highest amount of *amoA*, and slightly lower amounts of *norB* and *pmoA*, which was relatively in agreement with the co-existence of the members of ammonia-oxidizing *Nitrosomonas*/*Nitrosococcus*, nitrite-oxidizing *Nitrospira*, and methane-oxidizing *Methylococcus* on the basis of the 16S rRNA gene clone analysis and FISH observation. Comparison of the middle and lower mats showed similar levels of *pmoA* and *norB*, and less *amoA* in the lower mat. The results implied a significant population capable of oxidizing nitrite in the lower mat.

Discussion

When hot subsurface anoxic waters emerge into more moderate temperature, aerated oxic environments, they provide nutrient-rich environments for a variety of moderately thermophilic organisms. In this study, we have characterized three zones of a microbial mat community that occur in a subsurface geothermal stream without light. The various analytical techniques examining different aspects of the stream consistently supported the occurrence of shifts in the bacterial community structure and bacterial metabolic property in the mats from the three zones of the hot stream. This subsurface environment is an unusual one, high in methane and ammonia, relatively high in reduced-sulfur compounds (previously detected as 94 μM) (Takai et al. 2002), low salt, and a dark (non-sunlight) site at discharge. The results are consistent with methane as a dominant energy source, hydrogen being rapidly utilized, and ammonia and nitrite being more important in the site where temperature decreases (Fig. 4).

With regard to the bacterial community at the discharge site, it is to be noted that the two bacterial populations (relatives of thermophilic methanotroph within the *γ-Proteobacteria* (Bodrossy et al. 1999) and microaerobic hydrogen- and sulfur-oxidizing *Sulfurihydrogenibium* sp. within the order *Aquificales* (Takai et al. 2003)) were dominant. Both of these groups were also detected in the vein aquifer (Inagaki et al. 2003; Takai et al. 2002). The predominance of *Sulfurihydrogenibium* sp. agrees with the suggestion by Skirnisdottir et al. (2000) that sulfide concentration and occurrence of *Aquificales* group are related, as *Sulfurihydrogenibium* sp. is similar to the clones from the different sulfide-rich hot springs (Takai et al. 2002). The recovery of high numbers of particulate methane monooxygenase gene (*pmoA*) from the upper mat was consistent with the predominance of the thermophilic methanotroph and would be an important clue to estimate the in situ methanotrophic activity of the mat formation. The observation by molecular phylogenetic analyses was supported by the isolation of a novel thermophilic methane-oxidizer within the *γ-Proteobacteria* and hydrogen-oxidizers within the order *Aquificales* and *β-Proteobacteria*, which are closely related to the predominant phylotypes in the stream mat (unpublished data).

The predominant phylotypes in the microbial mat communities changed downstream to member of the *Nitrosomonas*/*Nitrosococcus* group in the *β-Proteobacteria* and the *Nitrospirae*. The sequences of the phylotypes HAuD-MB27 and HAuD-LB38 dominating in the middle and lower mats showed high similarities (98%) with that of *Nitrosomonas* sp. Nm107 (Purkhold et al. 2000) and *Nitrospira moscoviensis* (Ehrich et al. 1995), respectively. All of the cultured nitrifying bacteria are mesophilic nitrifiers usually growing up to approximately 40°C, although the in situ temperature of the water was above 50°C even in the lower stream. Therefore, it seemed difficult from the rRNA gene phylogenetic characterization alone to identify *β-Proteobacteria* and the *Nitrosprirae* phylotypes in the middle and lower mats as thermophilic versions of the nitrifying bacterial communities. However, the phylogenetic analysis and quantification of the homogeneous *amoA* and heterogeneous *norB* genes, and chemical analysis of the mat-interstitial water further supported the abundant occurrence of nitrifying phylotypes. The chemistry of the mat-interstitial water seemed to show the transition in the formation of inorganic nitrogen compounds likely caused by microbial consumption of ammonium and production of nitrite and nitrate along the flow of the hot water stream. The constant level of sulfate content throughout the stream seemed to be related to the absence of relatives of microorganisms carrying out sulfur transformation in the mats, except for the facultatively sulfur-oxidizing *S. subterraneum* in the upper mat.

Methanotrophs are also expected to be involved in ammonia oxidation, although the reaction is not thought to generate energy for growth (Bedard and Knowles 1989). Since, unexpectedly, abundant *pmoA* was detected in the lower mat, the highest concentration of nitrite in the lower mat-interstitial water in spite of low detection of ammonia oxidizer's genes may be attributed to accessory ammonia-oxidation by methanotrophs. The presence of methanotrophs in the downstream was also demonstrated by the isolation of methane-oxidizing *Methylococcus* sp. and *Methylosinus* sp. from the mixture of middle and lower mats (unpublished data).

The estimation of gene copy numbers could provide significant information about community size in the environments. However, the estimated gene copy numbers do not necessarily represent the actual numbers of the cells in the communities. It was reported that methane-oxidizing *γ-Proteobacteria* and ammonia-oxi-

dizing *β-Proteobacteria* generally had several copies of *pmoA* or *amoA* in their genomic DNA (Hirota et al. 2000; Hommes et al. 1998; McTavish et al. 1993; Semrau et al. 1995; Stolyar et al. 1999). Given that *norB* is present as a single copy in the genome of the nitrite-oxidizing *Nitrospira* members, the magnitude of nitrite-oxidizing function evaluated by copy numbers of *norB* gene may be underestimated as compared with those of methane and ammonia oxidation.

Whole-cell FISH experiment strongly indicated the existence of the predominant phylotypes in the mats. FISH-counts have been reported to underestimate the total cell numbers in analyses of natural samples (Alfreider et al. 1996; Manz et al. 1993). Nevertheless, a relatively good agreement was obtained in this study in population ratios assessed by the employed methods (FISH-counts consistently overestimated the percentages predicted based on those estimated from the number of clones).

To conclude, the observed transitional microbial flora represents the alteration of the predominant microbial metabolic processes, in which the energy and carbon sources for survival and growth were obtained from the geothermally provided compounds. The predominant energy conversion and carbon fixation metabolisms changed from thermophilic methanotrophy and hydrogen- and sulfur-oxidizing chemolithoautotrophy at the discharging point to putative thermophilic ammonia- and nitrite-oxidizing chemoautotrophy downstream. Molecular oxygen might serve as the primary electron acceptor for these thermophilic methanotrophs and chemolithoautotrophs.

Molecular oxygen is anthropogenically introduced into the subsurface environments of the Hishikari gold mine by way of the mining operation; thus the microbial community in the hot water stream does not completely represent a naturally occurring subsurface microbial ecosystem. However, the various environmental constraints in the subsurface of the mine would designate the microbial community of the stream as a novel and unique subsurface microbial ecosystem. The terrestrial thermophilic microbial ecosystem without sunlight irradiation has been poorly investigated as compared to the deep-sea hydrothermal microbial ecosystem independent of photosynthesis. The abundant methanotrophs are notable in microbial communities occurring in geothermal environments. Perhaps it can be attributed to the unique geological settings of this mine harboring a methane-rich aquifer rather than to discharging in the subsurface without sunlight. The most outstanding feature in this study was the predominant occurrence of the thermophilic nitrifying chemolithoautotrophs of the *Nitrosomonas*/*Nitrosococcus* group and *Nitrospira* group, because obligately or facultatively phototrophic thermophiles have often been reported as alternatives in surface hot springs with wide ranges of temperature (45–70°C) and pH (5–9) (Hiraishi et al. 1999; Ruff-Roberts et al. 1994; Skirnisdottir et al. 2000; Ward et al. 1998). The potential susceptibility of ammonia oxidation reaction to UV and near-UV light (Hooper and Terry 1974; Shears and Wood 1985) may also cause the scarce occurrence of the nitrifying communities in the presence of sunlight. In surface springs, the cyanobacterium *Synechococcus* sp. is found to occur in more acidic environments like this mine stream (pH 5–6) (Ruff-Roberts et al. 1994; Ward et al. 1998). In an Icelandic subsurface hot spring (72°C), SUBT-7 clone which is affiliated to *Nitrospira* group but only distantly related to the genera *Nitrospira* has been abundant, however geochemical characteristics of the spring water have not been reported (Marteinsson et al. 2001). In the Hishikari gold mine, the accretional basement in the oceanic subduction zone might play an important role in providing ammonium and CO_2 as energy and carbon sources for the nitrifiers.

The pH of this stream (pH 4.8–5.1) was significantly lower than the other hot spring environments, although some common bacterial groups have been detected in the stream (Skirnisdottir et al. 2000; Yamamoto et al. 1998). The isolates obtained from the stream, which are closely related to the predominant phylotypes of the stream, exhibit an optimum pH for growth as moderately acidic or neutral (pH 6.0–7.5) (unpublished data; Takai et al. 2003). The pH has been generally considered as an important factor controlling the types of communities in the hot spring environments. However, the temperature and availability of energy sources seem to have been controlling the community in this stream. The preliminary experiment showed an increasing pH of the stream water sampled and transported to the laboratory, which is probably due to degassing of CO_2 from the water. Therefore the observed low pH of the stream water is likely attributed to abundant dissolved CO_2 that is an important carbon source for autotrophic microorganisms detected in the stream.

A relationship between an archaeal community and geochemical properties of the stream is not clear. Competitive PCR experiment and phylogenetic analysis of archaeal 16S rRNA genes in the mat DNA assemblage showed that a single crenarchaeotic member was abundant in the upper mat, and its abundance was equal to *Bacteria* (data not shown). Since physiological traits of the archaeal members can not be inferred at the present time, the effects by those metabolisms on the stream water chemistry are also unknown. In contrast to the abundance of *Archaea* in the upper mat, *Bacteria* was predominant in the middle and lower mats. Detailed archaeal community analysis is also significant theme for comprehensive understanding of the stream community. We intend to report the analysis of *Archaea* in a separate paper.

On a final note, this study sheds some light on the existence of many novel, yet-uncultivated bacterial phylotypes in the subsurface geothermal environment. The isolation and characterization of the thermophilic methanotrophs and nitrifiers may provide new insights into in situ biogeochemical processes and population dynamics of the microbial mats in the subsurface hot water stream.

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