Full Paper

Phylogenetic characterization of microbial mats and streamers from a Japanese alkaline hot spring with a thermal gradient

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(Received June 7, 2002; Accepted August 8, 2002)

Dense microbial mats and streamers of various colors developed in an alkaline-hot spring water at 48–76°C and ~0.077 mM sulfide in Nakabusa, Japan. The microbial community structures with a thermal gradient were compared by denaturing gradient gel electrophoresis (DGGE) analysis of the PCR-amplified 16S ribosomal RNA gene fragments. The sequence analysis revealed that a predominant cyanobacterial DGGE band phylogenetically related to *Synechococcus elongatus* was detected only from green mats at 48°C. Four DGGE bands were detected commonly from green mats at 48°C, orange mats at 58°C and brown mats at 60°C. The sequence analysis revealed that these were phylogenetically related to *Chloroflexaceae* group, *Rhodothermus* group, a candidate division OP10, and an unclassified bacterium. On the other hand, *Aquificae*-, *Thermodesulfobacteria*-, *Thermus* group-, and *Crenarchaeota*-like sequences were detected as a predominant component of DGGE profiling from the streamers only at temperatures over 66°C, but no phototrophic bacterial bands were detected. Thus, the microbial community structure above 60°C was drastically different from that at the lower temperatures. After the addition of hydrogen into in vitro gray streamers with in situ spring water, sulfide production markedly occurred in the presence of ambient sulfate at 66°C. This result suggests that in situ sulfide is partly produced by *Thermodesulfobacteria*-like sulfate-reducing bacteria in the streamers.

Key Words—in situ temperature; microbial mats; microbial streamers; PCR-DGGE; 16S rDNA; sulfide production

Introduction

Dense microbial mats and/or streamers of various colors (white, yellow, pink, purple, orange, red, green, etc.) develop in neutral or alkaline hot springs as follows: The color is determined by interaction between microbes in hot springs and physicochemical factors such as temperature, pH, sulfur and light (Brock, 1978;

Castenholz, 1988; Hanada et al., 1995; Hiraishi et al., 1999; Jørgensen and Nelson, 1988; Pierson, 1994; Skirnisdottir et al., 2000). Among them, in situ temperature, sulfide and light can be some of the key factors in developing microbial community structure. For instance, *Aquificales*-like organisms dominated within white or yellow sulfurturf mats, consisting mainly of microorganisms and sulfur compounds, in sulfide-rich hot springs with sulfide concentrations over 0.091 mM and temperature ranging from 52–72°C (Skirnisdottir et al., 2000; Yamamoto et al., 1998). On the other hand, orange or yellow *Chloroflexus* mats were formed in the sulfide-poor hot spring with sulfide concentrations less than 0.030 mM at 65–70°C (Skirnisdottir et al., 2000).

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Most hydrogen sulfide in hot springs is generated geothermally, but sulfate-reducing bacteria (SRB) can partly supply it as the result of their respiration of sulfate as electron acceptor (Henry et al., 1994; Sonne-Hansen and Ahring, 1999; Widdel, 1992). Studies on the rates of microbial sulfate reduction demonstrated that thermophilic sulfidegenesis occurred with high activity despite low concentrations of sulfate in terrestrial hot spring waters, sediments and bacterial mats with volcanic activity (Sonne-Hansen and Ahring, 1997; Zeikus et al., 1983). Among the thermophilic sulfate-reducing microorganisms, *Thermodesulfobacterium* and *Thermodesulfovibrio* species have been already isolated from terrestrial hot spring sediments and microbial mats (Henry et al., 1994; Sonne-Hansen and Ahring, 1999; Zeikus et al., 1983). Moreover, the molecular approach revealed that organisms related to the *Thermodesulfobacteria* were distributed in terrestrial hot springs, such as the Obsidian Pool at 75–95°C in Yellowstone National Park, USA (Hugenholtz et al., 1998), and in an alkaline Icelandic hot spring at approximately 75°C (Skirnisdottir et al., 2000). Although several organisms belonging to *Thermodesulfobacteria* have thus far been detected from sediments and microbial mats in hot springs, the information regarding the in situ sulfate reduction activity of this group is limited.

In the present study, we investigated the microbial community structures of various colored mats and streamers in a sulfide-containing alkaline hot spring with a thermal gradient in Nakabusa, central Japan, using PCR-denaturing gradient gel electrophoresis (DGGE) with the primer sets for the domain of *Bacteria*, *Archaea* and the phylum *Cyanobacteria*. Green (48°C), orange (58°C), brown (60°C) mats and white streamers (73°C) were developed on the barrier wall for preventing landslides under running hot spring source water.

Materials and Methods

Study area and collection of microbial mat and water. The study area (36°23'15"N, 137°45'00"E) was Nakabusa, a slightly alkaline, sulfide-containing hot spring located in Nagano Prefecture, Japan. The hot spring microbial mats and streamers were collected from site 1-A to -E where the in situ temperatures were 48, 58, 60, 73, and 76°C, respectively, in July 2000, and from site 2 at 66°C in November 2000

Fig. 1. Photograph showing the sampling sites of the Nakabusa hot spring, Japan.

I, Site 1; II, Site 1-A, Site 1-B, Site 1-C, Site 1-D; III, Site 1-E; IV, Site 2-F.

(Fig. 1 and Table 1). The distance between the sites is approximately 50 m. The microbial mat sample was kept in a sterile plastic tube (50 ml) with spring water to prevent the oxidation of samples. Spring water was collected into a plastic tube without the headspace, immediately filtrated with Millex®-GP (pore size, 0.2 µm, Millipore) for inorganic analyses and fixed with the same volume of 90 mM zinc acetate solution for determination of sulfide concentration. All capped tubes were stored on ice and transferred to our laboratory. In situ spring water for sulfide production experiments of microbial mats was filtrated with GP mixed esters of cellulose membrane (pore size, 0.22 µm, Millipore) in the laboratory, and then stored at 4°C until use. The microbial mats for PCR-DGGE analysis were collected in a 1.5 ml centrifuge tube and then frozen at –20°C. The mat samples for morphology were checked with phase-contrast microscopy (Axioplan 2, ZEISS).

Extraction of nucleic acids. After homogenizing microbial mats with homogenization pestles on ice, the nucleic acids were extracted with sodium dodecylsulfate and proteinase K and then purified with hexadecyltrimethylammonium bromide (CTAB), chloroform-isoamyl alcohol (24:1 vol:vol), and phenol-chloroform-isoamyl alcohol (25:24:1 vol:vol:vol) as described by Wilson (1990). The extracted nucleic acids were precipitated with isopropanol, washed with 70% ethanol precipitation and then stored at –20°C until PCR.

PCR amplification of 16S rDNA. DNA fragments

Table 1. Physico-chemical and morphological characteristics of hot springs and biomats, Nakabusa, Japan.

Sites	Temperature (°C)	Color of mats/streamers	Thickness of mats/streamers (mm)	Major morphotype of bacteria	pH
Site 1					
A	48	Green Orange	Mat (5–10)	Filaments, Rods	N.D.
B	58	Orange	Mat (5–10)	Filaments, Rods	N.D.
C	60	Brown	Mat (2–3)	Rods	N.D.
D	73	White	Streamer (1–3)	Filaments, Rods	7.3
E	76	Gray White	Slime streamer (30–50)	Filaments, Rods	8.5
Site 2					
F	66	Orange Gray	Slime streamer (30–50)	Filaments, Rods	8.7

N.D. indicates not determined.

encoding 16S rRNA of the domain *Bacteria*, the domain *Archaea*, and the phylum *Cyanobacteria* were amplified using three sets of primers as follows: 341F with GC-clamp (341F, 5'-CCTACGGGAGGCAGCAG-3'; GC-clamp, 5'-CGCCCGCCGCGCCCGCGCC-CGTCCCGCCGCCCCGCCCCG-3') and 907R (5'-CCGTCAATTCCTTTTTRAGTTT-3') for the domain *Bacteria* (Muyzer et al., 1993, 1996), ARC344F with GC-clamp (5'-ACGGGGYGCAGCAGGCGCGA-3') and ARC915R (5'-GTGCTCCCCCGCCAATTCCT-3') for the domain *Archaea* (Raskin et al., 1994; Stahl and Amann, 1991), and CYA106F with GC-clamp (5'-CG-GACGGGTGAGTAACGCGTGA-3') and CYA781R-(a,b) (a, 5'-GACTACTGGGGTATCTAATCCCATT-3'; b, 5'-GACTACAGGGGTATCTAATCCCTTT-3') for the cyanobacteria (Nübel et al., 1997). PCR procedures for the bacterial, archaeal, and cyanobacterial primers were carried out as described by Muyzer et al. (1993), Casamayor et al. (2000), and Nübel et al. (1997), respectively. PCR amplifications were performed with 100 µl volumes containing 1–10 ng of template DNA, 1×EX *Taq* buffer (TaKaRa Shuzo, Kyoto, Japan), 250 µM each of deoxynucleoside triphosphate, 25 pmol each of the primer, 2.5 U of EX *Taq* DNA polymerase (TaKaRa Shuzo), and 2 drops of mineral oil (Sigma, Steinheim, Germany). PCR products were analyzed by electrophoresis in 2% (wt vol⁻¹) Nusieve 3:1 agarose (FMC, Rockland, ME, USA) gels containing ethidium bromide (1 µg ml⁻¹).

DGGE analysis of 16S rDNA fragments. DGGE was performed as described by Muyzer et al. (1996) using D-gene and D-code systems (Bio-Rad Laboratories, Hercules, CA, USA) with a 1.5-mm gel width. Approximately 500 ng of PCR products were applied directly onto 6% (wt vol⁻¹) polyacrylamide gels in 0.5× TAE buffer (40 mM Tris, 20 mM Acetic Acid, 1 mM EDTA, pH 8.3). Denaturing gradients were formed with 6% (wt vol⁻¹) acrylamide stock solution containing 0 and 80% denaturant (5.6 M urea [Bio-Rad, Laboratories] and 32% [vol vol⁻¹] formamide deionized [Bio-Rad Laboratories]). Electrophoresis was run in 1×TAE buffer (40 mM Tris, 20 mM Acetic Acid, 1 mM EDTA, pH 8.3) at the voltage of 200 V and 60°C for 4 h. After electrophoresis, the gels were incubated for 10 min in ethidium bromide (1.0 mg L⁻¹), rinsed for 10 min in distilled water, and then photographed with UV transillumination (wavelength, 312 nm) with a charge-coupled device camera (Image Server; ATTO, Tokyo, Japan). The digitized images were processed with the NIH Image software (National Institute of Health, Bethesda, Md.).

DGGE bands were excised from the gels, and reamplified using primers and PCR amplification as described above. The PCR products from the second amplification were electrophoresed again in a DGGE gel to check the purity of the bands, and then purified using QIAquick™ PCR purification kit (Qiagen, Hilden, Germany).

Sequence and phylogenetic analysis. The sequence of the DGGE bands was determined for both strands using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). Extension products were analyzed on an ABI model 377 automated sequencer (Applied Biosystems). Sequences from DGGE bands were entered into the BLAST programs of the National Center for Biotechnology Information (Altschul et al., 1997) in order to identify phylogenetic relatives. Sequence alignments with parts from 16S rRNA sequences of reference bacteria from GenBank and matrices of evolutionary distance were performed using CLUSTAL W program (Thompson et al., 1994). Phylogenetic trees were constructed from the evolutionary distance using Tree View software (Page, 1996).

Sulfide production test of microbial gray slime streamer. The in situ spring water was degassed under N_2 gas. Approximately 10 g of gray slime streamer taken from site 2 in November was mixed with approximately 30 ml of in situ spring water in a serum bottle (50 ml), which was sealed with a butyl rubber stopper under a headspace of N_2 gas. After incubation at 66°C for 73 min without shaking, sulfate (final concentration, approximately 5 mM) was added to the slime mixture and then lactate (final concentration, approximately 0.5 mM), 12 ml of H_2 - CO_2 (80:20 [vol:vol]) gas, and acetate (0.5 mM) were added sequentially. The subsamples (0.2 ml of medium water) were withdrawn with sterile nitrogen-flushed syringes. Sulfide concentrations in the water were measured colorimetrically according to the methylene blue formation reaction method (Cline, 1969).

Analytical methods. In situ temperature and pH of hot spring water were measured with the electrode method using a pH meter (WM-22EP, TOA Electronics, Tokyo, Japan). Sulfate concentrations were determined by ion chromatography (Dionex, Sunnyvale, CA, USA). Dissolved sulfide concentrations were measured colorimetrically as described above after fixing in situ with zinc acetate solution. Total organic carbon (TOC) was determined with a TOC analyzer (TOC-5000, Shimadzu, Japan).

Nucleotide sequence accession numbers. The rRNA gene sequences were submitted to GenBank and have been assigned the following accession numbers: AB075780 to AB075804.

Results

Physico-chemical and microscopic-morphological characteristics of hot spring mats

As summarized in Table 1, microbial mats and streamers of various colors developed in the hot spring pool and on the walls depending on the in situ water temperature. The concentrations of sulfide, SO_4^{2-} , Cl^- , and TOC in the hot spring pool at site 1 and site 2 in November, 2000 were respectively as follows: sulfide, 0.077 mM, 0.027 mM; SO_4^{2-} , 246 μ M, 218 μ M; Cl^- , 1.07 mM, 0.95 mM, and TOC, 0.415 mg L^{-1} , 0.393 mg L^{-1} .

At site 1-E (76°C), the gray-white hair-like slime streamer developed at approximately 10 cm depth from the surface of an alkalized spring pool (pH of 8.5) (Fig. 1-III). The in situ spring water temperature on the barrier to prevent landslides depended on the distance from the chink established subadjacently by the running spring source water. Dense microbial mats and streamers (site 1-A, B, C, and D) developed on the wall with different colors: green at site 1-A (48°C), orange at site 1-B (58°C), brown at site 1-C (60°C) and white at site 1-D (73°C) (Fig. 1-II). The green mat at site 1-A (48°C) underlay an orange layer. On the other hand, gray-orange hair-like streamers developed in effusing spring water at site 2-F (66°C) from an outlet pipe of the common spring source (Fig. 1-IV). The major morphotype of cells consisted of filaments and/or rods of different sizes in all samples. In the green mats (48°C), the filamentous cells were covered with the *Synechococcus*-shaped cyanobacterial ones.

DGGE band profiles of microbial mats

Figure 2 shows the 16S rDNA based DGGE profiles of microbial mats and streamers developed at various in situ temperatures. First, we found drastic differences in the profiles of the DGGE bands from the mats and/or streamers developed below 60°C and one growing above 60°C. The DGGE bands of NAB4, 5, and 6 were commonly found growing below 60°C, however, the NAB11, 13, 14, and 15 were found growing only in mats above 60°C. These results indicate that the microbial community structures over 60°C are drastically different from those at the lower temperatures. Second, the predominant DGGE bands (NAB12, 13, 14, and 15) obtained from the mat at site 1-E (76°C) indicated a different migration from those (NAB10, 11) of the streamer at site 1-D (73°C) on the

Fig. 2. DGGE profiles among microbial mats and streamers at various in situ temperatures by using three sets of primers for the domain *Bacteria*, phylum *Cyanobacteria* and the domain *Archaea*.

The bands labeled with number were sequenced. A1, NAB1; A2, NAB2; A3, NAB3; A4, NAB4; A5, NAB5; A6, NAB6; A7, NAB7; A8, NAB8; C9, NAB9; D10, NAB10; D11, NAB11; E12, NAB12; E13, NAB13; E14, NAB14; E15, NAB15; F16, NAB16; F17, NAB17; F18, NAB18; F19, NAB19; F20, NAB20; F21, NAB21; F22, NAB22; A23, NAB23; D24, NAB24; F25, NAB25; F26, NAB26.

DGGE gel, even though the in situ water temperatures were similar at both sites. A single cyanobacterial band (NAB23) was detected only at site 1-A and B at less than 58°C. The archaeal bands (NAB24, 25, and 26) were observed from the white streamers at site 1-D (73°C) and orange-gray streamers at site 2-F (66°C).

Phylogenetic analysis of 16S rDNA fragments of DGGE bands

The bands NAB10 and 16 were closely related to *Aquificae* clones derived from sulfur mats (Skirnisdottir et al., 2000; Yamamoto et al., 1998) (Fig. 3). *Thermodesulfobacteria*-like bands (NAB12, 13, 19, and 20) showed the highest sequence similarity to OPB45 obtained from sediments in the Obsidian Pool (75- to 95°C) in Yellowstone National Park, USA (Hugenholtz et al., 1998) (Fig. 4). NAB11, 14, 15, 21, and 22 be-

longed to the *Thermus* group (Table 2). Three *Chloroflexi* bands, NAB7, 8, and 9, were closely related to *Roseiflexus castenholzii* (Hanada et al., 2002), *Chloroflexus aurantiacus* (Pierson and Castenholz, 1974) and *Thermomicrobium roseum* (Oyaizu et al., 1987), respectively. As shown in Table 2, the bands NAB4, 17, 18, 5, and 6 were closely related to *Eubacterium* sp. (Ward et al., 1992), *Rhodothermus marinus* NR-32 (Silva et al., 2000), and molecular clones OPB80 and 90 belonging to the candidate division OP10 (Hugenholtz et al., 1998), respectively. Three archaeal bands (NAB24, 25, and 26) belonged to *Crenarchaeota* (Fig. 5).

Sulfide production from microbial gray streamers at site 2-F (66°C)

To clarify the potential sulfate reduction by thermophilic SRB within the gray slime streamer at site 2-F (66°C), sulfide production was monitored after the addition of substrates to a streamer consisting of several phylotypes of *Aquificae*, *Thermodesulfobacteria*, *Thermus* group, and *Crenarchaeota* as shown in Fig. 2 and Table 2. Sulfide concentration did not increase for 166 min of incubation in the presence of 5 mM sulfate. After the addition of hydrogen, marked sulfide production occurred, but lactate and acetate did not affect it.

Discussion

In situ temperature and sulfide as environmental factors to determine microbial community structure

As shown in Table 2, we found remarkable differences in the microbial community structure between mats and streamers below or above 60°C in an alkaline hot spring, Nakabusa. *Synechococcus* DGGE bands occurred at site 1-A (48°C) and site 1-B (58°C), however *Synechococcus lividus*, which can grow at temperatures of up to 70–74°C (Meeks and Castenholz, 1971), was not detected in Nakabusa. *Chloroflexus* mats developed at site 1-B (48°C) and site 1-C (58°C), whereas *Aquificae* streamers were found only at site 1-D (73°C). A similar result of changes in the community structures with a thermal gradient was obtained from the quinone profiles of microbial mats in a hot spring environment (Hiraishi et al., 1999). Therefore, *in situ* temperature should be a key environmental factor in Nakabusa. On the other hand, the sulfide concentration in the hot spring water of site 1-E was of moderate level (0.077 mM), compared to the sulfide-

Fig. 3. Neighbor-joining phylogenetic tree of bacterial DGGE bands (approximately 430 bp) obtained from the mats and streamers in Nakabusa, Nagano, Japan.

The scale bar represents an estimated 10% sequence divergence. Numbers before branch points indicate bootstrap values determined from 1,000 iterations of the neighbor-joining calculation.

rich hot springs (0.094 to 0.187 mm) in Iceland and Japan (Skirnisdottir et al., 2000; Yamamoto et al., 1998). Based on the PCR-DGGE analysis, the *Thermodesulfobacteria* dominated within the gray-white slime streamer in the moderate sulfide spring pool at site 1-E (Table 2). In contrast to the moderate sulfide hot spring in Nakabusa, an *Aquificae* mat developed in the sulfide-rich spring at 52–72°C (Skirnisdottir et al., 2000; Yamamoto et al., 1998). Thus, it is likely that the sulfide concentration in hot spring water is also a key environmental factor for the dominant microorganisms

within mats and streamers due to their specific adaptation to the sulfide level.

Comparison of community compositions between two types of slime streamers at site 1-D (73°C) and site 1-E (76°C)

The *Thermodesulfobacteria* did not occur as a dominant DGGE band from the white streamers (*Aquificae* and *Crenarchaeota*) on the wall (site 1-D) at 73°C (Fig. 2, Table 2). Based on the cloning and sequencing of 16S rRNA genes, 18% of the bacteria library derived

Fig. 4. Phylogenetic tree of archaeal DGGE bands from the white streamers and the gray streamers based on partial 16S rRNA gene sequences (approximately 470 bp).

from sulfur mats in Iceland corresponded to the *Thermodesulfobacteria* (Skirnisdottir et al., 2000). The possible reason for the difference in this abundance is as follows: anaerobic conditions did not easily develop within the white streamers in Nakabusa, because the hot spring water running on substrata of the barrier was shallow enough to diffuse oxygen from atmosphere. Several strains belonging to *Aquificae* are microaerophilic, obligately chemolithotrophic bacteria to utilize molecular hydrogen or sulfur as energy donors (Argano, 1992). Most members of the family *Desulfurococcaceae* grow chemolithoautotrophically by sulfur reduction (Burggraf et al., 1997). Therefore, sulfur

can be one of the important primary energy sources and/or electron acceptors in white streamers at site 1-F (73°C). Maki (1991) suggested that the sausage-shaped bacteria predominating within sulfur mats had an important role in the biological oxidation of dissolved sulfide in hot spring effluents with molecular oxygen as their electron acceptor. Moreover, Yamamoto et al. (1998) demonstrated that the sausage-shaped bacteria belong to *Aquificae*. Dissolved sulfide in the running spring water over sulfur-turf seems to be oxidized to elemental sulfur by molecular oxygen and/or *Aquificae*.

On the other hand, no DGGE band corresponding to

Table 2. Summary of the 16S rRNA sequences identified within mats and streamers in hot springs, Nakabusa, Japan.

Type sequences	Closest database match (%)	Affiliate group	Reference	DGGE band intensities						
				Site and in situ temperature (°C)						
				1-A 48	1-B 58	1-C 60	2-F 66	1-D 73	1-E 76	
<i>Bacteria</i>										
NAB10, 16	GANI, NAK, SRI (95, 99)	<i>Aquificae</i>	1, 2				+	+++		
NAB12, 13, 19, 20	OPB45 (95–98)	<i>Thermodesulfobacteria</i>	3				++	+	+++	
NAB11, 14, 15, 22	<i>Thermus</i> sp. (96–97)	<i>Thermus/Deinococcus</i> group	4				+++	+++	++	
NAB21	<i>Thermus antranikianii</i> (99)	<i>Thermus/Deinococcus</i> group	5				++			
NAB9	<i>Thermomicrobium roseum</i> (96)	<i>Chloroflexi</i>	6			+				
NAB7	<i>Roseiflexus castenholzii</i> (96)	<i>Chloroflexi</i>	7	++	++					
NAB8	<i>Chloroflexus aurantiacus</i> (98)	<i>Chloroflexi</i>	8	+	+					
NAB5	<i>Rhodothermus marinus</i> NR-32 (87)	CFB group	9	+	++	++				
NAB6	OPB80, 90 (96)	Candidate division OP10	10	+	+	++				
NAB4, 17, 18	<i>Eubacterium</i> sp. (94, 96, 90)	<i>Firmicutes</i>	11	+++	+++	+++	++			
NAB1	Clone 11–25 (86)	Unresolved	12	+						
NAB3	SJA-35 (89)	Unresolved	13	+						
NAB2, 23	<i>Synechococcus elongatus</i> (91, 99)	<i>Cyanobacteria</i>		+++	+					
<i>Archaea</i>										
NAB24	<i>Staphylothermus marinus</i> (95)	<i>Crenarchaeota</i>	14				+++	+++		
NAB25	TOYO-20-Dm (99)	<i>Crenarchaeota</i>	15				++			
NAB26	<i>Sulfolobococcus zilligii</i> (95)	<i>Crenarchaeota</i>	16				++			

+++ , Strong; ++ , substantial; + , recognizable; no mark, not recognizable.

Reference 1, Skirnisdottir et al., 2000; 2, Yamamoto et al., 1998; 3 and 10, Hugenholtz et al., 1998; 4, Reichenbach, 1991; 5, Chung et al., 2000; 6, Oyaizu et al., 1987; 7, Hanada et al., 2002; 8, Pierson and Castenholz, 1974; 9, Silva et al., 2000; 11, Ward et al., 1997; 12, Ludwig et al., 1992; 13, Von Wintzingerode et al., 1999; 14, Burggraf et al., 1997; 15, Nakagawa et al., 2002; 16, Hensel et al., 1997.

Fig. 5. Sulfide production from gray slime streamers (site 2).

After preincubation at 66°C for 73 min, sulfate, lactate, hydrogen gas, and acetate were sequentially added to the serum bottle (50 ml) containing approximately 10 g of the streamer.

Aquificae and *Crenarchaeota* was detected from the gray-white slime streamers (*Thermodesulfobacteria*) submerged in the pool at site 1-E (76°C). Sulfide in the pool did not seem to be oxidized to elemental sulfur by molecular oxygen, because gray-white slime streamers were permanently submerged in the anoxic hot spring water. The moderate concentration of sulfide in spring water might not be suitable for *Aquificae*.

Occurrence of *Cytophaga-Flavobacterium-Bacteroides* group and candidate division OP10

Strains of the *Cytophaga-Flavobacterium-Bacteroides* are associated with the degradation of complex organic matter and occur frequently in marine and freshwater environments (Reichenbach, 1991). The band NAB5 was related to *Rhodothermus marinus* NR-32 isolated from shallow marine hot springs (Silva et al., 2000) but the sequence similarity was 87%, suggesting a different genus. Several organisms belonging to the CFB group were detected from hot springs in Yellowstone National Park, USA (Hugenholtz et al., 1998), a deep-sea hydrothermal vent on the Mid-Atlantic Ridge (Reysenbach et al., 2000), and a shallow submarine hydrothermal vent in the Aegean Sea near the island of Milos (Greece) (Sievert et al., 2000). The phylogenetic affiliation of these organisms has adapted to the thermophile habitats in both marine and freshwater environments. In addition, several phylogroups belonging to candidate division OP10 were cultured from activated sludges (Bond et al., 1995), a hydrocarbon- and chlorinated-solvent-contaminated aquifer (Dojka et al., 1998), an anaerobic trichlorobenzene-transforming microbial consortium (Von

Wintzingerode et al., 1999), as well as hot spring sediments in Yellowstone National Park, USA (Hugenholtz et al., 1998).

Biological sulfide production by *Thermodesulfobacteria*

The DGGE bands (NAB19 and 20) belonged to the *Thermodesulfobacteria* occurring as a predominant member within gray slime streamers at site 2-F (66°C). The sulfate concentration was 218 µM in the hot spring (site 2). The low half-saturation constant (K_m value) for sulfate at 70°C of *Thermodesulfobacterium hveragerdense* is 3.1 µM (Sonne-Hansen et al., 1999), showing its high affinity to sulfate as known in freshwater SRB species (Ingvorsen and Jørgensen, 1984). Thus, sulfate in Nakabusa hot spring is not a limiting factor for *in situ* sulfate reduction by *Thermodesulfobacteria*.

Sulfide markedly increased immediately after the addition of hydrogen (Fig. 6). The K_m value of hydrogen at 70°C for *Thermodesulfobacterium hveragerdense* is 1.9 µM (Sonne-Hansen et al., 1999). Thus, thermophilic sulfate reducers within the gray slime streamers (site 2) in Nakabusa appear to utilize H₂. Hydrogen and various organic substances such as acetate and lactate might be produced through fermentation by *Thermus* and/or *Crenarchaeotas*. Some strains belonging to *Thermus* (Kieft et al., 1999) and *Desulfurococcaceae* (Burggraf et al., 1997) can produce sulfide by sulfur respiration. However, the sulfide production by sulfur respiration might be limited, since elemental sulfurs were not observed abundantly within the streamer, compared to white streamers containing *Aquificae* as a dominant member at site 1-D. Possibly, these results indicate that sulfate-reducing *Thermodesulfobacteria* species (NAB19, 20) are responsible for the biological sulfide production in the gray slime streamer. However, relatively little is known about sulfide formation via the action of thermophilic sulfate-reducing bacteria in terrestrial hot spring environments. Hence, it is essential to investigate the sulfate reduction rate of thermophilic sulfate-reducing microbes with $^{35}\text{SO}_4^{2-}$.

Conclusion

We showed that the microbial community structure at temperatures over 60°C was drastically different from that at the lower temperatures in the moderate-sulfide hot spring in Nakabusa. DGGE analysis based on PCR-amplification of 16S rRNA genes represents the most abundant PCR products and reflects the

abundance of genes in the target samples. However, several pitfalls and potential biases of PCR-dependent techniques have been previously described (Von Wintzingerode et al., 1997). Thus, abundance of filamentous cells and rods within the microbial mats and streamers could be investigated in more detail through the 16S rRNA-based application with a specific DNA probe. Furthermore, the present study indicates the possible implication of the *Thermodesulfobacteria* in biological sulfide production. Further study on microbial composition within microbial streamers using a molecular approach coupled with physiological characterization should provide a more comprehensive understanding of the in situ hot spring ecosystem.

Acknowledgments

We thank Kenji Kato, Hiroyuki Yamamoto, and Hisaya Kojima for their advice and technical assistance. We are also grateful to Katsumi Matsuura and Mitsunori Yamada for their valuable discussion. This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) to M.F. (12440219) and in part by MEXT through the Special Coordination Fund "Archaean Park" project.

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