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原　　著

Bacterial Community Structures in the Solfataric-Acidic Ponds in the Kirishima Geothermal Area, Kagoshima Prefecture

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鹿児島県霧島地域の酸性硫黄源泉における細菌群集構造の解析

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要　　旨

温度や化学成分が異なる温泉間で細菌群集構造を比較するため，鹿児島県霧島地域に分布する4つの酸性硫黄泉における細菌群集構造を，16S rRNA 遺伝子クローン解析法を用いて解析した．選択した温泉の温度および総化学成分量は次の通りである．1) Pond-A：93°C，1,679 mg L^{-1}，2) Pond-B：66°C，2,248 mg L^{-1}，3) Pond-C：88°C，198 mg L^{-1}，4) Pond-D：67°C，340 mg L^{-1}．相同性検索による種の同定を行った結果，4つの温泉由来の計372クローンは35系統群に分類され，何れの温泉においてもγ-Proteobacteria 綱が優占していた．最も多様性が高かったのは，温度と総化学成分量が共に相対的に低い Pond-D であり，この温泉では *Acinetobacter junii* に近縁な種が全クローンの37%を占めていた．Pond-D と同程度の温度ながら総化学成分量が相対的に高い Pond-B では，*Acidithiobacillus caldus* の近縁種およびδ-Proteobacteria 綱に属する未培養細菌が優占していた．一方，温度が相対的に高い Pond-A と C では *Acinetobacter johnsonii* に近縁な種が全クローンの57%以上を占めていた．本研究により，霧島地域の温度や化学成分が異なる酸性硫黄泉間における，細菌の群集構造および多様性の具体的な違いが明らかとなった．

キーワード：霧島温泉群，細菌，群集構造，多様性，化学成分，16S rRNA 遺伝子

Abstract

The bacterial 16S rRNA gene composition and environmental characteristics of four distinct solfataric-acidic ponds in the Kirishima geothermal area, Kagoshima Prefecture,

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Japan, were compared. The four ponds were selected based on differences in temperature and the total concentration of examined chemical components : 1) Pond-A : 93℃ and 1679 mg L^{-1} ; 2) Pond-B : 66℃ and 2248 mg L^{-1} ; 3) Pond-C : 88℃ and 198 mg L^{-1} ; and 4) Pond-D : 67℃ and 340 mg L^{-1}. In total, 372 clones of the 16S rRNA gene were classified into 35 phylotypes. The dominant bacterial group was the class γ-Proteobacteria. Bacterial diversity was greatest in Pond-D, and the dominant phylotype detected (37% of all clones) was closely related to *Acinetobacter junii*. Pond-B had the highest relative total concentration of examined chemical components, and the bacterial community was dominated by a phylotype closely related to *Acidithiobacillus caldus* as well as an uncultured species of δ-Proteobacteria. Pond-A and Pond-C had the highest relative temperatures and were dominated by a phylotype closely related to *Acinetobacter johnsonii* (accounted for more than 57% of the identified clones). This study highlights the different bacterial species composition and biodiversity present in solfataric-acidic ponds characterized by different temperatures and chemical components.

Key words : Kirishima geothermal area, bacteria, community structure, diversity, chemical component, 16S rRNA

1. Introduction

Extreme environments are unique locations for studying how organisms interact with and adapt to their surroundings. In particular, some high temperature environments such as terrestrial hot springs and oceanic hydrothermal vents may resemble the volcanic habitats that are thought to have existed on early Earth (Pace, 1991 ; Miller and Lazcano, 1995 ; Baross, 1998). Indeed, some of the bacterial and archaeal lineages identified from hot springs appear to be related to lineages close to the root of the phylogenetic tree (Pace, 1997).

Culture-dependent methods have traditionally been the primary means of surveying microbial diversity. However, these methods may underestimate the diversity of microorganisms and can potentially provide unrealistic descriptions of the microbial community structure. Utilization of molecular methods targeting the small-subunit (SSU ; 16S or 18S) rRNA gene in environmental samples has revealed great diversity of uncultured microbes in the natural environment. Given these new molecular findings, it is currently assumed that cultured species only account for 1% or less of all prokaryotes present on Earth (Amann *et al*., 1995).

Prokaryotes are divided into two domains, the Bacteria and the Archaea, based on 16S rRNA gene phylogenetic analysis (Woese and Fox, 1977 ; Woese *et al*., 1990). Hot spring prokaryotic microbial communities have been extensively studied using the 16S rRNA gene in areas such as Yellowstone National Park in the United States (Barns *et al*., 1994 ; Barns *et al*., 1996 ; Hugenholtz *et al*., 1998 ; Reysenbach *et al*., 2000 ; Blank *et al*., 2002 ; Meyer-Dombard *et al*., 2005), Kamchatka hot springs in Russia (Perevalova *et al*., 2008), Montserrat and Saint Lucia in the islands of the Lesser Antilles (Burton and Norris, 2000 ; Stout *et al*., 2009), Icelandic hot springs (Perevalova *et al*, 2008 ; Kvist *et al*., 2007), Mt. Unzen hot springs in Japan (Takai and Sako, 1999), Ohwakudani hot springs in Japan (Kato *et al*., 2011), Pisciarelli hot springs in Italy (Kvist *et al*., 2005), Bor Khlueng hot springs in Thailand (Kanokratana *et al*., 2004), the Wai-o-tapu geothermal area in New Zealand (Childs *et al*., 2008), and the Tengchong hot springs in China (Song *et al*., 2010). These pioneering studies have improved our understanding of prokaryotic communities living in high temperature environments. However, despite decades of research, we still know little

about the relationship between the environmental characteristics of a given hot springs and its prokaryotic community. It is important to identify the environmental factors that affect prokaryotic community structures in individual hot spring habitats. Temperature has perhaps received the most attention, but other potential constraining factors include pH, oxidation redox potential, elemental composition, and organic matter composition, among others.

We have surveyed a relatively wide geothermal field, the Kirishima geothermal area in Japan, and have found that this field contains many acidic ponds of various temperatures and chemical compositions. The temperature and concentration of chemical components in these ponds ranges from 63 to 94℃ and from 92 to 2,248 mg L^{-1}, respectively. Recently, we investigated the archaeal community structures in these ponds and found that the archaeal species diversity and composition differed between ponds (Satoh *et al*., 2013). However, little is known about the distribution of bacterial communities among these ponds and how this is affected by larger environmental variables (e.g., temperature and chemical compositions). In this study, the bacterial community structures and diversity of four distinct solfataric-acidic ponds in the Kirishima geothermal area, Kagoshima Prefecture, Japan, were compared by 16S rRNA gene phylogenetic analysis.

2. Experiments

2.1 Sample collection and analysis of chemical composition

The ponds investigated in this study were all located in a 1 km^2 field in/near the region of the Tearai hot spring (Tsuyuki, 1980). This district is situated 3 km southwest of the Ohnami-Ike volcanic crater lake, in the Kirishima geothermal area, Kagoshima Prefecture (Fig. 1, Table 1).

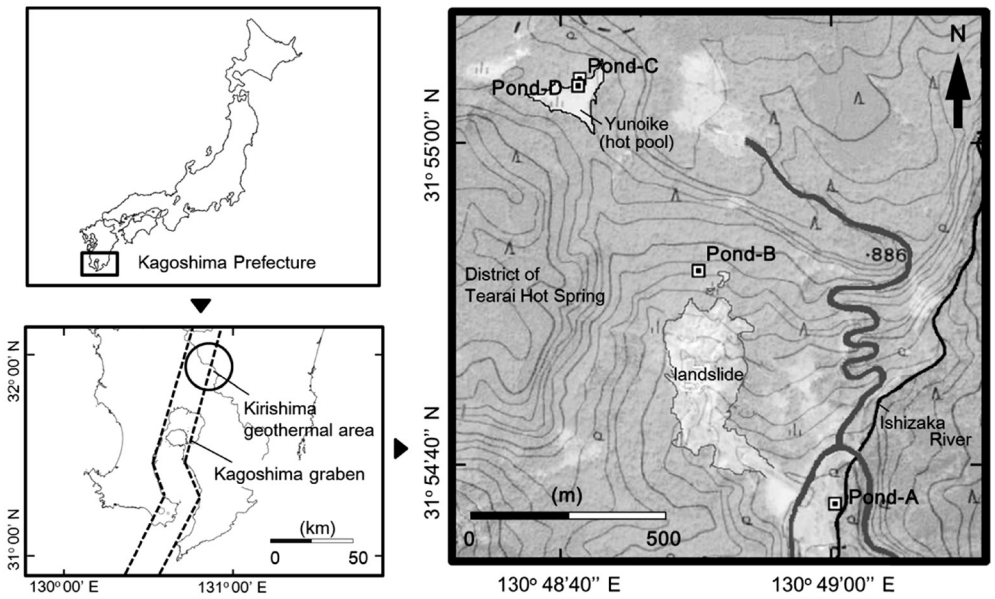

Fig. 1　Map of the sampling sites in/near the region of the Tearai hot spring, the Kirishima geothermal area, Kagoshima Prefecture.

Table 1　Characteristics of sampling sites, pond waters and sediments in the Kirishima geothermal area.

	Pond-A		Pond-B		Pond-C		Pond-D	
Temperature (℃)	93		66		88		67	
pH	2.6		2.0		2.4		2.3	
Chemical concentration (mg L^{-1})/composition (%)								
Fe	388.9	23	1149	51	9.630	5	27.18	8
S	663.2	40	702.8	31	59.76	30	61.90	18
Al	433.6	26	287.9	13	14.57	7	2.021	1
Mg	86.74	5	46.77	2	0.001	0	43.35	13
Si	47.88	3	45.52	2	103.9	53	148.4	44
Ca	54.81	3	10.88	0	7.498	4	39.26	12
P	2.85	0	4.711	0	1.265	1	1.266	0
Na	0.001	0	0.001	0	0.001	0	8.442	2
K	0.001	0	0.001	0	0.001	0	7.384	2
As	9.079	0	1.137	0	0.879	0	0.856	0
Total	1,679	100	2,248	100	197.5	100	340.1	100
Latitude (N)	31°54′37.7″		31°54′52.4″		31°55′05.0″		31°55′04.5″	
Longitude (E)	130°49′00.6″		130°48′50.3″		130°48′41.1″		130°48′41.0″	
Altitude (m)	759		842		884		885	
Color of sediments	light brown		light brown		gray		gray	

Detection limit is 0.001 mg L^{-1}.

The Kirishima geothermal region has been characterized by extensive volcanic activity since the Pleistocene epoch and this is continuing : this activity has resulted in the deposition of a thick pile of volcanic rocks (Goko, 2000). The Kirishima volcano, which is one of the largest Quaternary volcanoes in Japan, is part of the northern section of the Kagoshima graben, a volcano-tectonic depression (Tsuyuki, 1969) caused by the subduction of the Philippine Sea plate. The volcano occupies a 20 km × 30 km area that is elongated in the northwest-to-southeast direction and contains more than 20 small volcanoes (Imura *et al.*, 2001).

The sampling location within the Kirishima geothermal area is located on private land, and thus, the area is not usually exposed to human activity. We obtained permission from an owner of the land to sample the hot springs and pond water as well as soil and various other samples of organisms native to the area. There are many hot springs and muddy ponds present in the area, and these have a variety of temperatures and elemental compositions.

Muddy water samples from each pond were collected in sterile 100 mL glass bottles. The temperature and pH of the samples were measured at each sampling site. Part of each sample was filtered using a 0.22 μm membrane filter (Asahi Glass) and used for analysis of the chemical composition, which was performed by inductively coupled plasma optical emission spectroscopy (ICPS-7000 Ver.2, Shimadzu). We selected four ponds characterized by a wide range of temperatures and chemical compositions for the bacterial community analysis.

2. 2　16S rRNA gene clone libraries and sequencing

Environmental DNA was extracted from 10 g of each muddy water sample using the UltraClean

Soil DNA Kit Mega Prep (Mo Bio Laboratories) according to the manufacturer's instructions.

The purified DNA was then used as the template for amplification of the bacterial 16S rRNA gene using the bacteria-specific primer B27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and the universal primer U1492R (5′-GGYTACCTTGTTACGACTT-3′). The PCR conditions included an initial denaturation step at 94℃ for 3 min, followed by 35 cycles of denaturation at 94℃ for 30 s, annealing at 58℃ for 30 s, and extension at 72℃ for 2 min using *Ex Taq* DNA polymerase (Takara Bio). This was followed by a final extension step at 72℃ for 10 min.

The PCR products were purified using a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and ligated into the pT7 Blue T-Vector (Novagen). *Escherichia coli* DH5α cells were transformed with the plasmid library and plated onto LB plates including 100 μg mL^{-1} ampicillin, 40 μg mL^{-1} X-gal, and 0.5 mM IPTG. Blue/white selection was performed by randomly picking and subculturing individual white colonies in 100 μL of 2×YT medium containing 100 μg mL^{-1} ampicillin in a 96-well plate at 37℃ overnight. The inserted 16S rRNA gene was amplified using 1 μL of the culture as the template with the forward primer T7P-F (5′-TAATACGACTCACTA-TAGGG-3′) and reverse primer T7U-R (5′-GTTTTCCCAGTCACGACGT-3′). About 800 bp of the 5′-region of each 16S rRNA gene clone was sequenced using the aforementioned bacteria-specific primer B27F and used for the taxonomic and phylogenetic analyses.

2. 3　Identification of 16S rRNA gene clones and phylogenetic analysis

16S rRNA gene sequences were edited using MEGA5 (Molecular Evolutionary Genetics Analysis, http : //www.megasoftware.net/) (Tamura *et al*., 2011). We also searched for chimera sequences by manually checking the sequence alignments using GENETYX Ver.10.0.3 software (Genetyx). Clones with 97% or greater sequence similarity were treated as a phylotype. The representative sequences of each phylotype were compared with 16S rRNA gene sequences published in the National Center for Biotechnology Information DNA database using BLAST (BLASTN ; http : //www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1990) to identify individual clones. The representative sequences of each phylotype and related sequences in the GenBank database were aligned using CLUSTALW Ver.1.83 (Thompson *et al*., 1994). A maximum likelihood tree including bootstrap probabilities (1000 samplings) was constructed using MEGA5.

2. 4　Statistical analyses

Measurements of diversity ideally include richness, which is the number of different species or groups present, and evenness, which is the distribution of those groups (Hurlbert, 1971 ; Stirling and Wilsey, 2001). The Shannon–Weaver index (Shannon *et al*., 1949), $H' = -\Sigma(pi)(\ln pi)$, and Simpson's reciprocal index (Simpson, 1949), $1/D$, where $D = \Sigma(pi)^2$ and pi is the proportion of phylotypes i relative to the total number of phylotypes, both consider richness and evenness (Stirling and Wilsey, 2001 ; Stout *et al*., 2009). In this study, the Shannon–Weaver index and Simpson's reciprocal index were calculated using ESTIMATES 8.0 (Colwell, 2006). Evenness ($J' = H'/\ln S$) was also calculated (Pielou, 1969). ESTIMATES 8.0 was also used to calculate the Chao1 nonparametric richness estimator (Chao, 1987) and the abundance-based coverage estimator of species richness (ACE) (Chao *et al*., 2000). These coverage estimators determine the number of probable phylotypes in the environment compared with the number observed in the

sample. Homologous coverage (biodiversity coverage) C was determined using the following equation : $C=1-(N/n)$, where N is the number of phylotype sequences detected and n is the total number of clones analyzed (Good, 1953 ; Singleton *et al*., 2001). Statistical analysis also included principal components analysis, which was used to determine correlations among bacterial diversity and environmental factors including temperature and chemical composition. Canonical correlation analysis was also used to detect correlations between bacterial groups and temperature or chemical components using XLSTAT software (Addinsoft, New York, NY).

2.5 Nucleotide sequence accession numbers

Representative nucleotide sequences of the phylotypes are available in the DDBJ/EMBL/GenBank databases under accession numbers AB762419–AB762465.

3. Results and Discussion

3.1 Water chemistry

The four ponds in the Kirishima geothermal area were selected based on their different temperatures and total concentration of examined chemical components : 1) Pond-A : 93℃ and 1,679 mg L^{-1} ; 2) Pond-B : 66℃ and 2,248 mg L^{-1} ; 3) Pond-C : 88℃ and 198 mg L^{-1} ; and 4) Pond-D : 67℃ and 340 mg L^{-1}. The characteristics of the sampling sites and these ponds are shown in Table 1. The pH value of the ponds ranged from 2.0–2.6. In the ponds with higher concentrations of the examined chemical components, the concentration and percentage of Fe, S, and Al, in particular, were higher than in the other ponds.

To clarify the relationships between temperature and chemical composition or among the chemical components of the four ponds, Pearson's correlation coefficients (r) were calculated (Table 2). Several chemical components were found to be correlated with each other. For

Table 2　Correlation matrix showing r values for Pearson's correlation among factors in the pond waters of the Kirishima geothermal area.

Variables	Temp.	Fe	S	Al	Mg	Si	Ca	P	Na	K	As	Total conc.	Shannon
Temp.	1.00	−0.40	0.05	0.31	0.12	−0.29	0.29	−0.29	−0.55	−0.55	−0.01	−0.14	−0.34
Fe	−0.40	1.00	0.84	0.62	0.34	−0.76	−0.26	**0.99**	−0.46	−0.46	**0.90**	**0.92**	−0.68
S	0.05	0.84	1.00	**0.95**	0.71	**−0.93**	0.21	**0.91**	−0.57	−0.57	**0.99**	**0.98**	−0.74
Al	0.31	0.62	**0.95**	1.00	0.82	**−0.90**	0.44	0.72	−0.57	−0.57	**0.90**	0.87	−0.68
Mg	0.12	0.34	0.71	0.82	1.00	−0.49	0.82	0.44	−0.02	−0.02	0.61	0.64	−0.14
Si	−0.29	−0.76	**−0.93**	**−0.90**	−0.49	1.00	0.00	−0.83	0.84	0.84	**−0.94**	−0.89	**0.93**
Ca	0.29	−0.26	0.21	0.44	0.82	0.00	1.00	−0.15	0.33	0.33	0.07	0.08	0.32
P	−0.29	**0.99**	**0.91**	0.72	0.44	−0.83	−0.15	1.00	−0.51	−0.51	**0.95**	**0.97**	−0.72
Na	−0.55	−0.46	−0.57	−0.57	−0.02	0.84	0.33	−0.51	1.00	1.00	−0.62	−0.51	**0.96**
K	−0.55	−0.46	−0.57	−0.57	−0.02	0.84	0.33	−0.51	1.00	1.00	−0.62	−0.51	**0.96**
As	−0.01	**0.90**	**0.99**	**0.90**	0.61	**−0.94**	0.07	**0.95**	−0.62	−0.62	1.00	**0.99**	−0.79
Total conc.	−0.14	**0.92**	**0.98**	0.87	0.64	−0.89	0.08	**0.97**	−0.51	−0.51	**0.99**	1.00	−0.71
Shannon	−0.34	−0.68	−0.74	−0.68	−0.14	**0.93**	0.32	−0.72	**0.96**	**0.96**	−0.79	−0.71	1.00

Values in bold are different from 0 with a significance level α=0.10. The only Shannon-Weaver index for bacterial clone libraries is shown. Temp., Total conc. and Shannon indicate Temperature, total concentration of examined chemical components and Shannon-Weaver index, respectively.

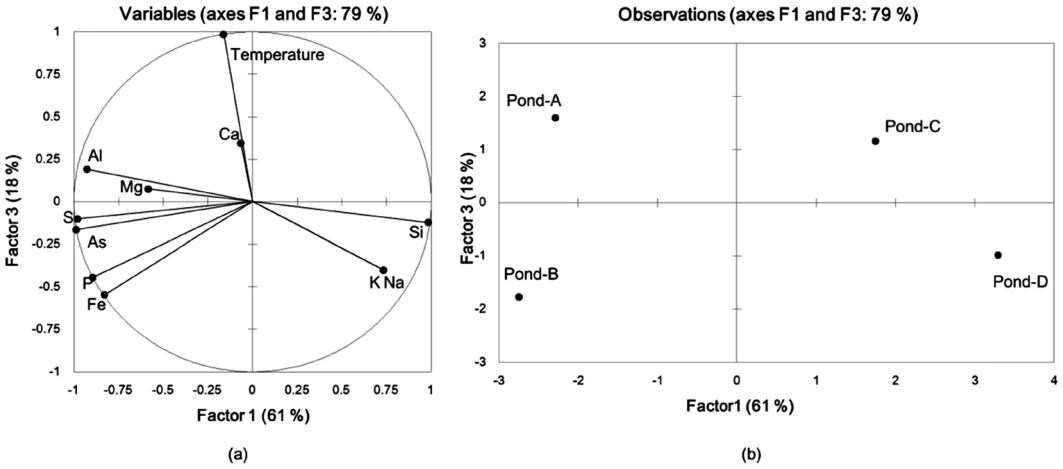

Fig. 2　Principal components analysis showing the environmental variables of the four ponds. (a) Factor loadings on principal components 1 and 3, (b) relationships between the four ponds and the principal components.

example, Fe was strongly correlated with P, and As, and S was strongly correlated with Al, Si, P, and As. The total concentration of the examined chemical components was also strongly correlated with Fe, S, P, and As. Temperature was not statistically correlated with any of the chemical components.

We also attempted to perform the principal components analysis to distinguish the four ponds based on environmental variables (Fig. 2). All the variables characterizing the sites were explained by three principal factors : Factor 1 (F1) 61%, Factor 2 (F2) 21%, and Factor 3 (F3) 18%. F1 was strongly loaded by Si (positively) and S, As, and Al (negatively) ; F2 was strongly loaded by Ca ; and F3 was strongly loaded by temperature. In this analysis, we focused on the principal components 1 and 3 (PC1 and PC3), which affected F1 and F3, respectively. Strong contributions to PC1 were made by S, As, Al, and Si and moderate contributions were made by P, Fe, K, and Na. Temperature contributed strongly to PC3 (Fig. 2a). These environmental variables were defining characteristics of the four ponds (Fig. 2b), and we therefore discuss the bacterial community structures by concentrating on these elements and the different temperatures of the ponds in the following sections.

3.2　Bacterial 16S rRNA gene clone libraries

The 16S rRNA gene clone libraries of domain Bacteria were successfully constructed using environmental DNA extracted from four muddy water samples. A total of 372 clones (Pond-A : 95, Pond-B : 94, Pond-C : 92, Pond-D : 91 clones) of the bacterial 16S rRNA gene were analyzed. No chimerical sequences were detected. The clones were classified into 35 phylotypes on the basis of the sequence similarity values, and these consisted of 10 classes : Flavobacteria, γ-Proteobacteria, β-Proteobacteria, α-Proteobacteria, Nitrospirae, δ-Proteobacteria, Bacilli, Actinobacteria, Thermotogae, and Aquificae (Table 3, Fig. 3).

Table 3　Affiliation and closest published species or clones of 35 phylotypes of bacteria detected in the ponds of the Kirishima geothermal area.

Phylotypes	Affiliation	Closest species or clones (accession number)	16S rRNA gene similarity (%)	Number of clones detected from each site			
				Pond -A	Pond -B	Pond -C	Pond -D
	class Flavobacteria						
ST16B10-59 (=ST15B2-2)	*Elizabethkingia miricola*	*Elizabethkingia miricola* (EU375848)	99.0			3	6
ST8B3-52	*Chryseobacterium aquaticum*	*Chryseobacterium aquaticum* (AM748690)	100.0	2			
	class γ-Proteobacteria						
ST8B3-2 (=ST16B3-5)	*Acinetobacter johnsonii*	*Acinetobacter johnsonii* (NR044975)	98.9	64		52	
ST15B2-3	*Acinetobacter junii*	*Acinetobacter junii* (NR026208)	99.7				34
ST8B3-33 (=ST2B3-83)	*Acinetobacter* sp.	Subsurface groundwater clone BANW433 (DQ264432)	96.8	1	1		
ST2B3-1 (=ST15B3-22)	*Acidithiobacillus caldus*	*Acidithiobacillus caldus* (NR026517)	99.6		41		4
ST8B3-10	*Pseudomonas poae*	*Pseudomonas poae* (AJ492829)	99.4	7			
ST8B3-13	Uncultured *Pseudomonadaceae*	Maple sap clone 100p3_613 (FJ934668)	95.4	1			
ST16B4-80 (=ST15B8-1)	Uncultured Pseudomonadales	Subsurface groundwater clone BANW563 (DQ264531)	97.1			1	2
ST15B8-95	Uncultured Pseudomonadales	Coastal urnaban watershed clone C01JMA (JF692239)	95.3				2
ST8B3-37	Uncultured Pseudomonadales	Subsurface groundwater clone BANW416 (DQ264418)	98.0	2			
ST8B3-15	Uncultured Pseudomonadales	Lake stream water clone D-79 (HQ860678)	94.4	1			
ST2B3-60	Uncultured γ-Proteobacteria	Bioleaching pulp with pH < 2.0 clone zy-5 (EF672753)	92.5		1		
ST15B2-44	Uncultured γ-Proteobacteria	*Acinetobacter junii* (NR026208)	90.8				1
	class β-Proteobacteria						
ST8B3-23 (=ST16B3-18)	*Acidovorax temperans*	*Acidovorax temperans* (NR028715)	99.2	3		11	
ST16B3-9 (=ST15B2-50)	*Delftia tsuruhatensis*	*Delftia tsuruhatensis* (NR024786)	99.5			8	5
ST16B3-16 (=ST15B3-82)	*Naxibacter alkalitolerans*	*Massilia alkalitolerans* (AY679161)	98.0			3	3
ST16B4-10	*Paracoccus marinus*	*Paracoccus marinus* (AB185957)	98.4			6	
ST8B3-40	*Curvibacter lanceolatus*	*Curvibacter lanceolatus* (NR024702)	99.5	1			
ST2B4-26	*Ralstonia pickettii*	*Ralstonia pickettii* (NR043152)	100.0		1		
ST8B3-18	*Methylophilus leisingeri*	*Methylophilus leisingeri* (NR041258)	99.5	1			
ST16B5-42 (=ST15B2-13)	Uncultured *Comamonadaceae*	Spacecraft assembly clean room *Delftia* sp. clone GI5-13-D06 (FJ192433)	98.5			1	2
ST8B3-46	Uncultured *Methylophilaceae*	Uranium-contaminated aquifer clone 1013-28-CG9 (AY532564)	93.4	1			
ST8B3-58	Uncultured *Methylophilaceae*	River site β-proteobacterium clone RBE2CI-98 (EF111184)	91.1	1			
	class α-Proteobacteria						
ST2B3-49	*Acidicaldus organivorans*	*Acidicaldus organivorans* (AY140238)	99.6		1		
	class Nitrospirae						
ST2B3-57	Uncultured Nitrospirales	Acid mine drainage sediment clone H50 (DQ328622)	99.6		1		
	class δ-Proteobacteria						
ST2B3-15	Uncultured δ-Proteobacteria	Extreme acid mine drainage clone BA71 (AF225447)	97.3		41		
	class Bacilli						
ST2B3-24 (=ST15B2-14)	*Staphylococcus epidermidis*	*Staphylococcus epidermidis* (NR036904)	100.0		1		13
ST16B3-94	Uncultured *Paenibacillaceae*	*Bacillus* sp. YNPRH6P-1 (AF465647)	99.2			7	
ST2B3-86	Uncultured Bacillales	Drinking bulk water clone SW-3S_A04 (JX286150)	99.5		3		
ST8B3-7	Uncultured Bacillales	Banana plantation soil clone WB128 (JX133663)	88.0	5			
	class Actinobacteria						
ST8B3-32 (=ST15B9-35)	*Propionibacterium acnes*	*Propionibacterium acnes* (NR040847)	99.3	5			10
ST2B3-20	Uncultured Acidimicrobiales	Hot spring clone SK299 (AY882848)	99.2		1		
	class Thermotogae						
ST2B4-6 (=ST15B8-31)	Uncultured Thermotogae	Thermal spring sediment clone kma134 (HM149925)	98.6		2		1
	class Aquificae						
ST15B2-55	*Hydrogenobaculum* sp.	Hot spring *Hydrogenobaculum* sp. clone KOZ166 (EF156606)	97.6				8
Total				95	94	92	91

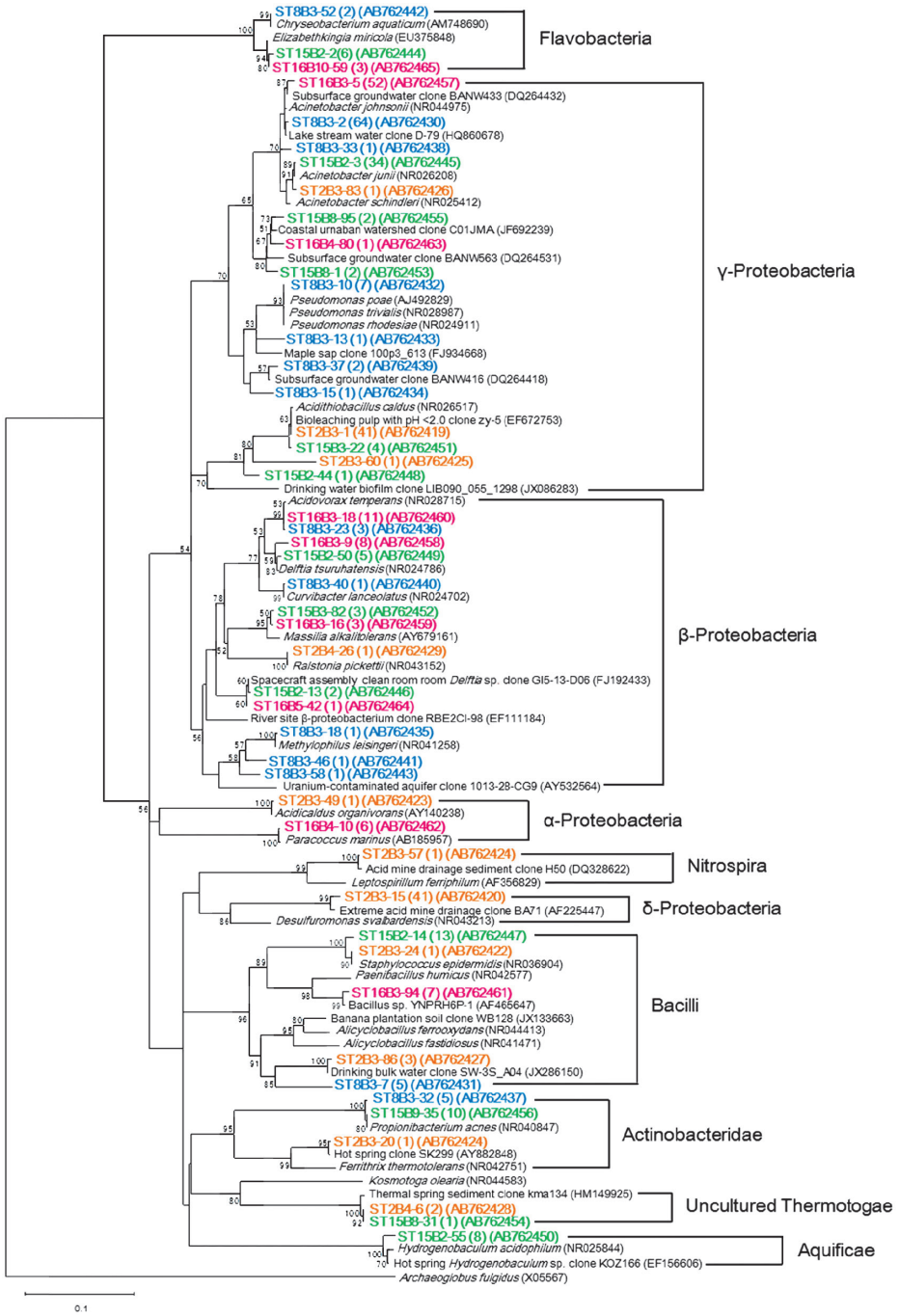

Fig. 3　Phylogenetic tree of bacterial 16S rRNA gene clones detected in the four ponds from the Kirishima geothermal area. Bootstrap values (>50%) based on 1000 replicates are indicated at nodes. The scale bar indicates the number of nucleotide substitutions per position. The number in parenthesis with next to the phylotype name represents the number of clones from each phylotype. The DNA database accession numbers are also indicated in parentheses. *Archaeoglobus fulgidus* was used as an outgroup species. The phylotype names derived from Ponds-A, B, C, and D are shown in blue, yellow, red, and green, respectively.

3.3 Bacterial community in Pond-A

Pond-A was characterized by relatively high temperature and high total concentration of the examined chemical components. Analysis of 16S rRNA gene sequence similarities of the 95 clones derived from the pond revealed 14 phylotypes, which was the largest number of phylotypes detected of all four ponds (Table 3). Of the sequences derived from this pond, 87% were very similar to those of cultured species (>98.9%), including *Chryseobacterium aquaticum* (Kim *et al*., 2008) from the class Flavobacteria ; *Acinetobacter johnsonii* (Bouvet and Grimont, 1986) and *Pseudomonas poae* (Behrendt *et al*., 2003) from the class γ-Proteobacteria ; *Acidovorax temperans* (Willems *et al*., 1990), *Curvibacter lanceolatus* (Ding and Yokota, 2004) and *Methylophilus leisingeri* (Doronina and Trotsenko, 1994) from the class β-Proteobacteria ; and *Propionibacterium acnes* from the class Actinobacteria (Douglas and Gunter, 1946 ; Moore and Cato, 1963 ; Bojar and Holland, 2004). The largest number of clones was assigned to a single phylotype, ST8B3-2, which accounted for 67% of all clones derived from this pond. This phylotype was also dominant in Pond-C and was very similar to the sequence of *A. johnsonii* (98.9%), which is an aerobic, gram-negative, heterotrophic bacteria with an optimal growing temperature of 15–30℃ ; no growth occurs at 37℃. The genus *Acinetonbacter* is widely distributed in soil, water (Baumann, 1968), and sewage (Warskow and Juni, 1972). The second dominant phylotype was ST8B3-10, which was very similar to the *P. poae* sequence (99.4%). This species is an aerobic, gram-negative, heterotrophic, fluorescent bacteria that has an optimal growth temperature of 21℃ ; no growth occurs at 41℃. The bacteria was isolated from the phyllosphere of grasses (Behrendt *et al*., 2003). These were unexpected results because these mesophilic microbes should not have been able to grow in Pond-A given its high temperature. At the moment, we have no reasonable explanation for these findings, but it is known that some species with closely related 16S rRNA gene sequences have different optimal growth temperatures. For example, we recently described a novel *Paenibacillus* species that is the only thermophilic strain of the genus *Paenibacillus*, which has to date only consisted of mesophilic species (Ueda *et al*., 2013). On the other hand, the remaining 13% of all clones derived from Pond-A were classified into seven phylotypes, and they did not show any significant similarity with any cultured species. The Shannon–Weaver index score for Pond-A was the third highest among these four ponds ; it was lower than those of Pond-C and Pond-D but higher than that of Pond-B (Table 4).

3.4 Bacterial community in Pond-B

Pond-B was characterized by a relatively low temperature and a high total concentration of the examined chemical components ; it had the lowest Shannon–Weaver index diversity score of all four ponds (Table 4). A total of 94 clones were derived from Pond-B, and these were determined to constitute 11 phylotypes (Table 3). Nearly half of the sequences in this pond showed a significantly close relationship with one of the following four cultured species (>99.6%) : *Acidithiobacillus caldus* (Hallberg and Lindstörm, 1994 ; Kelly and Wood, 2000) from the class γ-Proteobacteria ; *Ralstonia pickettii* (Ralston *et al*., 1973 ; Yabuuchi *et al*., 1995) from the class β-Proteobacteria ; *Acidicaldus organivorans* (Johnson *et al*., 2006) from the class α-Proteobacteria ; and *Staphylococcus epidermidis* from the class Bacilli (Schleifer and Kloos, 1975). Almost all the clones were assigned to a single phylotype, ST2B3-1, which was very similar to the sequence of

A. caldus (99.6%) ; these clones accounted for 44% of all clones derived from this pond. *A. caldus* is an aerobic, gram-negative, moderately thermophilic, sulfur-oxidizing acidophile with an optimal growth pH of 2–2.5 and temperature of 45℃. This species is also capable of chemolithotrophic growth on reduced sulfur and molecular hydrogen. On the other hand, 53% of the Pond-B clones constituted seven phylotypes that showed no significant similarity with any cultured species ; this was the highest percentage of cultured species among the four ponds. Most of the uncultured clones were assigned to the phylotype ST2B3-15, which affiliated with the class δ-Proteobacteria (Fig. 3). This phylotype showed the closest match to a published environmental clone BA71, which was detected from a lithotrophic biofilm at an extreme acid mine drainage site (DNA database Accession No. AF225447) (Bond *et al*., 2000). The clones of this phylotype were the most dominant in this pond, similar to the aforementioned ST2B3-1 phylotype.

3.5 Bacterial community in Pond-C

Pond-C was another pond with a relatively high temperature, and 92 clones were derived from this pond. These were classified into nine phylotypes, which is the lowest value of species richness among the four ponds (Table 4). The diversity index score in Pond-C was the second highest among the four ponds, i.e., it was lower than that of Pond-D but higher than those of Pond-A and Pond-B. Ninety percent of the sequences from this pond were very similar to the following cultured species (>98.0%) : *Elizabethkingia miricola* (Kim *et al*., 2005) from the class Flavobacteria ; *A. johnsonii* from the class γ-Proteobacteria ; and *A. temperans*, *Delftia tsuruhatensis* (Shigematsu *et al*., 2003), *Massilia alkalitolerans* (Kämpfer *et al*., 2011) and *Paracoccus marinus* (Khan *et al*., 2008) from the class β-Proteobacteria (Table 3). Most clones were assigned to a single phylotype, ST8B3-2, which was also dominant in Pond-A, and accounted for 57% of the clones derived from Pond-C. The second most dominant phylotype was ST8B3-23, which was very similar to the *A. temperans* sequence (99.2%). *A. temperans* is an aerobic, gram-negative bacteria that, has been reported as an abundant member of activated sludge microbial communities (Willems *et al*., 1990 ; Heijstra *et al*., 2009). On the other hand, the remaining 10% of the clones in Pond-C were allocated to three phylotypes that did not show any significant similarity with any cultured species.

3.6 Bacterial community in Pond-D

Pond-D, which was characterized by a relatively low temperature and a low total concentration of the examined chemical components, was the most diverse of the four ponds, as assessed by the Shannon–Weaver index and Simpson's reciprocal index (Table 4). A total of 91 clones were derived from this pond and these consisted of 13 phylotypes. Eighty-two percent of the sequences from this pond were very similar to those of the following cultured species (>98.0%) : *E. miricola* from the class Flavobacteria ; *A. junii* (Bouvet and Grimont, 1986) and *A. caldus* from the class γ-Proteobacteria ; *D. tsuruhatensis* and *M. alkalitolerans* from the class β-Proteobacteria ; and *S. epidermidis* from the class Bacilli and *P. acnes* of the class Actinobacteria (Table 3). A single phylotype, ST15B2-3, contributed 37% of all clones derived from this pond. This phylotype was only detected in this pond and was very similar to the *A. junii* sequence (99.7%). *A. junii* is an aerobic, gram-negative, heterotrophic bacteria with an optimal growth

temperature of 15–30℃ ; no growth occurs at 44℃. The second most dominant phylotype was ST2B3-24, which accounted for 14% of the clones in this pond. This phylotype was very similar to the *S. epidermidis* sequence (100.0%). *S. epidermidis* is an aerobic, gram-positive, heterotrophic bacteria that is ubiquitous in the environment. It has been isolated from human skin ; animal products such as meat, milk, and cheese ; and other sources including soil, sand, seawater, freshwater, dust, and air (Kloos *et al*., 1991 ; Wieser and Busse, 2000). On the other hand, 18% of the clones from Pond-D consisted of six phylotypes that did not show any significant similarity to any cultured species. Most of these uncultured clones were assigned to the ST15B2-55 phylotype, which is affiliated with the class Aquificae (Fig. 3). This phylotype was only detected in this pond and showed the closest match to a published environmental clone KOZ166 (97.6%) detected from Yellowstone National Park (DNA database Acc. No. EF156606).

3. 7 Bacterial diversity and community structure in relation to different temperatures and different total concentrations of the examined chemical components

At least 85% of the 16S rRNA gene sequences from each pond could be analyzed since the homologous coverage values were 0.85 or above for all ponds (Table 4). When diversity was compared for ponds of different temperatures (Temp. approx. 90℃, Pond-A + Pond-C vs. Temp. approx. 70℃, Pond-B + Pond-D), the lower temperature ponds showed higher diversity according to the Shannon–Weaver index and Simpson's reciprocal index values (Table 4). On the other hand, when ponds with different concentrations of the examined chemical components were compared, the diversity indices for the ponds with lower concentrations of the chemical components (Pond-C + Pond-D, Total conc. $<350\,mg\,L^{-1}$) were higher than those for the ponds with higher concentrations (Pond-A + Pond-B, Total conc. $>1{,}600\,mg\,L^{-1}$). As a result, the bacterial diversity was highest in the pond characterized by a lower temperature and a lower concentration of chemical components (Pond-D). In contrast, the combination of higher temperature and

Table 4　Diversity index scores for clone libraries of bacteria detected in the ponds of the Kirishima geothermal area.

Sample	Shannon	Simpson	Rich	Even	S_{ACE}	S_{Chao1}	Coverage	Total clone number
Pond-A	1.38	2.14	14	0.521	23.8	26.3	0.85	95
Pond-B	1.25	2.61	11	0.523	28.8	21.5	0.88	94
Pond-C	1.48	2.83	9	0.676	10.2	10.0	0.90	92
Pond-D	2.04	5.12	13	0.796	14.2	13.3	0.86	91
Temp. approx. 90℃ (Pond-A + Pond-C)	1.66	2.51	21	0.544	31.0	41.3	0.89	187
Temp. approx. 70℃ (Pond-B + Pond-D)	2.23	6.42	21	0.733	29.4	29.2	0.89	185
Total conc. $>1600\,mgL^{-1}$ (Pond-A + Pond-B)	2.00	4.70	24	0.630	42.9	42.0	0.87	189
Total conc. $<350\,mgL^{-1}$ (Pond-C + Pond-D)	2.33	7.09	17	0.823	17.9	17.5	0.91	183

Diversity index scores measured were Shannon–Weaver (Shannon), Simpson's reciprocal index (Simpson), Richness (Rich), Evenness (Even), the coverage estomators S_{ACE} and S_{Chao1} and the homologous coverage. Total conc. indicates total concentration of examined chemical components.

higher total concentration of the examined chemical components (Pond-A) resulted in the lowest diversity in this study.

With regard to species composition and distribution, these were different in ponds characterized by different temperatures and different total concentrations of the examined chemical components. As shown in Table 3, the dominant bacterial group across all ponds was the class γ-Proteobacteria. At a lower taxonomic level, within the class γ-Proteobacteria, phylotype ST8B3-2, which is very similar to the *A. johnsonii* sequence (98.9%) and is affiliated with the order Pseudomonadales, was only detected in the higher temperature ponds (Pond-A + Pond-C). On the other hand, phylotype ST2B3-1, which is very similar to the *A. caldus* sequence (99.6%) and is affiliated with the order Acidithiobacillales, was only detected in the lower temperature ponds (Pond-B + Pond-D). These results suggest that the species composition and distribution within the class γ-Proteobacteria differs for higher and lower temperature ponds. On the other hand, the species composition and distribution also varied between ponds with different total concentrations of the examined chemical components. Phylotype ST8B3-33, which is affiliated with the genus *Acinetobacter* spp., was only detected in the ponds with higher concentrations of the total examined chemical components (Pond-A + Pond-B). In contrast, phylotypes such as ST16B3-9, ST16B3-16, and ST16B5-42, which are affiliated with the order Burkholderiales, were only detected in ponds with lower total concentrations of the examined chemical components (Pond-C + Pond-D).

3.8 Geochemistry and bacterial diversity or group correlations

As shown in Table 2, bacterial diversity was statistically correlated with Si, Na, and K. To clarify the relationships between bacterial groups and the temperature or chemical composition of the four ponds, canonical correlation analysis was performed (Fig. 4). Specific bacterial groups

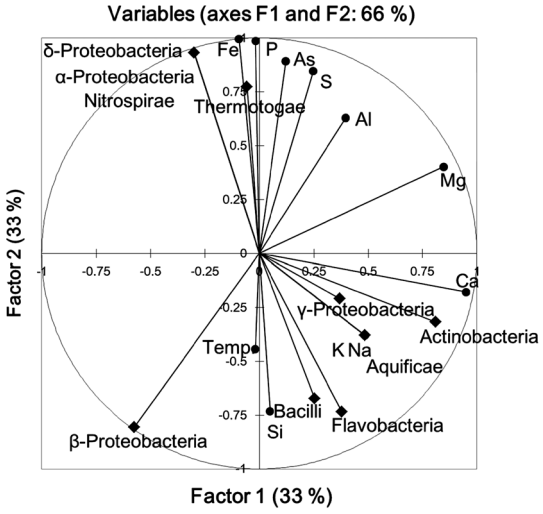

Fig. 4　Canonical correlation analysis showing correlations between environmental factors and proportions of individual bacterial groups. Bacterial groups are shown in abbreviations in a rhombus shape. Environmental factors are shown in circles.

were found to be correlated with particular factors : the classes δ-Proteobacteria, α-Proteobacteria, and Nitrospirae were strongly correlated with Fe and P, the classes Bacilli and Flavobacteria with Si, Na, and K, and Actinobacteria with Na and K. On the other hand, the classes γ-Proteobacteria and Thermotogae were positively and negatively correlated with temperature, respectively. These statistical analyses indicated that there are correlations between bacterial diversity and environmental factors, and these will allow us to begin tracing trends in environmental effects on bacterial diversity. However, linking these types of studies with culture-based studies will provide more insight into how specific elements affect bacterial communities.

4. Conclusion

In this study, 16S rRNA gene phylogenetic analysis was performed to compare the bacterial community structure and diversity of four distinct solfataric-acidic ponds in the Kirishima geothermal area, Kagoshima Prefecture, Japan. The four ponds displayed a wide range of temperatures and chemical compositions. Principal components analysis showed that these four ponds were clearly distinguished by different chemical compositions and temperatures. In general, the dominant bacterial group in the four ponds was the class γ-Proteobacteria followed by the classes β-Proteobacteria, δ-Proteobacteria, Bacilli, and Actinobacteria. Members of the classes Flavobacteria, Aquificae, Thermotogae, α-Proteobacteria, and Nitrospirae were also detected, but they were not as abundant. On the other hand, the bacterial diversity and community composition at the species level was clearly different among ponds with different temperatures and chemical compositions. Bacterial diversity was most affected by temperature, and species composition appeared to be affected by both chemical composition and temperature. Although other environmental factors could also have influenced the bacterial community structure, the present data will be helpful for improving our understanding of the bacterial ecology in the solfataric-acidic ponds. In addition, the 16S rRNA gene clones that showed no significant similarity with any cultured species should allow us to isolate some novel bacterial species via culturing experiments.

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