Prokaryotic Communities and Biodiversity in the Highly Acidic Hot Springs

高温強酸性温泉における原核生物の群集構造および生物多様性

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SYNOPSIS

温度や化学成分が異なる温泉間で原核生物の群集構造および生物多様性を評価・比較するため、鹿児島県霧島地域に分布する4つの強酸性温泉における細菌および古細菌群集構造を、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、1) Pond-A: 93℃、57.9 mmol L^{-1}、2) Pond-B: 66℃、73.4 mmol L^{-1}、3) Pond-C: 88℃、6.73 mmol L^{-1}、4) Pond-D: 67℃、11.5 mmol L^{-1} であり、これらの温泉は主成分分析により温度および化学成分が大きく異なることが示された。4つの温泉由来の計372細菌クローンは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。最も細菌の種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。この温泉における細菌の遺伝的多様性は最も高い値を示していたが、種多様性は最も低い値を示していた。温度が相対的に高い Pond-A と Pond-C では、*Acinetobacter johnsonii* が全クローンの57%以上を占めていた。一方、計431 古細菌クローンは 26 系統群に分類された。古細菌の遺伝的および種の多様性が最も高かったのは Pond-B で、この温泉では Sulfolobales 目に属する古細菌が52%を占めていた。Pond-D も比較的高い生物多様性を示したが、ここで優占していたのは uncultured thermoacidic spring clone group (UTSCG)で全体の58%を占めていた。温度が相対的に高い Pond-A と Pond-C における古細菌の種多様性はいずれも相対的に低かったが、遺伝的多様性は大きく異なり、Pond-A で最も低い値を示した。また、これら2つの温泉間における優占種も大きく異なり、Pond-A では Sulfolobales 目に属する古細菌が89%、Pond-C では Crenarchaeota 門に属する未培養古細菌が全体の99%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強酸性温泉間において、原核生物である細菌および古細菌の群集構造および生物多様性の具体的な違いが明らかとなった。

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 & 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 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 natural environment. Given these new findings, it is currently assumed that cultured species only account for 1% or less of all prokaryotes present on Earth (Amann *et al*., 1995).

Hot spring microbial communities have been extensively studied using phylogenetic analysis of the 16S rRNA gene in areas such as Yellowstone National Park in the United States, Kamchatka hot springs in Russia, the island of the Lesser Antilles, Icelandic hot springs, Mt. Unzen hot springs in Japan, Ohwakudani hot springs in Japan, Pisciarelli hot springs in Italy, Bor Khlueng hot springs in Thailand, the Wai-o-tapu geothermal area in New Zealand, and the Tengchong hot springs in China. 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, 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 3.07 to 82.4 m mol L^{-1}, respectively. However, little is known about the distribution of prokaryotic communities among these ponds and how this is affected by larger environmental variables (e.g., temperature and chemical compositions). In this study, the bacterial and archaeal 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.

Materials and Methods

Study site

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. The altitude in this area generally ranged from 800-1000 m (Goko, 2000). 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 (Tsu-

yuki, 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). Extensive volcanic activity occurred from the Pleistocene epoch to the present and has resulted in the deposition of a thick pile of volcanic rocks (Goko, 2000).

Sampling

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. The sampling was conducted in July 2005, February and June 2006. Surface Muddy water samples from each 21 pond were collected in sterile glass bottles. The temperature and pH of the samples were measured at each sampling site. Samples were brought back to the laboratory and part of each sample was filtered using a 0.22 μm membrane filter (Asahi Glass). Water samples were stored at $4^{o}C$ after acid treatment for analysis of the chemical composition. Muddy water samples were stored at $25^{o}C$ for the prokaryotic community analysis.

Measurement of dissolved elemental concentrations

The chemical composition targeting 12 elements (Fe, S, Al, Mg, Si, Ca, P, Na, K, As, Rb, and Cs) in water samples at 21 sampling site were quantified using the inductively coupled plasma optical emission spectroscopy. These elements were top elements after the qualitative analysis on 72 elements.

Analyses of bacterial community structures

The four ponds displayed a wide range of temperatures and chemical compositions were selected for the bacterial community analysis. 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 the amplification of the bacterial 16S rRNA gene using the bacteria-specific primer B27F (5′-AGAGTTTGATCCT GGCTCAG-3′) and the universal primer U1492R (5′-GGYTACCTTGTTACGACTT-3′). The PCR conditions included an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 2 min using Ex *Taq* DNA polymerase (Takara Bio). This was followed by a final extension step at 72°C for 10 min.

The PCR products were purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and were 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°C overnight. The inserted 16S rRNA gene was amplified using 1 μL of the culture as the template with the forward primer T7P-F (5′-TAATACGACTCACTATAGGG-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 analysis.

16S rRNA gene sequences were edited using MEGA5 (Molecular Evolutionary Genetics Analysis) (Tamura *et al*., 2011). Chimera sequences were searched 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 BLASTN (BLASTN; http://www.ncbi.nlm.nih.gov/BLAST/) 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). The maximum likelihood tree including bootstrap probabilities (1000 samplings) was constructed using the MEGA5.

Analyses of archaeal community structures

Analysis of the archaeal community structure at each four pond was performed as well as the bacterial community analysis. However, the archaeal 16S rRNA gene was amplified by using the archaea-specific primer A21F (5′-TTCCGGTTGATCCYGCCGGA-3′) and the universal primer U1492R (5′-GGYTACCTTGTTACGACTT-3′). In addition, the PCR conditions were modified as follows: an initial denaturation step at 94°C for 3min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2min using Ex *Taq* DNA polymerase. This was followed by a final extension step at 72°C for 10min. About 800 bp of the 5′-region of each archaeal 16S rRNA gene clone was sequenced using the aforementioned archaea-specific primer A21F and used for the taxonomic and phylogenetic analysis.

Diversity indices

The Shannon–Weaver diversity index (Shannon *et al*., 1949), $H' = -\Sigma$ (*pi*) (ln *pi*) and *pi* is the proportion of phylotypes *i* relative to the total number of phylotypes was calculated. Evenness ($J' = H'/$ ln S) were also calculated (Pielou, 1969). θπ, the average sequence divergence was also calculated (Martin, 2002). 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).

Results and Discussion

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°C and 57.9 m mol L^{-1}; 2) Pond-B: 66°C and 73.4 m mol L^{-1}; 3) Pond-C: 88°C and 6.73 m mol L^{-1}; and 4) Pond-D: 67°C and 11.5 m mol L^{-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, Rb, and Cs in particular, were higher than in the other ponds.

Bacterial communities and diversity of four solfataric acidic ponds with different temperatures and chemical compositions

A total of 372 clones of the bacterial 16S rRNA gene were analyzed. The clones were classified into 35 phylotypes on the basis of the sequence similarity values, and these consisted of 10 classes. 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 1).

Pond-A was characterized by relatively high temperature and high total concentration of the examined chemical components. Ninety-five clones derived from Pond-A revealed 14 phylotypes, which was the largest number of phylotypes detected of all four ponds (Table 1). Eighty-seven percent se-

quences derived this pond were very similar to those of cultured species (>98.9%), *Chryseobacterium aquaticum* from the class Flavobacteria; *Acinetobacter johnsonii* and *Pseudomonas poae* from the class γ-Proteobacteria; *Acidovorax temperans*, *Curvibacter lanceolatus* and *Methylophilus leisingeri* from the class β-Proteobacteria; and *Propionibacterium acnes* from the class Actinobacteria. 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°C; no growth occurs at 37°C. The genus *Acinetonbacter* is widely distributed in soil, water (Baumann, 1968), and sewage (Warskow & Juni, 1972). 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 similarities with any cultured species.

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 1). A total of 94 clones were determined to constitute 11 phylotypes. 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* from the class γ-Proteobacteria; *Ralstonia pickettii* from the class β-Proteobacteria; *Acidicaldus organivorans* from the class α-Proteobacteria; and *Staphylococcus epidermidis* from the class Bacilli. 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 °C. This species is also capable of chemolithotrophic growth on reduced sulfur and molecular hydrogen. On the other hand, 53% of the all Pond-B clones constituted seven phylopypes 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. 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.

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 1). Ninety percent of the sequences from this pond were very similar to the following six cultured species (>98.0%): *Elizabethkingia miricola* from the class Flavobacteria; *A. johnsonii* from the class γ-Proteobacteria; and *A. temperans*, *Delftia tsuruhatensis*, *Massilia alkalitolerans* and *Paracoccus marinus* from the class β-Proteobacteria. 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. 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.

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 (Table 1). A total of 91

clones consisted of 13 phylotypes. Eighty-two percent of the sequences from this pond were very similar to those of the following seven cultured species (>98.0%): *E. miricola* from the class Flavobacteria; *A. junii* 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* from the class Actinobacteria. 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°C; no growth occurs at 44°C. 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.

Table 1 Diversity index scores for bacterial clone libraries.

Sample	θ_π	Shannon	Rich	Even	Coverage	Total clone number
Pond-A	116	1.38	14	0.521	0.85	95
Pond-B	151	1.25	11	0.523	0.88	94
Pond-C	122	1.48	9	0.676	0.90	92
Pond-D	145	2.04	13	0.796	0.86	91

Diversity indices measured were gene diversity (θ_π), Shannon-Weaver (Shannon), Richness (Rich), Evenness (Even) and homologous coverage.

Archaeal communities and diversity of four solfataric acidic ponds with different temperatures and chemical compositions

A total of 431 clones were classified into 26 phylotypes on the basis of sequence similarity values, consisting of 25 crenarchaeal phylotypes and a single euryarchaeal one. At least 85% of the 16S rRNA gene sequences from each pond could be analyzed since the homologous coverage values were 0.88 or above for all ponds (Table 2).

A total of 106 clones derived from Pond-A consisted of five phylotypes of the phylum Crenarchaeota (Table 2). The 5% and 7% sequences of this pond were highly similar to those of cultured species (>98.0%): *Caldivirga maquilingensis* and *Vulcanisaeta distributa* from the order Thermoproteales, respectively. The type strains of both species were hyperthermophilic archaea optimally growing at above 85°C, and they were originally isolated from acidic hot springs in Philippines and Japan, respectively (Itoh *et al*., 1999; Itoh *et al*., 2002). On the other hand, the remaining 89% of all clones derived from Pond-A were classified into three phylotypes, and they did not show any significant similarity with any cultured species. Almost all the clones were assigned to a single phylotype, ST8A1-12, which affiliated with the order Sulfolobales. This phylotype showed 95-96% sequence similarity with published environmental clones, NAKO74-07 and HS3wa 52 detected from Nakabusa hot spring, Japan (DNA database Accession no. AB366602) (Kimura *et al*., 2010) and Tatung Volcano hot spring, Taiwan (DNA database Accession no. FJ797311). This pond was the most diverse of the four ponds, as assessed by the Shannon-Weaver index.

In contrast to the Pond-A, the largest number of phylotypes was detected in Pond-B, resulting that the diversity index score and evenness value in this pond were highest among the four ponds (Table 2). A total of 112 clones consisted of 14 phylotypes that were classified into the following six groups: the order Sulfolobales, Acidilobales, Thermoproteales, and three uncultured crenarchaeal groups. Twenty-one percent of the total clones were closely related to any of five cultured species (>98.0%): *Sulfolobus solfataricus*, *Metallosphaera sedula*, *Acidianus brierleyi*, *Caldisphaera lagunensis*, and *Caldivirga maquilingensis*. *S. solfataricus*, *M. sedula*, and *A. brierleyi* are facultatively chemolithoautotrophic aerobes and require elemental sulfur or sulfidic ores. These species and their close

relatives have been isolated from acidic Solfatara fields around the world (Huber & Stetter, 2001). *C. lagunensis* and *C. maquilingensis* are heterotrophic anaerobes. Their growths are stimulated or constrained by the presence of sulfur as an electron acceptor. On the other hand, the remaining 79% of all clones derived from Pond-B were classified into nine phylotypes, and they did not show any significant similarity with any cultured species. Nearly half of these uncultured clones were assigned as a phylotype ST2A1-5. This phylotype was most dominant (35%) in Pond-B and was phylogenetically distant not only from any cultured species but also from any published environmental clones. This novel phylotype belonged to a cluster in the order Sulfolobales. This cluster also harbored another Pond-B phylotype ST2A1-32, which showed 98% 16S rRNA gene sequence similarity with published environmental clone, LH2wa 90 detected from Taiwanese hot spring (DNA database Acc. no. FJ797343). The phylotype ST2A1-8 belonging to the uncultured thermoacidic spring clone group (UTSCG) (Kato *et al.*, 2011) was secondary dominant in Pond-B and it shared 18% of the total Pond-B clones. Interestingly, phylotypes similar to ST2A1-8 were also frequently detected in Pond-C and Pond-D, suggesting that this crenarchaeal species survive relatively wide range of temperature and dissolved elemental composition in acidic hot ponds. There might be unfavorable factors in Pond-A for the presence of UTSCG. The phylotype ST2A1-25 was thirdly dominant in Pond-B and was placed into the sister cluster of the hot water crenarchaeotic group II (HWCG II) (Takai & Sako, 1999; Schrenck *et al.*, 2003; Nunoura *et al.*, 2005) with phylotype ST2A1-52 and published environmental clone SK859 detected from acidic hot spring in Yellowstone National Park (DNA database Acc. No. DQ834111). We call this cluster as HWCGVI in this study. The phylotype ST2A1-25 was also dominant in Pond-C.

A total of 109 clones derived from Pond-C were classified into six phylotypes as follows: *Thermocladium modestius* of the order Thermoproteales, four uncultured crenarchaeal phylotypes and uncultured euryarchaeal phylotypes (Table 2). The only one clone was affiliated with *T. modestius.* The type strain of this species was originally isolated from solfataric mud at Noji-onsen, Japan, and is an anaerobic heterotroph growing optimally around 75°C, pH 4.0 (Itoh *et al.*, 1998). As mentioned in the previous section, the uncultured phylotypes ST2A1-8 of UTSCG and ST2A1-25 of HWCG VI were dominant in the clone library constructed for this pond sample. These two phylotypes shared 81% in total of the Pond-C clones. Three phylotypes sharing 56% of Pond-C clones were affiliated with uncultured thermoacidic spring clone group (UTSCG).

A total of 104 clones derived from Pond-D consisted of nine phylotypes (Table 2). The phylotype sharing 20% of the total clones was related to *Caldisphaera draconis* with 95% sequence similarity of 16S rRNA gene. This species is chemoorganotrophic anaerobe isolated from acidic hot spring in Yellowstone National Park (Boyd *et al.*, 2007). Other phylotypes showed no significant similarity with any cultured species. The most frequent phylotype was ST2A1-8 affiliated with UTSCG and it shared 50% of the total clone in this pond. In contrast to the archaeal communities in other three ponds, the secondary dominant uncultured phylotype (ST16A1-50) was affiliated with the phylum Euryarchaeota and showed 99% sequence similarity of 16S rRNA gene with thermal spring clone kmc048 detected from Kamchatka hot springs in Russia (DNA database Acc. no. HM150106). This phylotype shared 16% of the total clones in this pond.

Table 2. Diversity index scores for archaeal clone libraries.

Sample	θ_π	Shannon	Rich	Even	Coverage	Total clone number
Pond-A	60	0.53	5	0.332	0.95	106
Pond-B	122	2.06	14	0.780	0.88	112
Pond-C	116	1.23	6	0.687	0.94	109
Pond-D	113	1.45	9	0.659	0.91	104

Diversity indices measured were gene diversity (θ_π), Shannon-Weaver (Shannon), Richness (Rich), Evenness (Even) and homologous coverage.

Conclusion

In this study, 16S rRNA gene phylogenetic analysis was performed to compare the bacterial and archaeal community structure and biodiversity 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. The bacterial and archaeal biodiversity and community composition at species level was clearly different among the ponds. Although other environmental factors could also have influenced the bacterial and archaeal community structure and biodiversity, the present data will be helpful for improving our understanding of the prokaryotic 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 and archaeal species via culturing experiments.

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