ASTROBIOLOGY
Volume 14, Number 3, 2014

DOI: 10.1089/ast.2013.1008

Research Article

Microbial Community Structures of Novel Icelandic Hot Spring Systems Revealed by PhyloChip G3 Analysis

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Abstract

Microbial community profiles of recently formed hot spring systems ranging in temperatures from 57°C to 100°C and pH values from 2 to 4 in Hveragerði (Iceland) were analyzed with PhyloChip G3 technology. In total, 1173 bacterial operational taxonomic units (OTUs) spanning 576 subfamilies and 38 archaeal OTUs covering 32 subfamilies were observed. As expected, the hyperthermophilic (~100°C) spring system exhibited both low microbial biomass and diversity when compared to thermophilic (~60°C) springs. Ordination analysis revealed distinct bacterial and archaeal diversity in geographically distinct hot springs. Slight variations in temperature (from 57°C to 64°C) within the interconnected pools led to a marked fluctuation in microbial abundance and diversity. Correlation and PERMANOVA tests provided evidence that temperature was the key environmental factor responsible for microbial community dynamics, while pH, H_2S, and SO_2 influenced the abundance of specific microbial groups. When archaeal community composition was analyzed, the majority of detected OTUs correlated negatively with temperature, and few correlated positively with pH. Key Words: Microbial diversity—PhyloChip G3—Acidophilic—Thermophilic—Hot springs—Iceland. Astrobiology 14, xxx–xxx.

1. Introduction

Molecular techniques continuously widen the scope of microbial diversity studies and initiate fundamental biological research of extreme terrestrial environments, the most common of which are hot springs (Hugenholtz *et al.,* 1998; Kanokratana *et al.,* 2004), solfatara (Kvist *et al.,* 2007), hydrothermal vents (Martin *et al.,* 2008), and geothermally heated soils (Marteinsson *et al.,* 2001a). Thermophilic and hyperthermophilic microorganisms are of particular interest to microbiologists searching for bioactive compounds as well as hot spring habitats to examine molecular or mineral evidence of ancestral extremophiles. Typically, these hot springs contain fluids laden with dissolved mineral ions, which precipitate and create mineral deposits. With temperatures reaching above 100°C, Icelandic hot springs are some of the hottest in the world and are therefore ideal for studying microbial life under extreme conditions (Barth, 1950).

Due to their characteristic overall low biomass, exploring hot spring ecosystems with conventional methods is challenging. Prior to developments in molecular techniques, cultivation assays and microscopy were employed to study thermal spring systems (Shivvers and Brock, 1973; Shima and Suzuki, 1993). Although these techniques have advanced our understanding of life in extremes (see Stetter, 1982; Stetter *et al.,* 1981), they prove inadequate when discerning microbial community structure and interaction (Blank *et al.,* 2002; Mori *et al.,* 2008; Kublanov *et al.,* 2009). Since the 1990s, molecular methods such as ribosomal ribonucleic acid (rRNA) gene sequencing via radiolabeling have been used to examine terrestrial hot spring microbial communities (Stahl *et al.,* 1985; Ward *et al.,* 1990). This approach, along with subsequent conventional cloning and Sanger sequencing, has led to the discovery of novel uncultivated hyperthermophilic microorganisms (Reysenbach *et al.,* 1994; Barns *et al.,* 1996; Pace, 1997).

DNA microarray approaches like the PhyloChip have been demonstrated to accurately measure microbial community

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composition in extremely low biomass samples, such as those found in spacecraft assembly clean rooms (Cooper *et al.,* 2011; Venkateswaran *et al.,* 2012). As PhyloChip analysis can detect the presence of rare microorganisms at a proportional fraction of less than 10^{-4} abundance compared to the total sample, it can be used to measure microbial community structures in various environments (Brodie *et al.,* 2006; La Duc *et al.,* 2009; Hazen *et al.,* 2010). Furthermore, it was documented that even the earlier second-generation (G2) PhyloChip offered better bacterial and archaeal diversity coverage depth than short read pyrosequencing (~243 bp) when applied to acidophilic (pH 2.7) and mesophilic (29°C) spring samples from the Colombian Andes (Bohorquez *et al.,* 2012). The third-generation (G3) PhyloChip employed in this study implements more than 1 million probes targeting over 59,000 bacterial and archaeal 16S rRNA genes (DeSantis *et al.,* 2007; Hazen *et al.,* 2010).

The molecular microbial community structure of neutral pH hot springs has been assessed in Iceland (Skírnisdóttir *et al.,* 2000; Marteinsson *et al.,* 2001a), Thailand (Kanokratana *et al.,* 2004), and other parts of the world (Tobler and Benning, 2011), as have acidophilic (pH 2.7) and mesophilic (29°C) springs in the Colombian Andes (Bohorquez *et al.,* 2012). However, to our knowledge, this study is the first attempt to elucidate the molecular microbial community structure of an extremely low biomass, acidophilic (pH 2–4), and hyperthermophilic (55–100°C) Icelandic hot spring system with a highly sensitive microarray technology. In addition, we measured total and viable microbial populations, using field-deployable rapid molecular assays that enabled the *in situ* estimation of microbial burden. Furthermore, this study compared the influence of various parameters on the presence and prevalence of community structure.

2. Materials and Methods

2.1. Field sites and sample collection

Samples were collected from three Icelandic hot springs in the same geothermal vent field in Hveragerði, Iceland, 45 km southeast of Reykjavík. The surrounding active geothermal area is part of the Hengill central volcano, which experiences frequent minor earthquakes. The hot spring system, which is approximately 20 m long, includes three interconnected pools (P1, P2, and P3). A fourth pool (P0) at the beginning of the system, with no surface connection to the other three, was used as the control site (Fig. 1). In addition, two other geographically distinct hot springs were sampled: Leirgerdur (L1) (~250 m northeast of P0) and the vegetated Hrifla spring (HS1) (~330 m northeast of P0; Fig. 1). The distance between L1 and HS1 is ~125 m.

2.2. Physical and chemical parameters measurement

The geographic location, physical and chemical characteristics of the hot spring systems examined during this study have already been described (Marteinsson *et al.,* 2013) and were used to interpret each parameter's influence on the abundance of microbial richness. Temperature was measured with an *in situ* probe developed by ÍSOR, Iceland Geo Survey, and ionic activity was measured with a pH meter (PHM220, Radiometer, Copenhagen, Denmark). The EGM-1 (by PP Systems, Hitchin, UK) device was used to

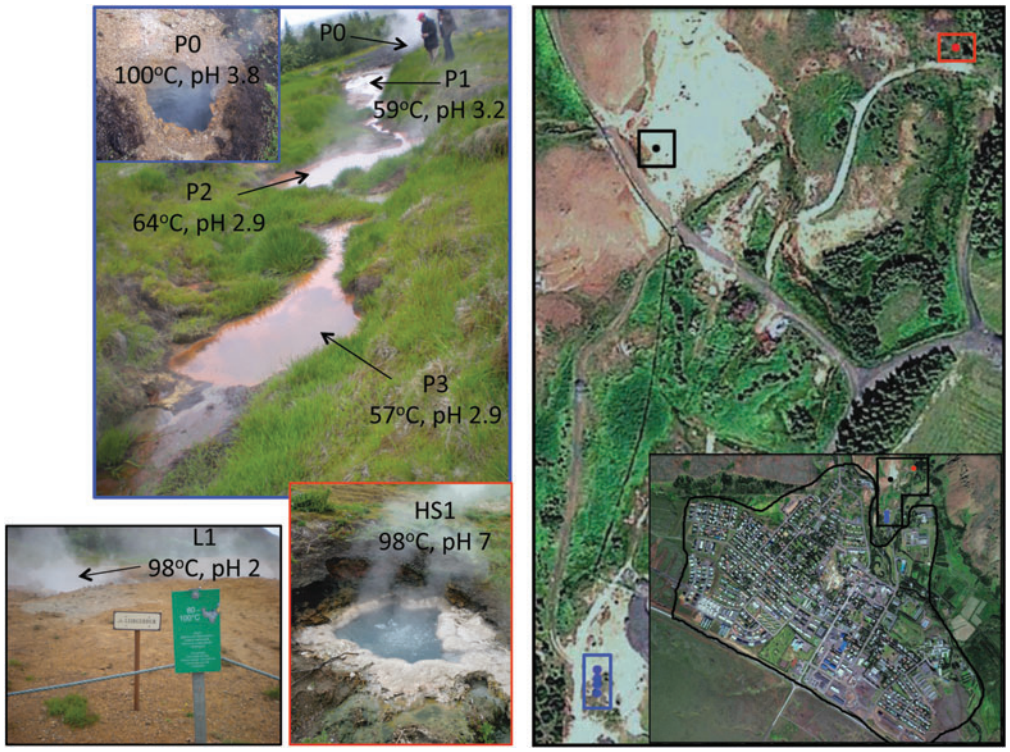

FIG. 1. Hot spring sampling sites of Hveragerði, Iceland. Borders of hot spring images correspond to the representative colored dots on the map to the right. Inset map shows outlined sampling location in reference to outlined Hveragerði town.

detect CO_2 (in ppm) through infrared analysis. SO_2 and H_2S concentrations were measured with an APSA 370 monitor (HORIBA, Kyoto, Japan). These measurements were carried out in two zones: the area directly associated with the hot springs (P1, P2, and P3 locations) and at a 20 m distance from the hot spring (P0) as the control site.

2.3. Sample characteristics

The physical, chemical, and microbiological characteristics of the Icelandic hot spring samples collected during this study are summarized in Table 1. The color of the three interconnected hot spring pools (P1, P2, and P3) and L1 was brick red, but the P0 hot spring was grayish-black in color. The HS1 hot spring was colorless with a neutral pH and high temperature (98°C). Likewise, the highly acidic L1 (pH 2) and P0 (pH 3.8) pools had high temperatures recorded at 98°C and 100°C, respectively. However, the other three interconnected acidic hot spring pools (P1, P2, and P3) had comparatively low temperatures (57–64°C). The temperature of the P2 hot spring pool was higher (64°C) than the adjacent hot spring pools (P1 and P3) due to having submerged hot water outlets. The location of L1 was unique in that there was no surrounding vegetation, while the HS1 spring was insulated from the surrounding vegetation by a basin (>20 cm in diameter) of silica precipitation (Fig. 1).

2.4. Sample processing

All samples were aseptically collected in sterile containers and disposables. A matrix of surface water slurry (50 mL of semiliquid mixture of a thin sloppy mud) and solid mud (50 g) was collected in triplicate from each sampling site. Slurry samples were collected with a sterile pipette and mud samples with a sterile scoop with a maximum depth of 25 cm deep into the sediment. The samples were then transported from the hot springs site to a hotel room in a cooling box at 4°C. The table surface used as a makeshift laboratory was cleaned with sterile 70% ethanol before sample processing in the hotel room. In total, six samples in triplicate were collected: four from hot spring pools (P0, P1, P2, and P3), one from the Leirgerdur (L1) hot spring, and one from the Hrifla spring (HS1). Appropriate controls were added to downstream molecular microbiological assays to ensure the cleanliness of the makeshift laboratory. By using a handheld adenosine triphosphate (ATP) instrument (Lumitester PD-10 and Lucipac-W, Kikkoman, Tokyo, Japan), *in situ* ATP measurements of the samples were carried out (data not shown). This approach enabled the selection of samples with measurable biomass. Furthermore, all collected samples were subjected to a high sensitivity ATP assay (see below) within 2 h of sampling in a makeshift laboratory near the sampling site before refrigeration or freezing. All samples were then transported to the Jet Propulsion Laboratory for further molecular analyses.

2.5. ATP assay

A bioluminescence assay was performed on all samples by using the CheckLite HS kit (Kikkoman) to determine the total ATP and intracellular ATP, as described previously (Venkateswaran *et al.,* 2003). To determine the total ATP (dead and viable microbes), 0.1 mL sample aliquots (four replicates; slurries) were combined with 0.1 mL of a cell lysing detergent (benzalkonium chloride) then incubated at room temperature for 1 min prior to adding 0.1 mL of

Table 1. Characteristics of the Six Hot Spring Sampling Sites of the Hveragerði, Iceland

Parameters	*P0*	*P1*	*P2*	*P3*	*L1*	*HS1*
Physical state	Mud	Slurry	Slurry	Slurry	Mud	Liquid
Color	Gray/black	Brick red	Brick red	Brick red	Brick red	Colorless
Temperature (°C)	100	59	64	57	98	98
pH	3.8	3.2	2.9	2.9	2.0	7.0
SO_2 (ppm)[a]	1.1×10^{-2}	3.4×10^{-3}	3.6×10^{-3}	3.6×10^{-3}	ND[b]	ND
H_2S (ppm)[a]	1.8×10^{0}	4.9×10^{-1}	1.4×10^{-2}	1.4×10^{-2}	ND	ND
CO_2 (ppm)[a]	3.996×10^{2}	3.902×10^{2}	3.887×10^{2}	ND	ND	ND
Presence of vegetation around	Yes	Yes	Yes	Yes	No	Yes
Total microbial population (RLU/mL)	BDL[c]	2.9×10^{6}	1.5×10^{6}	2.7×10^{6}	1.2×10^{6}	5.3×10^{4}
Total viable microbial population (RLU/mL)	BDL	4.0×10^{5}	1.4×10^{6}	1.0×10^{4}	1.4×10^{5}	3.7×10^{3}
Percent viable microbial population[d]	–[e]	14.1	94.8	0.4	12.2	7.0
Total bacterial population (16S rRNA copy number/mL)	BDL	1.0×10^{6}	2.8×10^{4}	4.0×10^{5}	3.4×10^{4}	8.7×10^{2}
Percent total bacterial population[f]	–	37.3	1.8	14.8	2.9	1.6
Bacterial PCR amplification (ng/μL)	BDL	117.06	81.51	117.86	BDL	BDL
Archaeal PCR amplification (ng/μL)	BDL	189.75	70.9	88.38	28.97	BDL
Bacterial subfamilies detected	8	313	127	318	7	8
Archaeal subfamilies detected	0	16	10	18	4	2

[a]Data from Marteinsson *et al.*, 2013.
[b]ND: Not determined.
[c]BDL: Below detection limit.
[d]Percent viable microbial population was calculated as (Internal ATP/Total ATP)×100.
[e]Percent viable microbial population was not determined, as both total microbial (Total ATP) and total viable microbial (Internal ATP) populations were below the detection limit. Percent total bacterial population was not determined, as total microbial population (Total ATP) and total bacterial population (bacterial qPCR) were below the detection limit.
[f]Percent total bacterial population was calculated as (16S rRNA copy numbers measured via bacterial qPCR/Total ATP)×100.

luciferin-luciferase reagent. The sample was mixed, and the resulting bioluminescence was measured with a luminometer (Kikkoman). To determine the intracellular ATP (viable microbes), 0.1 mL of an ATP-eliminating reagent (apyrase, adenosine deaminase) was added to a 1 mL portion of the sample, mixed, and incubated for 30 min to remove any extracellular ATP, after which the ATP assay was carried out as described above. As previously established, one relative light unit (RLU), the unit of measurement of ATP, was assumed to be approximately equal to one colony-forming unit (La Duc *et al.,* 2007).

2.6. qPCR assay

The frozen mud/slurries were thawed at room temperature, and 1 mL of subsample was aseptically transferred for DNA extraction. Nucleic acid from each sample was extracted in duplicate with a PowerSoil DNA Isolation Kit (Catalogue # 12888, MoBio Lab, Carlsbad, CA, USA) by using the manufacturer's protocol. A real-time quantitative polymerase chain reaction (qPCR) assay targeting the 16S rRNA gene was performed in triplicate with a qPCR instrument (BioRad CFX-9600, Hercules, CA, USA) to measure bacterial burden. Standards were prepared from known concentrations of PCR amplicon of the 16S rRNA gene from *Escherichia coli* spanning 10^8 to 10^2 gene copies/μL. Universal bacterial primers targeting the 16S rRNA gene, 1369F (5′-CGG TGA ATACGT TCY CGG-3′), and modified 1492R (5′-GGW TAC CTTGTT ACG ACT T-3′) were used for this analysis (Kwan *et al.,* 2011).

2.7. PhyloChip G3 analysis

Bacterial and archaeal 16S rRNA genes were amplified from DNA preparations of each sample. When having quantifiable DNA concentrations for P1, P2, and P3 (Qubit 1.0, Invitrogen, Grand Island, NY, USA), 3.0 ng of DNA was used per PCR reaction and amplified in eight replicate 25 μL reactions spanning a temperature gradient of 48–58°C, as previously described (Hazen *et al.,* 2010). For low-biomass samples irrespective of DNA concentration (Table 1), 4 μL of DNA was used for gradient PCR reactions. Archaeal amplification of 16S rRNA genes was performed with primers 4Fa and 1492R; bacterial amplicons were generated by using the 27F and 1492R primer pair (Hazen *et al.,* 2010). A total of 35 PCR reaction cycles was run for each gradient PCR. Bacterial PCR amplicon was concentrated to ~25 μL with Amicon Ultra-0.5 mL Centrifugal Filters (Millipore, Billerica, MA, USA). Archaeal PCR amplicon was concentrated as above, gel-extracted with the Qiagen MinElute Gel Extraction Kit (Qiagen, Valencia, CA, USA), and eluted in 15 μL of elution buffer. Subsequently, 1 μL of concentrated bacterial and archaeal amplicons were quantified by 2% agarose gel (Invitrogen, Carlsbad, CA, USA) prior to running PhyloChips. A maximum of 600 ng of PCR amplicon (500 ng bacterial and 100 ng archaeal) from each sample was used for PhyloChip analysis. However, when the bacterial PCR amplicon concentration was below 500 ng per a volume of 21.5 μL, a maximum of 21.5 μL of the PCR amplicons was used. A detailed explanation of the PhyloChip G3 assay and operational taxonomic unit (OTU) calling has been described elsewhere (DeSantis *et al.,* 2007; Hazen *et al.,* 2010). The OTU analysis is referred to as ''microbial richness or diversity'' in this communication. Hybridization intensities of OTUs were transformed ($\log_2$ * 1000) and are henceforth referred to as ''microbial abundance.''

2.8. PhyloChip data processing and statistical analysis

Ordination analysis (non-metric multidimensional scaling, NMDS) and PERMANOVA testing (at 999 permutations), based on abundance scores of OTUs and a Bray-Curtis distance, were performed in the R programming environment (Vegan and MASS package). Weighted principal component analysis (PCoA) was performed by using the FAST Unifrac interface in which OTUs were grouped into subfamilies and the number of different OTUs per subfamily served as weighting (Hamady *et al.,* 2010).

Pearson correlation of microbial richness or abundance values of individual OTUs with different environmental factors (pH, temperature) and chemical data (SO_2, H_2S) was performed in the R environment. The same software platform was used for generating heatmaps of OTUs showing significant correlations.

For phylogenetic tree construction, a representative OTU was manually selected from the respective subfamily. The 16S rRNA gene sequence of each representative OTU was retrieved from SILVA (Pruesse *et al.,* 2007), compiled in a multiple sequence alignment, and used to generate a neighbor-joining phylogenetic tree in MEGA 4 (Tamura *et al.,* 2011). Afterward, heatmaps (presence/absence of each representative OTU) were overlaid onto trees in iTOL (Letunic and Bork, 2011). OTUs of represented subfamilies were classified by using the Greengenes (DeSantis *et al.,* 2006) database in combination with the Ribosomal Database Project, and SILVA (DeSantis *et al.,* 2006; Pruesse *et al.,* 2007; Cole *et al.,* 2009). This classification scheme was also repeated later for all archaeal OTUs in the manuscript due to recent classification changes in the literature (Spang *et al.,* 2010).

3. Results

3.1. Microbial population

Total microbial populations, as measured by the ATP assay, were higher in P1, P2, P3, and L1 samples (~2.0×10^5 RLU/mL) compared to P0 (below detection level) and HS1 samples (5.3×10^4 RLU/mL). Low ATP content was correlated with the high temperature (>98°C) of the hot spring samples tested. The viable microbial population based on ATP measurements was as high as 95% in samples collected from P2 (pH 2.9, 64°C). In contrast, this percentage was very low in the P3 sample (0.4%) despite having a similar pH as P2 (Table 1). The combination of low pH (3.8) and high temperature (100°C) might have been the reason for the below-detection level of total microbial (ATP assay) and total bacterial (qPCR assay) population in P0 samples. As revealed by ATP assay, qPCR also showed higher bacterial populations for the other samples compared to the P0 and HS1 samples. The percent bacteria (qPCR-based) among the ATP-based total microbial population in all samples collected during this study ranged between ~2% and 37% (Table 1).

3.2. Microbial richness

An overview of the bacterial richness based on subfamilies in each sample classified by higher taxonomic level

is depicted in Fig. 2 (presence/absence of a subfamily in each sample). Despite extremely low ATP content in some of the samples (>98°C) studied (Table 1), the bacterial diversity measurement via the PhyloChip G3 method was successful in all six samples. In total, 1173 bacterial OTUs spanning 576 subfamilies were detected (Supplementary Table S1; Supplementary Data are available online at www.liebertonline.com/ast). All hyperthermophilic hot spring samples had a lower bacterial richness than thermophilic samples (Supplementary Fig. S1). Of the high-temperature samples—P0 (84 OTUs), HS1 (19 OTUs), and L1 (19 OTUs)—only 24 subfamilies were observed; three OTUs were shared between L1 and P0 samples and five OTUs between HS1 and P0. However, hundreds of subfamilies were observed in P1 (529 OTUs), P2 (168 OTUs), and P3 (674 OTUs). Comparatively, P2 was hotter than P1 and P3, both of which had greater microbial richness than P2. Furthermore, the bacterial richness of the P1 and P3 samples, with temperatures <59°C, was similar. The distribution of OTUs at higher taxonomic levels was dominated by Firmicutes (23%), followed by Betaproteobacteria (15%), Gammaproteobacteria (13%), Deltaproteobacteria (7%), Actinobacteria (6%), Alphaproteobacteria (5%), Acidobacteria (5%), and others. With reference to the archaeal richness, 38 OTUs covering 32 subfamilies were detected including Crenarchaeota (20 OTUs), Euryarchaeota (17 OTUs), and Thaumarchaeota (1 OTU) (Supplementary Table S1). Archaeal OTUs were found in all samples except in P0.

3.3. Environmental clustering

Ordination analysis based on NMDS and abundance scores of bacterial OTUs showed that hyperthermophilic hot spring samples clustered apart from thermophilic samples (Fig. 3). This could be attributed to the fact that the bacterial PCR amplicons amplified from hyperthermophilic samples were fewer in quantity (<100 ng per chip) than samples from thermophilic sites (500 ng per chip). Hence, high-temperature samples exhibited both weaker hybridization intensities and lower abundance scores. However, among the samples with lower temperatures, the bacterial community structure in P1 was comparatively more similar to P3 than P2 (Fig. 3).

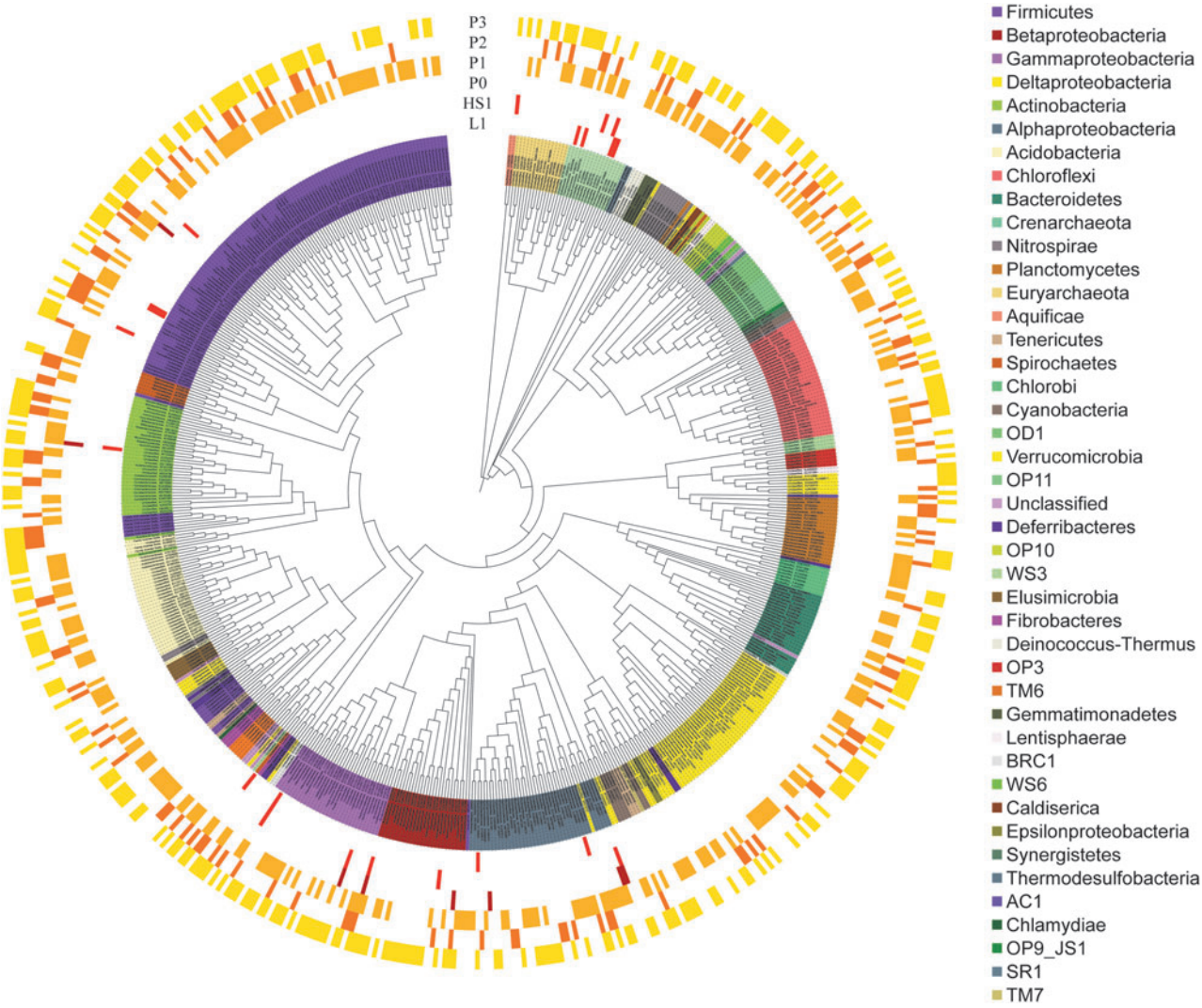

FIG. 2. Phylogenetic neighbor-joining tree of representative OTUs per subfamily detected in the samples. Each node shown in the iTOL circular tree is a representative OTU of a subfamily. Colors indicate the presence of a subfamily in the sample, which are arranged as rings around the tree. The branch lengths in the tree are ignored.

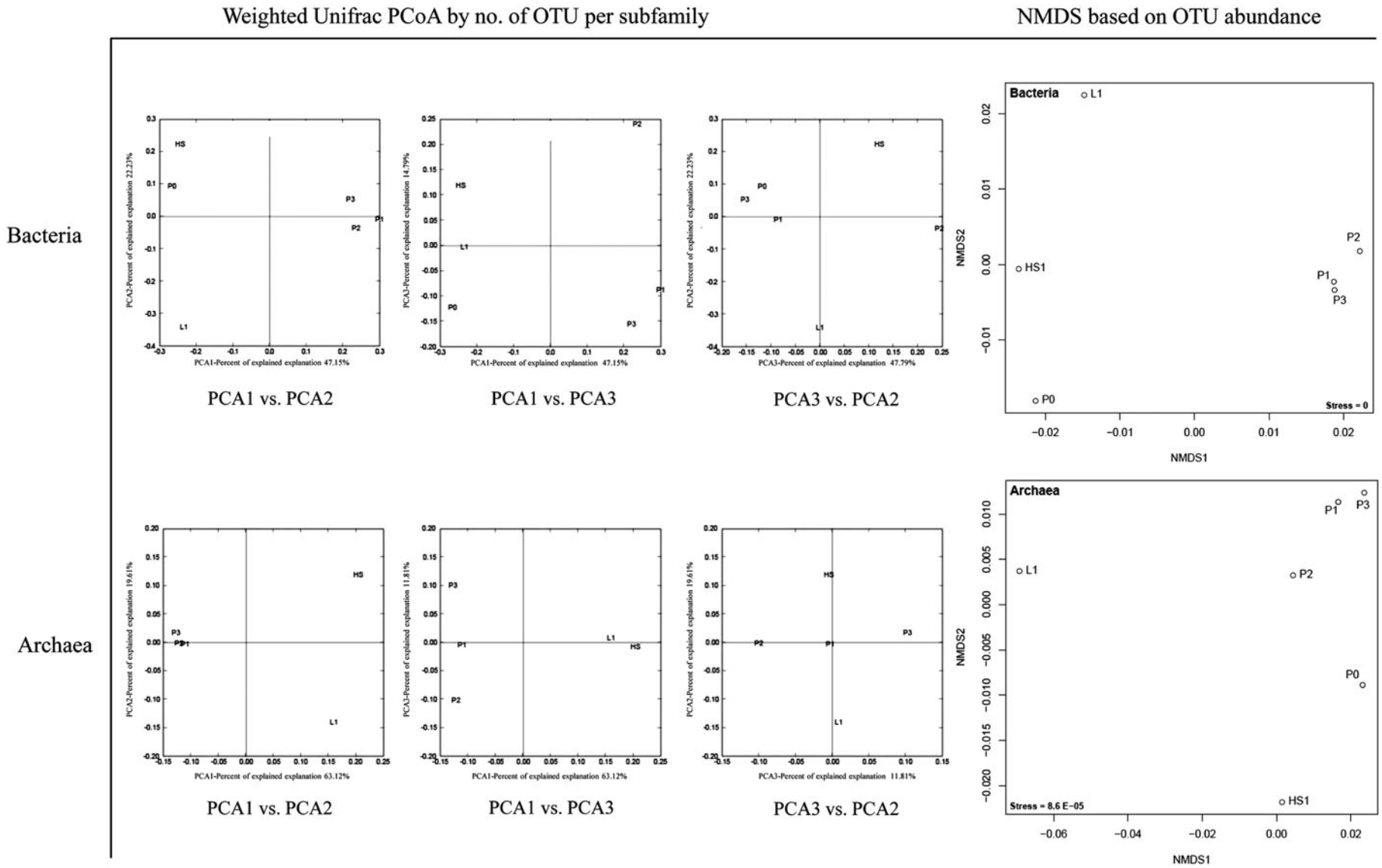

FIG. 3. Ordination methods to show microbial diversity relationships of samples taken from various hot springs in Iceland (Fig. 1, Table 1). Weighted Unifrac PCoA was performed by categorizing OTUs in subfamilies and using the number of OTUs as the weighting. Each subfamily had one representative OTU for phylogenetic relationship measurements. NMDS was based on a Bray Curtis distance of OTU abundances that were detected in at least one of the samples.

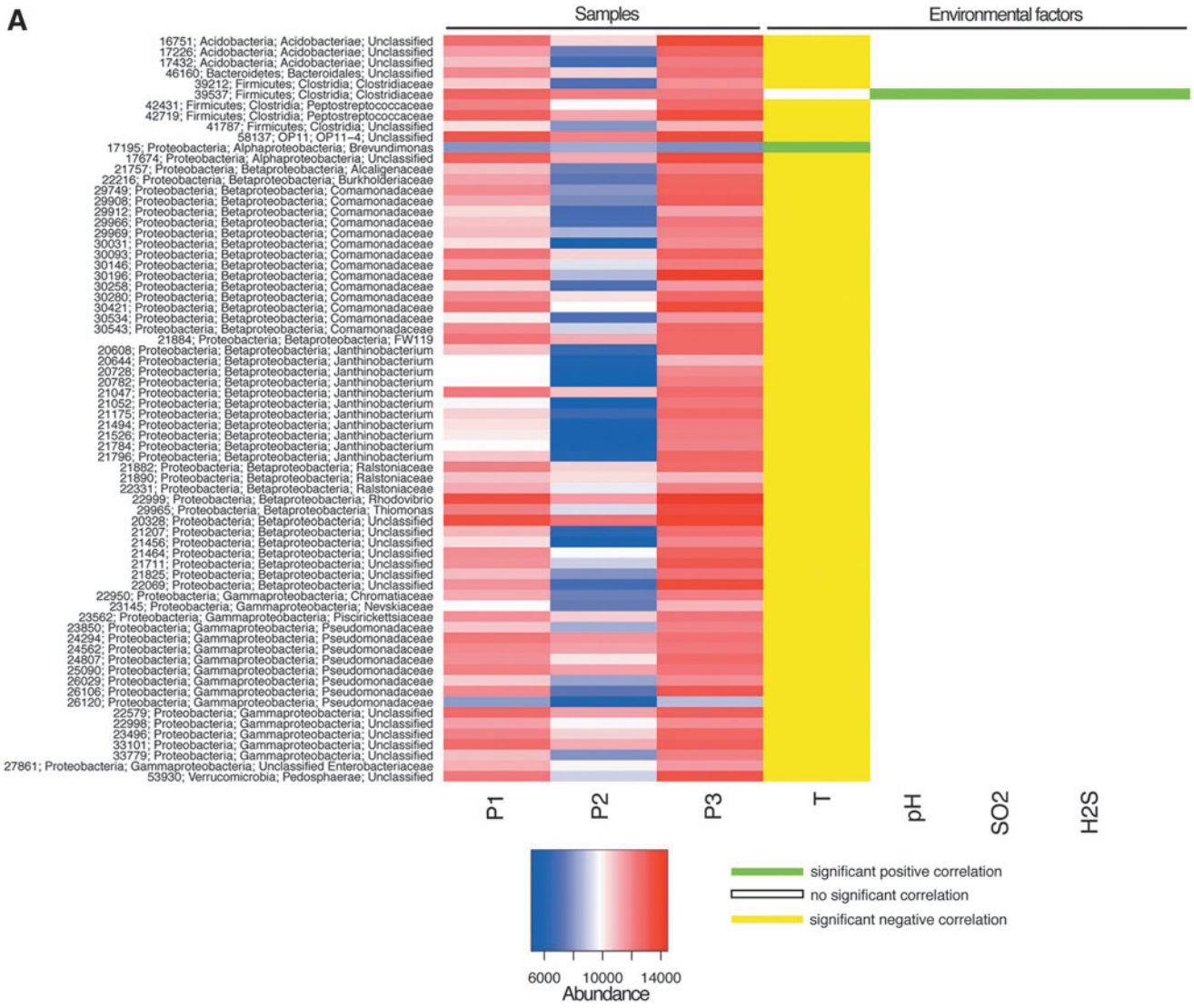

FIG. 4. (Continued).

The NMDS analysis for the archaeal community showed similar environmental clustering as that of bacteria (Fig. 3). With the exception of HS1 and P0, all samples had the same amount of archaeal PCR amplicon used for PhyloChip (100 ng), which allowed us to conclude that the archaeal communities in the P1, P2, and P3 samples were similar to each other but differed greatly from L1. An alternate ordination approach was performed based on the presence or absence of OTUs with one representative per subfamily by using weighted PCoA (Fig. 3). The PCoA-based clustering for the bacterial and archaeal microbial community was in accordance with the relationships observed in NMDS. Sample P0 was not included in PCoA, as no archaeal OTUs were present in this sample (Fig. 3).

3.4. Correlation of bacterial community with environmental factors

When bacterial subfamily richness was compared, temperature was the only environmental parameter having a significant correlation (Supplementary Table S2). Considering only thermophilic samples, both environmental clustering methods (NMDS and PCoA) suggested that sample P3 was more related to P1, even though P2 was located between P1 and P3 in the water streamlet. However, it should be noted that P2 was flanked by at least one visible hot spring that emanated into the streamlet (Fig. 1; data not shown), increasing its temperature. To understand the environmental factors that cause this dissimilarity, a Pearson's correlation was applied to the abundance values of individual OTUs that occurred at least once in the P samples (P0, P1, P2, and P3). Most of the bacteria present (700 out of 1158 OTUs) exhibited a significant negative correlation with temperature (p value < 0.05), whereas only one of them correlated positively (Caulobacterales–*Brevundimonas;* Fig. 4A).

Other environmental factors showed significant positive or negative influence on the prevalence of individual OTUs retrieved from P samples: pH (5 OTUs), SO_2 (458 OTUs), and H_2S (6 OTUs). However, less amount of bacterial PCR amplicon was hybridized for P0 than for P1, P2, and P3 due

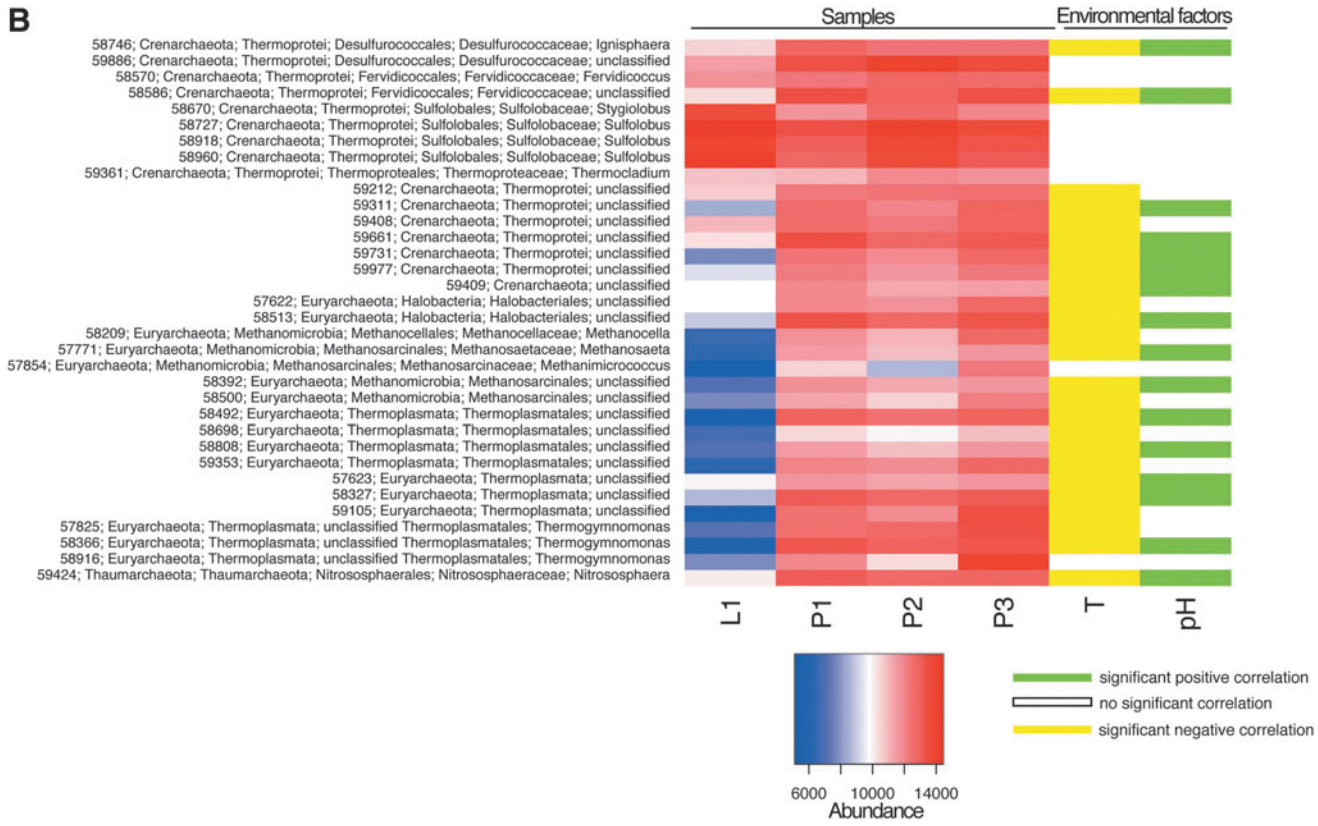

FIG. 4. (**A**) Heatmap of abundance values of bacterial OTUs detected in the P system. OTUs that showed a significant correlation between abundance scores in P1, P2, and P3 samples and of the environmental factors measured are shown. Classifications are at the phylum/class level. (**B**) Heatmap of abundance scores of all archaeal OTUs detected in P1, P2, P3, and L1 samples and their correlation with environmental factors (pH and temperature).

to a weak amplification rate, which could confound the correlation analysis performed above. Among the 1158 OTUs detected from the P system, 69 showed a significant correlation with temperature and abundance values when considering P1, P2, and P3 samples only. Additionally, one clostridial OTU correlated positively with pH, SO_2, and H_2S (Fig. 4A). As P1 and P3 samples were more similar in environmental clustering methods when compared to P2 (Fig. 3), the abundance values were averaged, and the percent increase or decrease in P1 and P3 (or P1-P3) compared to P2 was calculated (Supplementary Fig. S2). All bacterial OTUs showed an increase in relative abundance in P1-P3 samples, pointing to a possible absence of novel bacteria in P2 sample (5–7°C less). The OTUs representing genetic signatures of *Thermodesulfovibrio* and *Thiomonas* were detected in high relative abundance in P1 and P3, which might be characteristic to these pools.

3.5. Correlation of archaeal community with environmental factors

To clarify the influence of physical and chemical parameters on the distribution of archaea, statistical tests on different community profiling levels were employed. First, ordination analysis of the archaeal population revealed separate grouping of the interconnected P samples from both L1 and HS1 (Fig. 3). PERMANOVA testing based on the Bray-Curtis index of OTU abundances was completed for P1, P2, P3, and L1 samples and demonstrated that temperature had a significant influence on the archaeal community structure (p value = 0.04) but pH did not (p value = 0.17). Similarly, temperature showed a highly significant negative correlation with archaeal richness (Pearson's $r = -0.958$, p value = 0.003), while pH was insignificant (Pearson's $r = -0.414$, p value = 0.414, Supplementary Table S2). Second, an individual correlation analysis of each archaeal OTU with temperature and pH, respectively, was performed. Hybridization intensities of OTUs that were present in at least one of the above-mentioned samples were individually correlated with environmental factors across these samples. OTUs with significant correlation values were selected to construct a heatmap presenting their relative difference in hybridization intensity, correlation with the environmental factor, and their taxonomic affiliation (Fig. 4). In general, when archaeal OTU abundances of P1, P2, P3, and L1 samples were computed, 74% correlated negatively with temperature, and 47% correlated positively with pH (Fig. 4B). For instance, genetic signatures of mesophilic archaea, such as *Nitrososphaera*-related OTUs, were more abundant at higher pH and lower temperatures.

4. Discussion

There are numerous technological problems (sample collection, processing, and detection) that have precluded a comprehensive microbial census of ''low biomass''

extreme environments such as hot springs. A previously favored technique to describe the microbial composition of hot springs at a molecular level was the cloning and Sanger sequencing method (Marteinsson *et al.,* 2001a, 2001b; Hobel *et al.,* 2005; Kvist *et al.,* 2007). Many publications show the limitations of this technique, in particular, its low sensitivity due to the small amount of clones sequenced per sample (La Duc *et al.,* 2009; Hazen *et al.,* 2010). In a previous study, Thermotogales or Thermodesulfobacteria taxa were not detected from the samples of Hveragerði wastewater drain (~pH 9) or from Geysir sites (70–83°C, pH ~9) (Tobler and Benning, 2011). However, the present study identified Thermodesulfobacteria within the thermophilic P1, P2, and P3 systems, which corroborates that the PhyloChip G3 microarray is one of the most sensitive technologies among next-generation methods available for comprehensively measuring the microbial census (Hazen *et al.,* 2010; Venkateswaran *et al.,* 2012). The limit of detection for PhyloChip G3 is 2 p*M* of generated 16S rRNA gene amplicons. Even though some of the samples (>98°C) studied were extremely low in ATP content (a proxy for microbial biomass), the microbial diversity measurement via PhyloChip G3 method was successful in all samples examined.

Compared to other terrestrial habitats such as soil, where PhyloChip G3 analysis was able to detect more than 33,000 different OTUs (Mendes *et al.,* 2011), the samples reported here contained a very restricted community profile (1173 OTUs) that reflects the extreme nature of the environments studied. Supporting our assumption that hot springs of this nature are low in biomass, a recently concluded PhyloChip-based study reported the presence of only 4882 OTUs associated with a Chinese hot spring (Briggs *et al.,* 2013). As documented previously in Thailand's Bor Khlueng neutral pH hot spring systems (50–57°C), members of major phyla were well represented in the P1, P2, and P3 samples (Kanokratana *et al.,* 2004). Similarly, bacteria of high abundance (Aquificales, *Nitrospira,* and *Thermodesulfobacterium*) detected during this study were also shown to be major constituents in nearby sulfide-rich and silica-depositing Icelandic hot springs that were alkaliphilic (pH 8–10) and thermophilic (~65°C) (Tobler and Benning, 2011), as well as in other silica-precipitating hot springs in New Zealand (Childs *et al.,* 2008), Japan (Yamamoto *et al.,* 1998), and the USA (Blank *et al.,* 2002; Wilson *et al.,* 2008). In Hveragerði wastewater drain sample with a temperature of about 70°C, Aquificae represented about 11% of OTUs (Tobler and Benning, 2011). Though no Aquificae OTUs were detected in P0 (100°C), 16%, 5.3%, and 4.8% of all OTUs were members of Aquificae in L1 (98°C), HS1 (98°C), and P2 (64°C), respectively. Therefore, with the exception of P0, hot springs sampled with temperatures above 60°C showed greater percentages of Aquificae OTUs in our study. These observations are in agreement with earlier studies that suggested that chemolithotrophic organisms, belonging to the order Aquificales, dominate the bacterial communities in hot spring geothermal waters (Flores *et al.,* 2008; Boomer *et al.,* 2009).

In an independent parallel study, the same samples collected from P1, P2, and P3 pools were subjected to the denaturing gradient gel electrophoresis (DGGE) method; and the presence of Alphaproteobacteria, Betaproteobacteria, Actinobacteria, Bacilli, Clostridia, Aquificae, and unclassified bacteria was reported (Marteinsson *et al.,* 2013). However, PhyloChip G3 analysis revealed the presence of not only those bacterial taxa detected via DGGE but also additional lineages (Fig. 2, Supplementary Table S1) not previously detected in other hot spring systems (Hreggvidsson *et al.,* 2006; Flores *et al.,* 2008; Koskinen *et al.,* 2008). It is also worth mentioning that DGGE bands were not observed in hyperthermophilic P0, L1, or HS1 samples (Marteinsson *et al.,* 2013), whereas PhyloChip G3 revealed the presence of several microbial taxa (Fig. 2, Supplementary Table S1).

While temperature was the key environmental factor of all parameters measured, pH might have dictated the abundance of acidophilic bacteria. Also, the acidic nature of this hot spring system influenced its microbial diversity and structure. For instance, 84 OTUs dominated by Gammaproteobacteria were observed in the hot P0 sample (100°C) where pH was 3.8, but only 19 OTUs were observed in a similarly high-temperature but neutral pH sample (HS1). Most of the identified OTUs belonging to the acidophilic bacteria were from the P1, P2, and P3 systems. The acidophilic taxa classified during this study were either identified as Acidobacteria (56 OTUs) or belong to the members of Alphaproteobacteria (59 OTUs) and Nitrospirae (15 OTUs), whose sequences were previously retrieved from acidophilic environments. *Nitrospira*-related OTUs were found in the P1, P2, and P3 samples, whose presence was confirmed previously in a Hveragerði wastewater drain (~pH 9) (Tobler and Benning, 2011). *Nitrospira* was shown to be tolerant of low (~2.9) and moderately high (~9) pH possibly due to microhabitats in which other microbes provide a pH niche for these bacteria to survive (Altmann *et al.,* 2003). Similarly, members of the *Nitrospira* genus isolated from a hot spring in Nevada, USA, demonstrated a maximum growth temperature slightly above 63°C (similar to P2) (Lefèvre *et al.,* 2010). Thus, the presence of *Nitrospira* in the P1, P2, and P3 systems might be due to their preference to a thermophilic condition, along with the adaptation to the environment or coexisting microbes that might alter the pH of the microhabitat. In addition to *Nitrospira, Thermus* and *Bacillus* were previously found to be dominant microorganisms in hot spring geothermal waters (Hreggvidsson *et al.,* 2006).

Past studies have shown that Archaea flourish in pH, temperature, salinity, and oxygen-level extremes (Woese *et al.,* 1990; Kristjánsson and Hreggvidsson, 1995; Blochl *et al.,* 1997; Chen *et al.,* 2005; Stetter, 2006). However, recent studies have detected and cultivated many mesophilic and non-extremophilic archaea (de la Torre *et al.,* 2008; Tourna *et al.,* 2011), members of this domain are still largely unknown. It was reported that ammonia-oxidizing archaea like *Nitrososphaera* occur at moderate temperatures and were abundant in soil under both neutral pH conditions and thermophilic conditions (Hatzenpichler *et al.,* 2008; Tourna *et al.,* 2011; Spang *et al.,* 2012). Consequently, an enrichment of these archaea at lower temperatures and higher pH was in accordance with their physical and chemical properties. In contrast, three OTUs of the Sulfolobales (*Stygiolobus* sp. and *Sulfolobus* sp.) did not correlate negatively with temperature or positively with pH. For this archaeal order, a trend was observed in lieu of a significant relationship (Pearson's $r = 0.92$ for OTU 58670, p value 0.08); the Sulfolobales were enriched (higher abundance

values) in the L1 sample, which had higher temperature and lower pH. These archaea maintain low intracellular pH to keep Fe-S enzymes operative in central metabolic and bioenergetic pathways (Schafer *et al.,* 1999; Iwasaki and Oshima, 2001). Previous studies have reported Sulfolobales in hot springs at low pH levels, where they thrive chemolithotrophically by oxidizing sulfur (Shivvers and Brock, 1973; Brock, 1978; Kvist *et al.,* 2007). In contrast to the enrichment of the Sulfolobales, members of the methanogens showed a positive correlation of abundance with increasing pH. It is generally believed that a pH below 5 can inhibit the methanogenic activity in anaerobic biological systems (Kim *et al.,* 2004), but all samples included in the archaeal correlation analysis had a pH below 3.5. Their presence in these systems may be attributed to microhabitats provided by other microorganisms.

It has been demonstrated that the microbial community structure correlates with environmental geochemical parameters, such as temperature, salinity, pH, energy source availability, and geographical isolation (Petursdottir *et al.,* 2009). Likewise, when physical, chemical, and biological parameters of thermophilic but neutral pH Icelandic hot springs were characterized, temperature, salinity, and sinter growth rate were found to be the primary regulators of microbial abundance (Tobler and Benning, 2011; Tobler *et al.,* 2008). Since only microbial density and abundance were characterized in previous Icelandic hot spring studies (Tobler *et al.,* 2008), a more detailed look at the microbial diversity is necessary to understand the influence of the environmental factors measured. Results of this study provide evidence that temperature was the key abiotic factor responsible for microbial community dynamics, while pH, H_2S, and SO_2 influenced the abundance of specific microbial groups.

5. Conclusion

The combination of multiple analyses improves our ability to accurately assess the microbial structure and dynamics of low-biomass extreme environments. Metadata collection (physical and chemical attributes), microbial population estimation (ATP and qPCR assays), and microbial richness and abundance measurement (PhyloChip G3) carried out during this study enabled us to better understand the microbial population dynamics of the Hveragerði Icelandic hot spring system. Overall, this study, along with others, revealed that the microbial community structure correlates well with specific physical-chemical and geochemical parameters, including temperature, salinity, pH, and energy source availability. The Hveragerði Icelandic hot spring system may harbor sources of new bioactive compounds as well as novel microbial species and may merit future diversity mapping with emerging novel molecular technologies.

Acknowledgments

Part of the research described in this study was carried out at the Jet Propulsion Laboratory, California Institute of Technology, under contract with the National Aeronautics and Space Administration. A. Probst's contribution was supported by the German National Academic Foundation (Studienstiftung des deutschen Volkes). J. Krebs's participation was funded by a Caltech Amgen Scholars Fellowship awarded in 2011. The authors are grateful to the Coordination Action for Research Activities on life in Extreme Environments (CAREX) project funded by the European Commission. A special thanks to N. Walter, European Science Federation, for supporting P. Vaishampayan's travel to Iceland. We are also thankful to all the participants for their assistance in the Icelandic CAREX fieldwork.

Abbreviations

ATP, adenosine triphosphate; DGGE, denaturing gradient gel electrophoresis; NMDS, non-metric multidimensional scaling; OTUs, operational taxonomic units; PCoA, principal component analysis; qPCR, quantitative polymerase chain reaction; RLU, relative light unit; rRNA, ribosomal ribonucleic acid.

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Submitted 31 March 2013
Accepted 28 January 2014