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Culture-Dependent and Culture-Independent Characterization of Microbial Communities in Hot springs of Bursa, Turkey

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The microbial diversity was investigated in hot spring of Cekirge, Kemalpasa, Orhaneli and Oylat (Temperature: 48-68°C, pH 7.4-7.8) in Bursa, 2014 using an integrated approach that included enrichment culturing and 16S rRNA gene pyrosequencing. Enrichment cultures for both aerobic and anaerobic microorganisms were successfully obtained at temperatures ranging from 50°C to 70°C. An average of 1,296- 2,365 operational taxonomic units per sample observed. The bacterial community was composed of sequence reads moderately related (nucleotide identity > 90%) to 36 phyla and 6 candidate phyla, with Proteobacteria, Chloroflexi, Bacteroidetes and Chlorobi being dominant. This study results provide evidence of the necessity to combine both culture-dependent and culture-independent methods for better understanding of microbial communities in hot springs.

Keywords: enrichment culturing, pyrosequencing, microbial community, hot spring, Bursa.

Hydrothermal fluids create a suitable environment for many phototrophic and chemolithotrophic microorganisms[1, 2]. In these surroundings, mainly are microbial organisms as cyanobacteria and filamentous green non-sulfur bacteria[3], thermophilic denitrification bacteria[4] and especially *Crenarchaeota* from Archaea members[5, 6]. It has been known from many studies[7, 8] mainly from the one conducted in Yellowstone National Park which has more than ten thousand hot springs and in terrestrial hot springs like Tengchong in China, Kamchatka in Russia and in Pacific Ocean deep see hydrothermal vents, in hydrothermal springs in shallow sea waters of Papua New Guinea[9] that although having kinds that may be cultivated, the cultivation of most part of thermophiles has not been done yet.

Since classical microbiologic methods in isolation and cultivation studies are limited to enable information about high microbial variety of hydrothermal areas, today findings obtained with molecular methods through genomic DNA are preferred. Upon completion of gene sequence analysis of 16S rRNA gene in *Escherichia coli* in 1978[10], demand and usage of 16S rRNA gene in microbiology and microbial ecology have increased. Ribosomal RNA gene sequences have not been only used as marker genes to construction bacterial phylogenetic tree but also used to understanding microbial community composition. Due to lower sequencing expenses and big parallel capacities such as 454 pyrosequencing technique in the last decade, the number of rRNA gene sequence has significantly increased. Pyrosequencing has been used as a fast and effective instrument to compare microbial communities and for detailed analysis of them including pre-detection of microbial communities before metagenomic analysis[11]. Furthermore, generally rare members corresponding to more than half of natural environment are also result of evolutionary past and obviously have limitless genomic inventory source[12].

Turkey has a huge geothermal potential due to high numbers of orogenic, magmatic and volcanic activities as it lies on Alp-Himalaya

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orogenic belt [13]. It is at the 7th row in the world geothermal energy potential listing. Due to active faults and volcanism, there are over 600 geothermal springs mainly in Aegean Region, Northwest, Middle Anatolia, and East and Southeast Anatolia regions.

Cold springs and hot springs in the southern Marmara region have unique characteristics. The North Anatolian Fault Zone, is one of the most important fault zones in the world, is located in this region. With this study, microbial diversity has been determined with culture-dependent and culture-independent (454 pyrosequencing) for the first time in some of the Bursa´s hot springs using a holistic approach.

MATERIALS AND METHODS

Sample collection and analysis of fluid chemistry

Fluid samples were collected at Cekirge, Oylat, Orhaneli and Kemalpasa hot springs in March 2014, Bursa, Turkey (Fig. 1). Fluid samples were filtered (0.22 µm pore size Millipore filter) into acid-washed polypropylene bottles, and stored at 4°C until analysis. Fluid chemical and physical analyses were performed using the international standards suggested by the American Public Health Association (APHA)[14]. The temperature, pH and electrical conductivity of fluid were measured using a multiparameter analyser Multi 340i (WTW, Weilheim, Germany) in the field site. Total dissolved sulfide, dissolved oxygen, and total iron were measured in the field site by using a portable spectrophotometer (HACH model 2800) and reagent (Hach Co., Loveland, CO). Trace elements in these fluids were determined using a inductively coupled plasma mass spectrometry (ICP-MS). Chlorine, calcium, and magnesium values were detected with an ion chromatographer (ICS 1100, Thermo Scientific).

Enrichment culturing

The growth media for each site contained (per liter of distilled water): 0.1 g KCl, 0.3 g KH_2PO_4, 1 g NH_4Cl, , 20 mM NaNO3, , 0.1 g $CaCl_2.2H_2O$, 0.5 g $MgCl_2$. $6H_2O$, 1 g NaCl, 0.3 g K_2HPO_4, 1 g yeast extract, and 10 ml trace element solution [15]. A pH of 6.5, 7, and 7.5 were tested. The Hungate technique (1969) was used throughout [16]. Growth experiments were performed in triplicate using Hungate tubes containing basal medium. Water baths were used to incubate bacterial cultures at 50^{0}C and up to 75^{0}C. The strain was subcultured under the same experimental conditions before growth rates were determined.

DNA extraction, 454 Pyrosequencing and Sequence Analysis

Genomic DNA was extracted from the enrichment culture samples using the Fast DNA Spin Kit for Soil (MP Biomedicals, CA, USA) following the manufacturer's instructions. Final extracts were stored in an "80°C freezer until further analysis. For the pyrosequencing, the V3-V5 hypervariable region of the 16S rRNA gene was amplified using PCR as previously described[17], with Bact-338F/Bact-909R. A single-step 30 cycle PCR using the HotStar Taq Plus Master Mix Kit (Qiagen, Valencia, CA) was used with the following conditions: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute. Then, a final elongation step at 72°C for 5 minutes was performed. Following PCR, amplicons from three reactions for each sample were excised from gels, pooled, then purified using QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA). The samples were sequenced with a 454/Roche GS-FLX-Titanium system (Roche, Mannheim, Germany) in a commercial facility (Research and Testing Laboratories, Lubbock, TX).

Sequence analysis by combining the quantitative data into microbial ecology (QIIME), Mothur software v.1.33.0, and ribosomal database project (RDP II) programs were as described previously[18]. The sequences were deposited in the NCBI Sequence Read Archive under accession number SRXMA379513.

RESULTS AND DISCUSSION

There are many studies conducted about the geochemistry of the hot springs at Turkey in the past decade[19, 20, 21, 22]. Nowadays, our understanding of microbial ecology of hot springs in Turkey remain limited. This first study is describing the microbial composition of hot springs in Bursa (Turkey) by using enrichment culturing and high-throughput sequencing.

The results of the water geochemistry for all sampling sites are shown in Table 1. Average temperature of hot springs in this study was between 48^{0}C and 68^{0}C. The pH value was between

7.4 and 7.8. Comparing the value of calcium, magnesium and chlorine obtained in this study to rain water[23] and marine hydrothermal vent value[24] reported in other studies, it has been concluded that geothermal waters used in this study are meteoric origin because calcium, magnesium, sulfate, chlorine values in this study are highly over the values of rain waters but very lower than value of hydrothermal vent. Similarly hot springs of Simav regions were detected meteoric origin, similarly[25].

The pyrosequencing of samples generated a total 267,850 sequence reads after quality filtering and contaminant removal, representing 80% of the original data set. The sample library size ranged from 4,690 to 7,420 sequence reads using for further analysis. The Kemalpasa hot spring sample, observed species richness was 2,365 OTUs with highest values, followed by the Cekirge hot spring, Orhaneli hot spring and Oylat hot springs samples (Table 2). Shannon index between 3.98 and 4.92, and the Chao1 richness ranged from 1,890 to 2,365 (Table 2) in these hot springs. Several studies[26, 27, 28] have demonstrated that the terrestrial hot springs have a high level of bacterial diversity.

A total of 36 phyla and 6 candidate phyla were identified from in this study. Bacterial

Fig. 1. Map of Sample Locations in Bursa, Turkey (source:google earth)

Table 1. Field data and geochemistry of fluids collected at Cekirge, Oylat, Orhaneli and Kemalpasa hot springs

	Cekirge	Oylat	Orhaneli	Kemalpasa
On-site data				
Fluid Temp (°C)	48	45	68	50
pH	7.5	7.6	7.8	7.4
Conductivity µS	2146	1870	2423	1907
Major Ions				
Cl^- (ppm)	5.7	3.2	11	9.5
PO_4^{-3} (ppm)	<0.02	<0.02	<0.02	<0.02
NO_3- (ppm)	2.52	0.14	0.40	0.98
Na^+ (ppm)	30.90	22.70	19.03	185
NH_3-N (mg/L)	<0.05	n.a.	<0.05	0.07
K^+ (ppm)	4.72	3.86	15.70	5.93
Mg^{+2} (mg/L)	19	6.8	124	87
Ca^{+2} (mg/L)	67	123.4	412	464

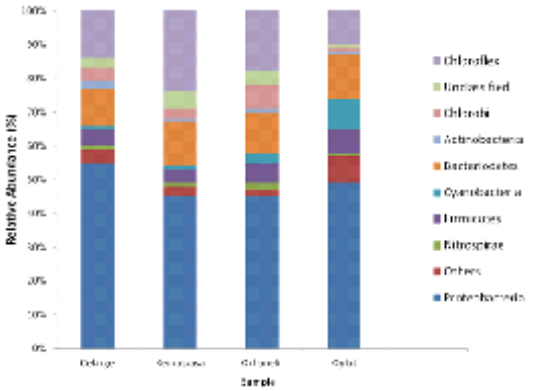

Fig. 2. Bacterial community composition of 454 sequence libraries

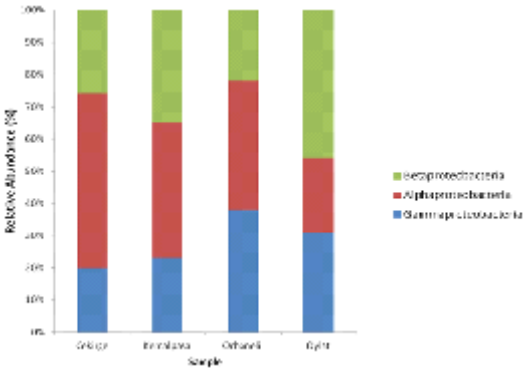

Fig. 3. Distribution of Proteobacteria class in 454-pyrosequencing libraries

Table 2. Summary of 454 –pyrotaq OTUs and diversity and richness estimates

Sample	Number of OTUs	Chao1	ACE	Shannon
Cekirge	1,896	2,810	3,812	4.73
Kemalpasa	2,365	2,641	4,106	4.92
Orhaneli	1,568	2,260	3,520	4.10
Oylat	1,296	1,890	2,856	3.98

[a] Calculated using ACE richness estimator

communities were dominated by *Proteobacteria*, with abundances ranging from 45% to 55 for all sampling sites (Figure 2). *Chloroflexi* and *Bacteroidetes* were second and third most abundant phylum, followed by *Firmicutes, Chlorobi* in Cekirge, Kemalpasa and Orhaneli hot springs samples. On the other hand, *Bacteroidetes* and *Chloroflexi* were second and third most abundant phylum, followed by *Cyanobacteria and Firmicutes* in sample of Oylat hot spring. The OTUs within *Proteobacteria* were distributed between *Alphaproteobacteria, Betaproteobacteria* and *Gammaproteobacteria* for each sample (Figure 3). Previous culture-independent based studies determined the most dominant phyla is *Proteobacteria*[29] and *Chloroflexi* and *Bacteroidetes*[30, 31], both of which were also detected by environmental sequence, stable isotope and lipid analyses. It has been determined that the most present bacteria genus were respectively *Thiofaba, Ignavibacterium, Chloroflexus, Gpl* and *Gp3* in this study. Previously identified cultured representatives of bacterial members of *Geobacillus* and *Aeribacillus*[32, 33] were detected by traditional isolation method. On the other hand, relative to these culture-dependent and clone library approaches, which are important to reveal variations of microbial communities in different hot springs of same geological region.

CONCLUSION

This research is the fisrt study about the characterization of microbial communities in Cekirge, Kemalpasa, Orhaneli and Oylat hot springs, Bursa, Turkey using enrichment culturing and 454-pyrosequencing. According to the results have shown that these fault-associated hot springs include high diversity of microorganism. The knowledge about microbial communities at genus level might be basement the further research about isolation of enzymes of thermophiles.

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