JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2015. Vol. **9**(1), p. 181-187

Combined T-RFLP and 16s rRNA Gene Library Analyse the Microbial Diversity in Changbai Mountain Hot Springs

Honglei Wang[1,3], Wei Bing[1], Xiaoli Zhou[1], Yan Feng[1,2]* and Zuoming Zhang[1]*

[1]Key Laboratory for Molecular Enzymology and Engineering, the Ministry of Education,
School of Life Science, Jilin University, Changchun, Jilin - 130012, China.
[2]State Key Laboratory for Microbial Metabolism and School of Life Sciences &
Biotechnology, Shanghai Jiao Tong University, Shanghai - 200240, China.
[3]College of Chemistry & Life Sciences, Changchun University of Technology,
Changchun, Jilin - 130012, China.

(Received: 10 May 2014; accepted: 24 June 2014)

Multiple factors can influence the overall community diversity of microorganisms in thermophilic mats. In this paper, two cultivation-independent molecular methods, i.e. terminal restriction fragment length polymorphism analysis and 16SrRNA gene library were used to analyse the microbial community structures of hot springs in the Changbai Mountains. This paper analysed the bacterial community compositions in the surface sediments of three hotsprings, and remarkable differences were observed among hot springs. The temperature impacted markedly the bacterial community structure in the hot spings. The chemoautotrophic bacteria were abundant in three separate hot springs, and the light autotrophic bacteria were dominant in the high-temperature hot springs. However, more comparative studies on different springs will be necessary. *Thermotogae, Thermus, Clostridium, Gallionellaceae* and *Pseudomonadales* were the dominant bacterial group in the clone library from the hot springs, whereas *Aquificales* appeared to be the dominated colonizers by T-RFLP. Combined detection is more effective for the research of microorganism community in the hot springs. In addition, the present study points to the interactions between the bacterial community composition and the sediment environmental characteristics, revealing the roles of microorganisms involved in the biogeochemical cycling of nutrient elements in the hot spring ecosystem.

Key words: Hot spring sediment; 16S rRNA gene library;
T-RFLP analysis; Phylogenetics; Biogeochemical cycles.

Terminal restriction fragment length polymorphism (T-RFLP) analysis technique is an emerging microbial molecular biology technique. As an ideal way to study microbial community characteristics, T-RFLP has been increasingly interested. The technology has been successfully applied to the comparative analysis of various microbial communities, microbial diversity and community structure characteristics of peculiar regions. Combined T-RFLP and 16S rRNA gene library technology provide the new means to study the microbial community diversity [1,2]. T-RFLP has a unique advantage to reveal the diversity of microbial community structure and provide continuously quantitative information, and 16S rRNA gene libraries accurate qualitative characteristics each other, so combined T-RFLR and 16S rRNA gene library can obtain the optimal information[3.]

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* To whom all correspondence should be addressed.
Tel: 86 155266 39178;
E-mail: zmzhangjlu@sina.com

Recently, thermophiles and the functional genomics of thermophilic bacteria are of increasing interests by scientists, because thermophilic enzymes have broad practical application and thermophilic microorganisms play an important role in the origin of life, evolution and adaptation mechanisms [4,5]. Sulfur hot springs, mesophilic springs and other geothermal environments are the major sources of thermophiles. Changbai Mountains locate in Jilin Province, China. It is a more ancient volcano mountain in the geological age and has a long history in geological evolution and ecology evolution possessing more primitive ecosystems. The Changbai Mountains embrace plenty of high-temperature hot springs, which are rich in thermophilic bacteria genes and species resources. Its ancient geological features, unique volcanic geological features and the special geographical environments determine the specific and primitive biological resources in the Changbai Mountain hot springs. However, they are rarely studied.

In the present study, T-RFLP and 16S rRNA gene library are used to investigate the Changbai Mountain hot springs thermophilic bacteria species so that discover new ultra-thermophilic species and genetic resources and provide a complementary to pulse ecology and geochemistry of Changbai Mountains. Using T-RFLP and 16S rRNA gene library technology, we would find out the microbial community structure in hot springs at different temperatures and provide an important reference for screening functional proteins and thermophilic bacteria. Furthermore, how the surrounding geological environment and climatic conditions influence the microbial community structure of Changbai Mountains hot springs is analysed.

MATERIALS AND METHODS

Sampling

Changbai Mountain is located in Northeast China (42°N, 128°E; altitude: 2189 meters). There are more than 100 hot springs within the Heavenly Lake (crater), named Julong hot springs. They are high-fever hot springs, the temperature of water is between 55°C~83°C, and the most spring temperature is above 60°C[6]. The Changbai Mountains are the regions of magma activity. The volcanic hot springs belong to sulfur hot springs and come from the depths of the earth. They are rich in carbonate ion, chloride ion and sulfate ion, and their pH is about 7.0. Three undisturbed geothermal sites located along a 0.5 square kilometers, including 3 hot springs: 53°C (12#), 74°C (17#) and 83°C (19#), respectively. Sampling was performed in July 2010 and November 2013. The samples mainly included the sludge of the bottom and hot spring water. At present, the hot springs was kept their original state to maintain its original nature and diversity of microbial resources.

Cloning and sequencing of bacteria gene library

The high molecular weight genomic DNA was extracted from each sampling site using the DNA extraction protocol [7]. The 16S rRNA PCR products derived from total nucleic acid were cloned using a TA cloning kit (Takara, Japan). Clones were randomly selected for further analysis. Sequencing of 16S rRNA clones was carried out by company (Huada Gene, China). The sequencing primers are 27F(5'-AGA GTT TGA TC2 CTG GCT CAG-3'); 1495R:(5'-CTA CGG CTA CCT TGT TAC G-3').

Sequence data, phylogenetic analysis and ecological indices

The 16S rRNA gene sequences (1,000 or 1,500 bp) were compared with the sequences of public databases by using NCBI mega Blast (http://www.ncbi.nlm.nih.gov/blast). Sequences, which were the highest matches in the Blast search, were obtained from GenBank database (http://www.ncbi.nih.gov/Genbank). To identify sequences in the clone libraries, all received sequences were checked with the ClustalX 1.83. In a total of 376 clones, 80 sequences were identified. Phylogenetic tree of retrieved clonal 16S rRNA gene sequences were constructed by using the neighbour-joining algorithm (PHYLIP) of MEGA5.1 software package.

T-RFLP analysis of 16S rRNA gene fragments and ecological indices

The bacterial hydroxylfluorescein (FAM) PCR primer synthesized by the Jikang Company (Shanghai, China) was used for T-RFLP analysis. The primer sequence was 27F-FAM: 5'-FAM-AGA GTT TGA TCC TGG CTC AG-3'. PCR amplification conditions were: 94°C 4 min; 32 cycles composed by 94°C 30 sec, 50°C 45 sec for annealing and 72°C

3 min; 72°C 10 min for final extension. The PCR products were digested using HaeIII and HhaI. The restriction fragment scanning was performed by the Jikang Company (Shanghai, China) where the ABI PRISM ® GeneScan Analysis Software Version 3.7 was used for scanning analysis.

Each restriction fragment (T-RF) of T-RFLP patterns was an operational taxonomic unit (OTU). The OTU abundance was calculated as referenced to the Saikaly method [7], i.e., the relative peak value (each T-RF peak value divided by the accumulated peak value) served as the OTU abundance and the peak less than 100 fluorescence units was excluded. The diversity indices including Shannon Diversity H' and Shannon Diversity E' were calculated using the BIO-DAP program (http://nhsbig.inhs. Uiuc. Edu/wes/populations.html). The similarity of three microbial communities was measured using the Jarccard formula [8] through on-line TAP T-RFLP analysis by the website http://mica.ibest.uidaho.edu/trflp.php. Combined with the 16S rRNA gene library, T-RFLP patterns were subject to qualitative analysis.

RESULTS

Diversity of 16S rRNA gene library

The 80 possible OTU from three hot springs were selected and sequenced, and then 47 different sequences of bacterial 16S rRNA genes were obtained to construct a phylogenetic tree (see Fig. 1). According to the tree, the microbes of the library were clustered into 7 phylum 17 genus: *Thermotogae*, *Thermus*, *Actinobacteria*, *Firmicutes*, *Proteobacteria*, *Aquificae* and *Bacteroidetes*. Where contains *Clostridium*, *Chloriflex*, *Desulfuromonadales*, *Erythrobacteraceae*, *Gallionellaceae*, *Pseudomonas*, *Sphingobacterii*, *Thermaceae*, *Flavobacteriaceae*, *Hydrogenothermaceae*, *Thermothrix*, *Xanthomonadaceae*. Some new genus can be often found by cultivation-independent analysis [9,10]. In this study, there were 21 unknown sequences less than 96% homology, indicating that there are unknown microbes in the Changbai Mountain hot springs. The 16S rRNA library can only reflect the diversity of species populations, not reflect the number of relationships, so the experiments used the T-RFLP method to analyse the habitat community composition.

Table 1. Analysis of microbial diversity based on T-RFL

Sample	H	Simpson	Inv-Simp	Richness	Evenness
site19	2.116	0.863	7.298	10.000	0.919
site17	2.031	0.853	6.821	9.000	0.924
site12	1.916	0.834	6.024	8.000	0.922

Structure of the bacterial community based on T-RFLP analysis

Based on the T-RFLP profiles (see Fig. 2), the bacterial diversity indices of three samples were calculated by the number of the terminal restriction fragments and its relative peak values (Table 1). As shown in Table 1, the 19# sample (83°C) had more abundant diversity and the most abundant community constitutes (diversity index H = 2.116). Terminal restriction fragments (T-rfs) length was analysed by the T-RFLP v1.0Beta 1 software of the Mica database, by referencing to the community distribution determined by the 16S rRNA gene library (see Fig. 3). The quantitative analysis for community structure was conducted in combination the phylogenetic tree with the T-RFLP data. As a consequence, the sum of *Thermus* and *Aquificales* were 19% (19 #), 14% (17 #) and 9% (12 #), respectively.

DISCUSSIONS

Combined the phylogenetic tree and T-RFLP results, we found the microbial community structure and metabolic types affected by the environment in the Changbai Mountain. In totally 17 genus, there were five genus in three hot springs, they were Thermotogae, Thermus, Clostridium, Gallionellaceae and Pseudomonadales. Those should be the indigenous microorganisms in hot spring microbial communities (see Figure 1). And the most ancient phylogenetic phylum, Thermus

Fig. 1. Phylogenetic tree of the bacterial 16S rRNA gene sequences received from of three hot springs. Part A: 12#; Part B 17#; PartC: 19#

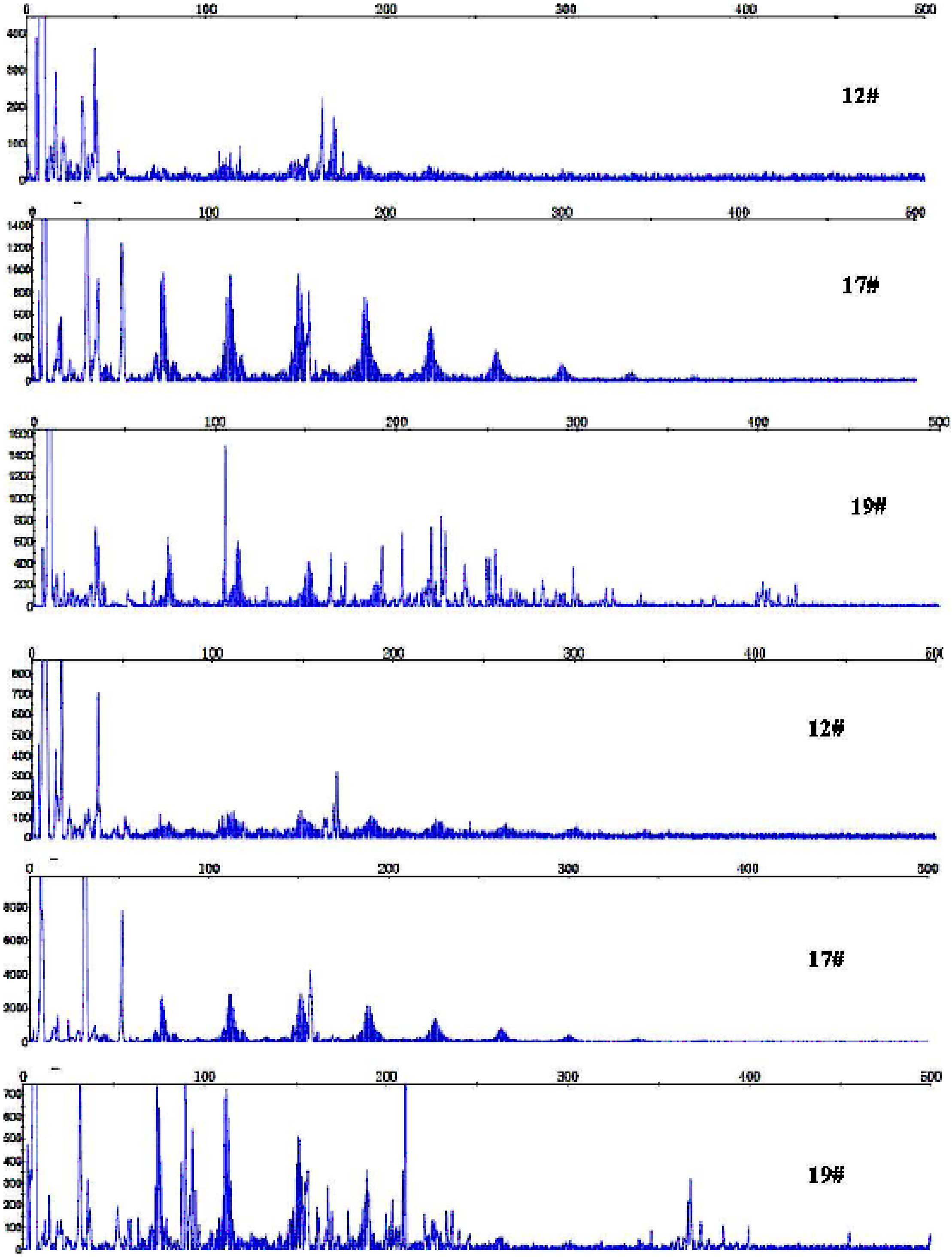

Fig. 2. T-RFLP profiles of three samples from the hot springs. A: *Hae*III digestion, B: *Hha*I digestion

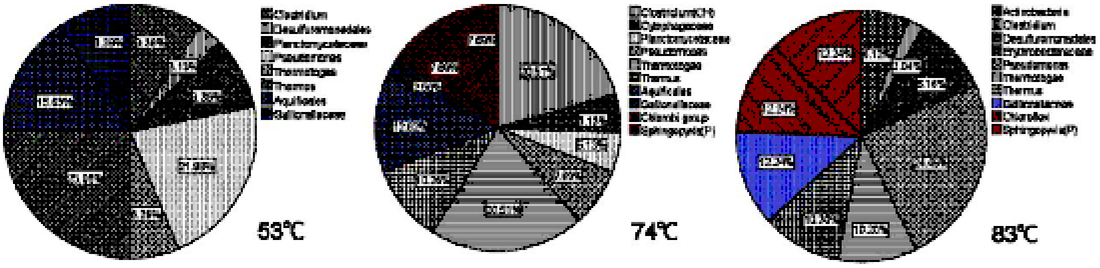

Fig. 3. The community distribution according to the results of 16SrDNA gene sequencing library

and Aquificales were 19% (19#) 14% (17 #) and 9% (12 #) in three hot springs. In a study, the stable isotope composition and distribution of tritium of hot springs were investigated and it found that the hot spring water was from atmospheric precipitation, and tritium decreased gradually from south to north, indicating that the groundwater migrated from south to north, that is to say, the higher the temperature of the region was, the older the water body was. Therefore, as the temperature decreased, the water body age became younger, resulting in a proportional decrease in ancient bacteria located in the root of phylogenetic tree.

Based on the metabolic type, *Rhodobacter, Sphingobacteriia* and *Chloroflexi*, which are autotrophic photosynthetic bacteria, were distributed in two hotter springs but not detected at low temperature hot springs (12#). Chemoautotrophic bacteria *Gallionellaceae* were detected at three samples. In hot springs with organic carbon scarcely, autotrophic bacteria are the primary producers, capable of carbon fixation, and serve as a nutrient source for heterotrophic bacteria. In the mesophilic springs of rich carbon, the light autotrophic bacteria are not dominant, only a portion of heterotrophic bacteria are involved in nutrient cycling.

Berthe-Corti has evaluated the microbial diversity of six hot springs in New Zealand using the Biolog method [12]. The results showed that temperature has a great influence on microbial community structure; when the temperature rises, the microbial substrate utilization is very restricted. Therefore, the autotrophic bacteria are dominant as influenced by the temperature limit.

Biogeochemical cycle refers to the transformation of nature substances driven by biochemical impetus in biosphere. All bioorganisms are involved in the biogeochemical cycling of matter, but microbes play an important role in the biogeochemical cycles for their wide distribution, diverse metabolic capacity and high enzyme activity. The material composition of the environment determines the composition of species in particular habitats. The content of HCO_3^-, Cl-, B and SO_4^{2-} (volatile matter of magma) is slightly higher in high-temperature hot springs and these compositions may work in the microbial carbon, sulfur and hydrogen cycles. This biological characteristic may pertain to the biogeochemical cycles.

In conclusions, the present study shows the bacterial community compositions in the superficial sediments of three hotsprings of the Changbai Mountains, and remarkable differences were observed among hot springs. Temperature impacted markedly the bacterial community structure in the hot spings. However, more comparative studies on different springs will be necessary. Thermotogae, Thermus, Clostridium, Gallionellaceae and Pseudomonadales were the dominant bacterial group in the clone library from the hot springs, whereas Aquificales appeared to be the dominated colonizers by T-RFLP. Combined detection is more effective for the research of microorganism community in the hot springs. In addition, the present study points to the interactions between the bacterial community composition and the sediment environmental characteristics, revealing the roles of microorganisms involved in the biogeochemical cycling of nutrient elements in the hotspring ecosystem.

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