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BACTERIAL PROFILES OF KARVACHAR HOT SPRING IDENTIFIED BY COMBINATION OF DIFFERENT MOLECULAR APPROACHES

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Molecular techniques, including denaturing gradient gel electrophoresis (DGGE), 16S rRNA genes clone library construction and metagenomic analysis, were used to describe the bacterial composition of the Karvachar geothermal spring. It was shown the predominance of bacteria belonging to the phyla Proteobacteria, Bacteroidetes, Firmicutes and Cyanobacteria in the studied spring. Representatives of phylum Firmicutes were not detected in the clone library, while DGGE profiling and metagenome analysis confirmed the presence of Firmicutes as one of the major components in the bacterial community.

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***Keywords*:** geothermal spring, DGGE, 16S rRNA genes clone library, metagenomics analysis, Proteobacteria, Bacteroidetes, Firmicutes, Cyanobacteria.

Introduction. High temperature environments including hot springs are habitats of thermophilic microbes, the diversity of which is currently being intensively studied. Thermophilic microorganisms are taxonomically diverse and belong to various phyla throughout the Tree of Life [1]. It has been reported that hot springs are inhabited thermophiles mainly belonging to the domains of Bacteria and Archaea [2]. Metagenomics provides the opportunity to reveal and explore the uncultivated fraction of the microbes in nature [3].

The development of different and combined culture-independent molecular techniques has largely expanded our understanding of the structural and functional diversity of microbial communities. All used molecular based methods have both advantages and drawbacks [4–6]. Given the limitations of different methods used, it is recommendable to use a combination of several molecular techniques, which will give rapid and reliable results [7].

Despite intensive studies of the microbiota of terrestrial hot springs worldwide, very little is known about the microbial diversity of thermal springs located at high elevation areas. Recently, geothermal springs located on the Armenian Highland have been the focus of attention of researchers to determine their structural and functional diversity [8–10]. The present study reports the bacterial composition of a geothermal spring located in Karvachar, Nagorno Kharabakh, identified using different approaches of molecular microbial ecology. Culture-

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independent studies such as denaturing gradient gel electrophoretic (DGGE) analysis, construction of clone libraries of 16S rRNA genes, and whole-genome shotgun sequencing using the Illumina HiSeq 2500 platform, were used to identify the dominant microbial populations of the studied spring.

Materials and Methods.

Study Sites and Sampling. Water/sediment samples were collected from terrestrial geothermal springs located in Karvachar, Nagorno Karabakh. The geographical location and elevation of these springs were determined using a portable GPS (Germin GPSMAP 64s). Water temperature, pH, and conductivity were measured *in situ* during the sampling using a portable combined pH/EC/TDS/Temperature tester (HANNA HI98129/HI98130).

DNA Extraction, PCR Amplification and DGGE Analysis. DNA was extracted from water/sediments by using the sodium dodecyl sulfate (SDS) lysis procedure modified according to the protocol of Dempster et al. [11].

The bacterial community structure in the samples was studied using PCR-DGGE. The extracted DNA was used as templates for amplification of the V3 region of bacterial 16S rRNA gene sequences using primers L340F with CG clamp and K517R [12]. The DGGE analysis of PCR products was performed using TV-400-DGGE System (Topac Inc., USA) with 8% (w/v) polyacrylamide gel (37.5:1 acrylamide/bisacrylamide) in 0.5×TAE (20 *mM* Tris-HCl, 10 *mM* Acetat, 0.5 *mM* EDTA) buffer and denaturants (100% denaturant contains 7 *M* urea and 40% deionized formamide). Electrophoresis was performed at a constant voltage of 20 *V* for 10 *min*, following by 200 *V* for 4 *h*. DGGE gels were stained with SYBR® Gold ("Invitrogen", USA) for 60 *min* and photographed on Gel DocXR system (Bio-Rad Laboratories). The DNA in the excised gel slices were incubated in 20 *mL* of MiliQ water at 4°*C* for 24 *h* and re-amplified by PCR with the aforementioned primer set.

Clone Library Construction and Sequencing. The extracted DNA was also used as a template for amplification of 16S rRNA genes by PCR using universal primers 27F and 1525R [8]. PCR products were cloned into chemically competent *E. coli* cells using CloneJET™ PCR Cloning Kit ("Ferments") according to the manufacture's recommendations. The plasmids were purified using GenElute™ Plasmid Miniprep Kit ("Sigma"). The presence of inserted genes was observed by 0.8% agarose gel-electrophoresis.

Whole-genome Shotgun Sequencing Using Illumina HiSeq 2500 Platform. DNA sequencing using Illumina Hiseq 2500 platform was done at the Michigan State University RTSF Genomics Core. Metagenomic libraries were prepared using the Illumina TruSeq Nano DNA Library Preparation Kit.

Sequencing and Bioinformatic Analysis. Amplicons obtained from DGGE gel and clone library were sequenced on ABI PRISM capillary sequencer according to the protocol of the ABI Prism BigDye Terminator kit ("Perkin Elmer"). The presence of chimeric sequences was determined using the DECIPHER web tool [13]. Closest matches for partial 16S rDNA sequences were identified using NCBI Nucleotide BLAST web service [14]. Sequencing from metagenomics libraries was performed in a 2×125 bp paired end format using HiSeq SBS v4 kit. Trimmomatic was used to exclude Illumina adapters and low quality bases. MG-RAST pipeline was used to annotate taxonomically obtained metagenomic datasets [15].

Results and Discussion. The temperature of water of the studied geothermal spring (with location of 40°17′41.7″N 46°27′50.0″E, and altitude of 1584 *m* AMSL) in the outlet was ~70°*C*. The studed geothermal spring belongs to the category of hot springs from low-temperature fields (mesothermal). The pH of water was circumneutral (7.3), conductivity was 4600 $\mu S \cdot cm^{-1}$.

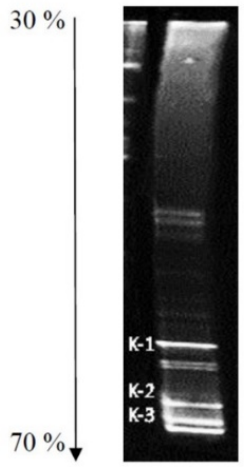

Fig. 1. Electrophoregram obtained by DGGE showing denaturant gradient. K-1–K-3 bands of different phylotypes.

Environmental DNA was extracted from water/sediment samples and 16S rRNA genes were successfully amplified by PCR and used for DGGE analysis and clone library construction. The results of DGGE analysis are shown in Fig. 1 and Table. Representatives of the phylum Firmicutes were the major component of the bacterial community of the Karvachar geothermal spring. According to the DGGE profile, the Karvachar geothermal spring was also colonized by uncultivated representatives of the phylum Bacteroidetes.

According to the results of sequence analysis of clones obtained from bacterial 16S rRNA gene libraries, the studied hot spring harbored representatives of the phyla Proteobacteria, Cyanobacteria, Bacteroidetes, Chloroflexi, Verrucomicrobia and Planctomycetes (Fig. 2). The dominating bacterial group was the phylum Proteobacteria. Another major group was the phylum Cyanobacteria, representatives of which are the most important primary producers in hot spring ecosystems. Unclassified bacteria accounted for 3% of all the detected phylotypes.

BLAST results of bacterial 16S rRNA gene sequences derived from excised DGGE bands

Band	Seq. length, bp	Closest match (accession no.)	Phylogenetic affiliation
K-1	134	uncultured clone B_OTU_1064 (KX031046)	Bacteroidetes
K-2	133	*Geobacillus* sp. (KU291217)	Firmicutes
K-3	131	*G. kaustophilus* (KY883609)	Firmicutes

Based on the data obtained from the whole-genome shotgun sequencing of environmental DNA using the Illumina HiSeq 2500 platform, bacterial phylotypes belonging to Actinobacteria, Alpha-, Beta-, Delta-, Epsilon- and Gammaproteobacteria, Bacteroidetes/Chlorobi, Firmicutes, Chlamydiae, Cyanobacteria/Melainabacteria and Synergistia were detected. Among these groups, Proteobacteria (Alpha-, Beta- and Gammaproteobacteria) and Firmicutes were the major components in the total bacterial sequence reads (Fig. 3). The sequences affiliated with Gammaproteobacteria were predominant (48.96% of all the Proteobacteria).

Around 10.3% of the total bacterial clone sequences were affiliated with some minor groups, such as Actinobacteria, Bacteroidetes/Chlorobi, Chlamydia, Cyanobacteria/Melainabacteria, Fusobacteria and Synergistia. Most of these sequences

were closely (98–99%) related to clones retrieved from water environments and different habitats [16]. Most of the Cyanobacteria detected were related to others previously reported in thermophilic environments [17]. Representatives of the genus *Rhodobacter* (purple non-sulfur anoxygenic phototrophs) and other phototrophic microbes were found to share these environments with the cyanobacteria.

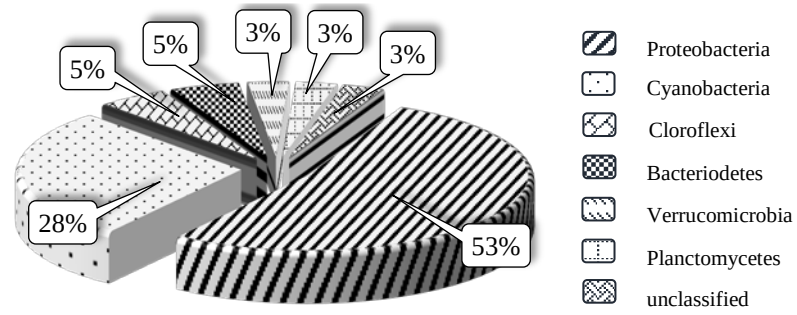

Fig. 2. Phylum level distribution of bacterial phylotypes obtained from clone library analysis.

These observations are consistent with many global studies indicating that thermophilic bacteria belonging to the phyla Proteobacteria, Cyanobacteria, Bacteroidetes and Firmicutes are abundant in mesothermal hot springs [18].

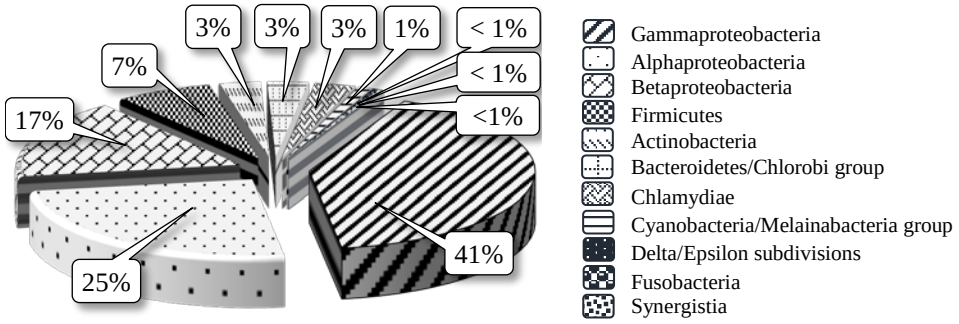

Fig. 3. Phylum level grouping of bacterial sequence reads obtained by shotgun sequencing using Illumina HiSeq 2500 platform.

According to BLASTn analysis, the obtained phylotypes shared less than 97% similarity with their closest matches in GenBank, indicating that the studied springs harbored a unique community including novel microbial species. Although most of the retrieved sequences were most similar to uncultured bacteria, some of them were phylogenetically related to environmental clones obtained from other geothermal springs. A comparison of the optimal growth temperature of the closest cultivated relatives of the microorganisms detected in the clone libraries, DGGE profiles, or methagenome suggested that most of the microorganisms, including microorganisms representing some of the most dominant groups, are likely able to grow at the temperature of the reservoir under consideration, and therefore should not be regarded as contaminants.

It was shown earlier that DGGE technique alone detects only dominant populations constituting more than 1% of microbial community [19]. To find out the remaining fraction of the microbial populations we appealed to additional molecular methods. The clone library construction applied in this study has several potential biases, in particular in the number of clones, which are not large enough to fully represent the microbial community. That was the reason for subsequently applying metagenomic approach to capture the complete profile of microbial diversity.

Representatives of the phylum Firmicutes were not detected in the clone library, while DGGE profiling of the same samples indicated presence of Firmicutes (genus *Geobacillus*) as one of the major components in the bacterial community of the Karvachar geothermal spring. This was confirmed by a metagenome analysis. The results of the clone library analysis, demonstrating the predominance of representatives of Proteobacteria, Bacteroidetes and Cyanobacteria, were in good agreement with metagenome profile. Thus, the combined application of different molecular approaches to describe the complete quantitative picture of microbial diversity is justified.

Conclusion. Here, we have focused on the bacterial composition of the Karvachar mesothermal spring using a combination of different molecular methods. The PCR-DGGE profile showed that the studied spring was colonized by bacteria belonging to the phyla Firmicutes and Bacteroidetes. The results of the clone library analysis corresponded to the methagenome analysis confirming the predominance of thermophilic bacteria belonging to phyla Proteobacteria (83%) and Cyanobacteria (7%). Most of the obtained bacterial sequences shared less than 97% identity with their closest match in GenBank indicating a unique community of studied environments. This study justified the synergy of different molecular approaches to better understand the microbial diversity of similar ecosystems.

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Հ. Հ. ՓԱՆՈՍՅԱՆ

ՔԱՐՎԱՃԱՌԻ ԵՐԿՐԱՋԵՐՄԱՅԻՆ ԱՂԲՅՈՒՐԻ ԲԱԿՏԵՐԻԱԿԱՆ ԿԱԶՄԻ ՈՐՈՇՈՒՄԸ ՄՈԼԵԿՈՒԼԱՅԻՆ ՏԱՐԲԵՐ ՄՈՏԵՑՈՒՄՆԵՐԻ ՀԱՄԱԴՐՈՒԹՅԱՄԲ

Մոլեկուլային մեթոդների, այդ թվում 16S ռՌՆԹ-ի գեների կլոնային գենադարանների ստեղծման, դենատուրացնող գրադիենտային ժել էլեկտրաֆորեզի (ԴԳԺԷ) և մետագենոմային վերլուծության միջոցով բացահայտվել է Քարվաճառի երկրաջերմային աղբյուրների բակտերիական համակեցության կազմը: Ցույց է տրվել հետազոտվող երկրաջերմային աղբյուրում Proteobacteria, Bacteroidetes, Firmicutes և Cyanobacteria ֆիլումներին պատկանող բակտերիաների գերակայությունը: Կլոնային գենադարանում Firmicutes-ին պատկանող ֆիլոտիպեր չեն հայտնաբերվել, սակայն ԴԳԺԷ-ն ու մետագենոմային վերլուծությունը հաստատել են դրանց, որպես բակտերիական համակեցության գերկաշռող բաղադրիչի առկայությունը:

О. А. ПАНОСЯН

БАКТЕРИАЛЬНЫЕ ПРОФИЛИ ГОРЯЧЕГО ИСТОЧНИКА КАРВАЧАР, ВЫЯВЛЕННЫЕ КОМБИНАЦИЕЙ РАЗЛИЧНЫХ МОЛЕКУЛЯРНЫХ ПОДХОДОВ

Молекулярные методы, в том числе денатурирующий градиентный гель-электрофорез (ДГГЭ), построение библиотеки клонов гена 16S рРНК и метагеномный анализ, были использованы для описания бактериального состава геотермального источника Карвачар. Показано преобладание бактерий, принадлежащих к филумам Proteobacteria, Bacteroidetes, Firmicutes и Cyanobacteria, в исследуемом источнике. Представители типа Firmicutes не были обнаружены в библиотеке клонов, в то время как профилирование ДГГЭ и анализ метагенома подтвердили присутствие Firmicutes как одного из основных компонентов в бактериальном сообществе.