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PHYLOGENETIC DIVERSITY OF THERMOPHILIC BACILLI ISOLATED FROM GEOTHERMAL SPRINGS OF NAGORNO KARABAKH

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Totally twenty three thermophilic bacilli strains were isolated from Karvachar (70ºC) and Zuar (42ºC) geothermal springs of Nagorno Karabakh. The comparison of generated 16S rDNA sequences of the isolates with the ones available in GenBank database indicates their relations to nine species distributed in four genera: *Aeribacillus* (*A. pallidus*), *Anoxybacillus* (*A. flavithermus*, *A. suryakundensis*, *A. rupiensis*, *Anoxybacillus* sp.), *Bacillus* (*B. licheniformis*, *B. simplex*, *B. cereus*), and *Geobacillus* (*G. toebii*). Representatives of genus *Anoxybacillus* was dominated in the Karvachar geothermal spring composing 67% of all detected isolates. Zuar spring was populated by representatives of *A. rupiensis* and *G. toebii*.

***Keywords*:** geothermal springs, thermophilic bacilli, *Bacillus*, *Anoxybacillus*, *Aeribacillus* and *Geobacillus*, 16S rRNA gene sequencing.

Introduction. Thermal springs are hot spots of biodiversity of microbes which can be utilized as source of novel genes, molecules and hydrolytic enzymes for white, grey, and red biotechnological sectors [1, 2]. These communities have attained the focus of applied research not only in terms of biotechnological prospects, but also to understand the use of primitive analogues of biomolecules existed during early Earth environments [1, 3]. Thermophilic microorganisms are not grouped into a separate taxonomic unit, but appear in various taxonomic groups and at various phylogenetic distances throughout the taxonomic system [4]. The taxonomy and especially the identification of thermophilic bacilli have generated considerable interest over recent decades. It was shown that representatives of the genus *Bacillus* and related genera (such *Aeribacillus, Anoxybacillus* and *Geobacillus)* to be the thermophilic aerobes most frequently isolated from terrestrial geothermal water environments [5–8]. The importance of these bacteria has increased, owing to their potential as a source of thermoenzymes, including xylanases, proteases, amylases, peroxidases, glucose isomerases, lipases and DNA restriction enzymes [9, 10]. Phenotypic and genotypic characterization of thermophilic bacilli has been carried out for many geothermal areas in different parts of the world, including Turkey [6, 11], Bulgaria [12], Morocco [13], Iran [14], Russia [15], Italy [16], New Zeland [17], China [18], Central Mongolia [19] and other countries.

Despite intensive studies on terrestrial thermal springs, very little is known about bacilli diversity of thermal springs at high elevation [20]. Geothermal springs

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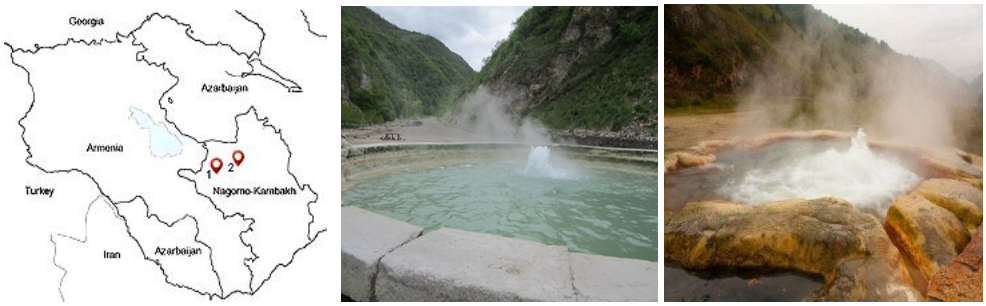
located in the Minor Caucasus still represent a challenge for searching of undescribed biotechnological resource. The geology of the region, where Armenia and Nagorno Karabakh (NK) are situated is complex, owing to accretion of terrains through plate-tectonic processes, and to ongoing tectonic activity and volcanism [21]. Numerous geothermal springs at high elevations of different geotectonic origin and with different physicochemical properties are found on the territory of Armenia and NK [22]. Recently microbiological investigations to evaluate bacilli distribution in some Armenian geothermal springs were carried out [23, 24]. Despite this progress very little is known about the diversity of bacilli thriving in NK geothermal springs. The present study reports the phylogenetic diversity based on 16S rRNA genes analysis of thermophilic bacilli isolated from geothermal springs distributed on the territory of NK.

Materials and Methods.

Study Sites and Sampling. Water/sediment samples were collected from two moderate temperature terrestrial geothermal (mesothermal) springs located on Karvachar and Zuar regone of NK (Fig. 1). Geographical location and elevation of these springs were determined using a portable GPS (GERMIN 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).



a b c

Fig. 1. Location of studied sites. Map of NK with the locations of studied geothermal springs 1 – Karvachar, 2 – Zuar (a); close to the map are photographs of source pools of Karvachar (b) and Zuar (c) geothermal springs.

Enrichment and Isolation. To enrich aerobic endospore-forming thermophilic bacteria slurry water and sediment samples (1 *g*) were inoculated in Nutrient Broth ("HiMedia") and incubated overnight at 55, 65 and 70º*C* with shaking at 150 *rpm*. Before inoculation all samples were treated at 80º*C* for 10 *min* aiming to isolate only spore-forming microorganism. Cultures showing different colony morphology was further purified by streaking samples on the same medium supplemented with agar (2%, w/v). All colonies obtained on plates were picked and purified by streaking onto the same medium at least three times. The subcultures were considered pure after microscopic observation of a single morphological type per culture. The subcultures' purity, cell morphology, motility, endospore location were determined by phase-contrast microscopy of freshly prepared wet mounts. All isolates were tested for their colony morphology, Gram reaction, thermophilic growth and catalase activity using common accepted methods [25].

DNA Extraction, Polymerase Chain Reaction (PCR) and Sequencing. DNA was extracted from pure isolates using GenEluteTM Bacterial Genomic DNA Kit ("Sigma") according to the manufacturer's recommendations and used as a template in the PCR assays. 16S rRNA genes were amplified using universal primer pairs 27f (5'-GAGTTTGATCCTGGCTCA-3') and 1525r (5'-GAAAGGAGGAGATCCAGCC-3') (*Escherichia coli* numbering). PCR mixtures used for amplification of sequences contained 10 *ng* DNA, 5 *μL* 10×PCR buffer, 5 *μL* 10 *mM* dNTP (dATP, dGTP, dCTP and dTTP), 1 *μL* each primer (25 *pmol*/*μL*), 1.5 *mM* $MgCl_2$, 0.2 *μL* Taq DNA polymerase, and sterile water up to the final volume of 50 *μL*. PCR amplifycation was completed using a DNA Engine thermocycler ("BIO RAD"). First, the templates were denaturized for 3 *min* at 96°*C* then 30 cycles of the following steps were completed: denaturation for 30 *s* at 96°*C*, annealing for 30 *s* at 55°*C*, and extension at 2.5 *min* at 72°*C*. The 30 cycles were followed by a final 10 *min* extension at 72°*C*. PCR products were viewed under UV light after standard ethidium bromide gel electrophoresis. PCR products were purified with GenElute™ PCR Cleanup Kit ("Sigma").

Sequencing and Phylogenetic Analysis. Sequencing of bacterial 16S rDNA amplicons were performed on ABI PRISM capillary sequencer according to the protocol of ABI Prism Big-Dye Terminator kit ("Perkin Elmer") using above mentioned primers. The presence of chimeric sequences was determined using the DECIPHER web tool (http://decipher.cee.wisc.edu/FindChimeras.html) [26]. Raw data of DNA sequences was analyzed with Chromas and BioEdit software. A nucleotide BLASTn search was performed in order to obtain information on the phylogenetically closest relatives (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/Blast).

Results and Discussion. The temperatures of water of studied geothermal springs in the outlet were 42°*C* for Zuar and 70°*C* for Karvachar. Both geothermal spring belong to the category of hot springs from low-temperature fields (mesothermal) and are characterized by neutral to alkaline pH and high concentration of dissolved minerals and gases. The location and physical-chemical parameters of studied geothermal springs are shown in Tab. 1.

Table 1

The location and physical-chemical parameters of geothermal springs distributed on the territory of NKR

Geothermal spring	Spring location	Altitude, *m* above sea level	pH	Conductivity, *μS*/*cm*	Temperature of outlet water, *T* °*C*
Karvachar	40°17′41.7″ N 46°27′50.0″ E	1584	7.3	4600	70
Zuar	40°02′47.6" N 46°14′09.3" E	1520	7.0	4300	42

Collected 5 water and adjacent sediment samples were analyzed to evaluate the total thermophilic aerobic endospore-forming bacterial abundance. Totally the 23 isolates with different colony morphologies were obtained from the studied geothermal springs. All the strains developed colonies within 24 *h* of incubation at 65–70°*C*, thereby indicating the fastidious nature of microorganisms in geothermal springs. All isolates were rod-shaped, gram-positive, endospore-forming, catalase-positive bacteria.

The aim of the present study is to reveal the phylogenetic diversity of isolated thermophilic aerobic endospore-forming bacteria based on their 16S rRNA gene analysis. For this purpose, 16S rRNA genes from the extracted DNA of each isolate were successfully amplified by PCR and further sequenced. A homology search was carried out by using the basic BLASTn search program at the NCBI website.

Table 2

The genus and species group of the isolates, the intragenic sequence similarity values and the number of bacteria belonging to these groups derived from 16S rRNA gene nucleotide sequences

Genus	16S rDNA (sub)grouping, (isolates)	Seq. length, bp	16S rDNA sequences similarities of the isolates to the closest relative %, |Accession №|	Q-ty of isolates
Karvachar geothermal spring				
Aeribacillus	1 – *A. pallidus* group (K-78)	943	99, |KF879315|	1
Anoxybacillus	1 – *A. flavithermus* group			
	subgroup-1 (K-1, K-33, K-35, K-80, K-83, K-97, K-98, KB-2, KS-1, KV-1)	839–1475	98–99, similarity to *A. flavithermus* AK1 |KC503890|	10
	subgroup-2 (K-99)	1447	99, similarity to *A. flavithermus* WL|FJ950739|	1
	subgroup-3 (K-103)	1550	99, similarity to *A. flavithermus* K1|CP000922|	1
	2 – *A. suryakundensis* group (K-102)	505	98, |KF772607|	1
	3 – unidentified group *Anoxybacillus* sp. (KC-3)	983	98, |KX784766| (98, similarity to type strain *A. geothermalis* |KJ722458|	1
Bacillus	1 – *B. licheniformis* group			
	subgroup-1 (Kt-3)	1471	95, similarity to *B. licheniformis* MV|LT669761|	1
	subgroup-2 (K-4)	768	99, similarity to *B. licheniformis* 30P3-4 |JN366771|	1
	subgroup-3 (K-15-1)	1457	99, *B. licheniformis* Q63 |KP686132|	1
	2 – *B. simplex group* (K-9, K-25)	1454–1455	99, |JF496323|	2
	3 – *B. cereus* group (K-26)	1462	99, |KF500919|	1
Zuar geothermal spring				
Anoxybacillus	1 – *A. rupiensis* (Z-2)	945	99, |AM988776|	1
Geobacillus	1 – *G. toebii* (Z-1)	797	99, |JQ929016|	1
			Total	23

Comparison of generated 16S rRNA gene sequences of isolates with those in GenBank database indicated that all of them belonged to Firmicutes phylum, Clostridium-Bacillus subphylum, group of *Bacillus*-like genera closely related to members of nine species of four genera: *Aeribacillus* (*A. pallidus*), *Anoxybacillus* (*A. flavithermus*, *A. suryakundensis*, *A. rupiensis*, *Anoxybacillus* sp.), *Bacillus* (*B. licheniformis*, *B. simplex* and *B. cereus*) and *Geobacillus* (*G. toebii*). The divergence of the species in obtained genera, their 16S rRNA gene sequence similarity values to their closest relatives and the number of the isolates belonging to the species groups (clusters) are given in Tab. 2.

Among the described species, the closest relatives of isolates K-1, K-33, K-35, K-80, K-83, K-97, K-98, K-99, K-103, KB-2, KS-1 and KV-1 were *A. flavithermus* with the sequence homology rate of 98–99%. Representatives of species *A. flavithermus* are known to be isolated from geothermal springs from different parts of the globe [17, 27]. Successive analysis of the amplified 16S rRNA gene revealed 98% phylogenetic relationship of isolate K-102 to type strain *A. suryakundensis* JS1(T), a facultative anaerobe, moderately thermophilic alkalitolerant bacilli, isolated from hot spring at Jharkhand, India [28]. Detection of thermophilic alkalitolerant bacilli is not surprising and is in a good agreement with physical-chemical conditions of studied spring.

A separate group contains isolate KC-3, 16S rDNA sequence of which was affiliated (98%) with *Anoxybacillus* sp. This isolate shared less than 98% similarity to type strain *A. geothermalis*, a facultatively anaerobic, endospore-forming bacterium isolated from mineral deposits in a geothermal station [29]. This suggests that isolate KC-3 may appear to be a novel anoxybacilli species.

The isolate K-78 exhibited 99% similarity by 16S rRNA gene to *A. pallidus* isolated from Moroccan hot springs [13].

All isolates from Karvachar geothermal spring that belonged to the genus *Bacillus* were thermotolerant microorganisms, among which *B. licheniformis* appeared as the dominating species. Isolates K-4 and K-15-1 had identical 16S rDNA sequences with 99% identity to the 16S rDNA of different strains of *B. licheniformis* (30P3-4 and Q63 respectively), while other isolate (Kt-3) shared a significant similarity (95%) to *B. licheniformis* strain MV. The isolate Kt-3 was thought to be novel species belonging to genus *Bacillus* as they showed very low sequence similarities to their closest relative. Another one, K-26, had 16S rRNA gene highly similar to *B. cereus* (99%). The 16S rDNA sequences of isolates K-9 and K-25 were identical (99%) to the sequences of *B. simplex*.

Only two isolates belonging to genera *Anoxybacillus* and *Geobacillus* have been isolated from Zuar geothermal spring. An isolate designated as Z-1 shared 99% similarity to *G. toebii* most frequently isolated obligate thermophiles from hot springs [23]. The isolate Z-2 demonstrated closest match with *A. rupiensis,* which was isolated from Rupi geothermal spring in Bulgaria [12].

Therefore, thermophilic microbiota in the Zuar geothermal spring is taxonomically less diverse than in the Karvachar spring. *Anoxybacillus* and *Bacillus* with their 14 (≈67%) and 7 (≈47%) isolates respectively, were the predominant genera in the Karvachar spring. Species *A. flavithermus* was dominated in the Karvachar geothermal spring composing more than 57% of all isolates. Although representatives of the genera *Aeribacillus* and *Geobacillus* are commonly considered the species most frequently isolated from similar habitats [11], both springs sampled in this study demonstrated significantly lower content of these species.

From the metabolic point of view, genera *Aeribacillus*, *Anoxybacillus* and *Geobacillus*, includes chemoorganotrophic, aerobic or facultative aerobic obligatory thermophiles [7, 11]. Their presence is in agreement with temperature regime of the studied thermal spring.

As part of the microbiota, thermophilic bacilli presumably make significant contributions in the biogeochemical cycles of the springs under extreme temperature conditions. Moreover, apart from the thermal conditions, abiotic factors such as high mineralization could act as limiting factors for microbial diversity and

biomass. Recent studies have also highlighted that other factors such as biogeography and geological history can also be important in determining the thermophilic diversity in geothermal springs [31]. Environmental conditions and the nutritional status available in a natural habitat may indicate toward the development of a particular group of microbial population. Environments, with a particular set of physical-chemical and edaphic conditions, allow natural selection of a few species that can dominate and multiply in the ecologically relevant niche. Low nutrient status, high mineralization and moderate temperature regime of studied springs allowed the development of a unique population dominated by a large number of bacilli including *Anoxybacillus* spp. Moreover, the sequence comparisons of some isolates demonstrated that they represented novel species among genus *Anoxybacillus* and *Bacilus* as they shared lower than 98% sequence similarity to all the described type species.

The results obtained show the importance of further investigation of the phylogenetic diversity of microbes in geothermal springs to discover and isolate new thermophilic species.

Conclusion. This study is one of the few published studies describing thermophilic microbes of geothermal springs in NK. Totally twenty three thermophilic bacilli strains were isolated from Karvachar and Zuar geothermal springs and were characterized. Phylogenetic analysis based on the nearly complete 16S rDNA sequences revealed that all the isolates obtained were affiliated with genera *Aeribacillus, Anoxybacillus, Bacillus* and *Geobacillus*. Representatives of *A. rupiensis* and *G. toebii* were only thermophilic microbes obtained from Zuar spring. It was shown that *A. flavithermus* was dominate species in the Karvachar geothermal spring. The relatively low similarity (<98%) of some isolates to the sequences in the bacterial 16S rRNA genes in GenBank indicates that the hot spring harbors unique microbial communities with potentially novel species.

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