Հայաստանի Գիտությունների Ազգային Ակադեմիա
Национальная Академия Наук Армении
National Academy of Sciences of Armenia

Հայաստանի Կենսաբանական Հանդես
Биологический Журнал Армении
Biological Journal of Armenia

•Փորձարարական և տեսական հոդվածներ •Экспериментальные и теоретические статьи•
•Experimental and theoretical articles•

Biolog. Journal of Armenia, 4 (62), 2010

PHYLOGENETIC DIVERSITY BASED ON 16S rRNA GENE SEQUENCE ANALYSIS OF AEROBIC THERMOPHILIC ENDOSPORE-FORMING BACTERIA ISOLATED FROM GEOTHERMAL SPRINGS IN ARMENIA

H.H. PANOSYAN

Yerevan State University, Faculty of Biology, Department of Microbiology and Plant and Microbe Biotechnology, Email: hpanosyan@yahoo.ca

Nineteen thermophilic aerobic endospore-forming bacteria were isolated from Armenian terrestrial hot (Arzakan) and warm (Akhourik) geothermal springs. The phylogenetic and taxonomic diversity of isolates was studied by the 16S rRNA gene (16S rDNA) analysis. The comparison of generated 16S rDNA sequences of the isolates with the ones available in GenBank database indicates their relation to phylum *Firmicutes*, subphylum *Clostridium-Bacillus*, group of *Bacillus*-like genera. The isolates are related to eleven species distributed in five genera: *Bacillus, Geobacillus, Sporosarcina, Paenibacillus,* and *Thermoactinomyces*. The thermophilic endospore-forming microflora was less diverse in the Akhourik spring and included representatives of genera *Bacillus* and *Thermoactinomyces*, with predominance of *Bacillus* species. Representatives of the genus *Geobacillus* were prevailing in the hot spring, in total constituting 50% of the detected isolates.

Geothermal springs − thermophiles − Bacillus and related genera − 16S rRNA gene (16S rDNA) sequence − phylogeny

Հայաստանի տաք (Ախուրիկ) և ջերմային (Արզական) հանքային աղբյուրներից մեկուսացվել են տասնինը թերմոֆիլ աերոբ էնդոսպոր առաջացնող բակտերիաներ և ըստ 16S ռՌՆԹ-ի գենի (16S ռԴՆԹ) նուկլեոտիդային հաջորդականությունների վերլուծության ուսումնասիրվել է դրանց տաքսոնոմիական և ֆիլոգենետիկական բազմազանությունը: Մեկուսացված կուլտուրաների 16S ռԴՆԹ-ի նուկլեոտիդային հաջորդականությունների համեմատումը GenBank-ի տվյալների բազայում առկա հաջորդականությունների հետ հաստատել է դրանց պատկանելիությունը *Firmicutes* ֆիլումին, *Clostridium-Bacillus* ենթաֆիլումին, *Bacillus* և ազգակից ցեղեր խմբին: Մեկուսացված շտամները նույնականացվել են որպես 11 տեսակներ՝ բաշխված *Bacillus, Geobacillus, Sporosarcina, Paenibacillus* և *Thermoactinomyces* ցեղերում: Թերմոֆիլ էնդոսպոր առաջացնող միկրոֆլորան Ախուրիկի հանքային աղբյուրում քիչ բազմազան է և ներառում է *Bacillus* և *Thermoactinomyces* ցեղերի ներկայացուցիչներ՝ *Bacillus* ցեղի տեսակների գե-րակշռմամբ: *Geobacillus* ցեղի տեսակները գերակշռում են ջերմային աղբյուրում՝ կազմելով մեկուսացված տեսակների 50%:

Երկրաջերմային աղբյուրներ – թերմոֆիլներ – Bacillus և ցեղակից տեսակներ – 16S ռՌՆԹ-ի գենի (16S ռԴՆԹ) հաջորդականություններ – ֆիլոգենիա

Из наземных теплых (Ахурик) и горячих (Арзакан) геотермальных источников Армении изолированы девятнадцать термофильных аэробных эндоспорообразующих бактерий. На основании анализа гена 16S рРНК (16S рДНК)

изучено их филогенетическое и таксономическое разнообразие. Сравнение последовательностей 16S рДНК изолятов с нуклеотидными последовательностями базы данных GenBank'а указывает на их принадлежность к филуму *Firmicutes*, к подфилуму *Clostridium-Bacillus*, к группе *Bacillus* с родственными родами. Изоляты отнесены к 11 видам пяти родов: *Bacillus, Geobacillus, Sporosarcina, Paenibacillus, Thermoactinomyces*. Термофильная эндоспорообразующая микрофлора менее разнообразна в теплом источнике и включает виды родов *Bacillus* и *Thermoactinomyces*, с доминированием представителей рода *Bacillus*. Представители рода *Geobacillus* доминируют в горячем источнике, составляя в целом 50% обнаруженных изолятов.

Геотермальные источники – термофилы– Bacillus и родственные роды– секвенс гена 16S рРНК (16S рДНК) – филогения

Microbial ecology of geothermal springs located in different parts of the world has been arising interest of scientists during the last decades [5, 10, 12, 19]. Natural geothermal springs are primarily associated with tectonically active zones. Numerous warm and hot mineral springs of different geotectonic origin and with different physical-chemical properties are found in Armenia, where evidence of recent active volcanism is still noticeable [1].

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 [2, 5, 9, 17]. Representatives of the genus *Bacillus* and related genera have been shown to be the thermophilic aerobes most frequently isolated from terrestrial geothermal water environments [10, 12, 23]. Numerical classification based on a series of phenetic characteristics is used for identification and classification of bacilli [14, 21]. At the present, the phylogenetic approach to *Bacillus* and related genera taxonomy is accomplished mainly by analysis of 16S rRNA genes (16S rDNA) [8, 16, 20, 25].

Distribution of some groups of extremophilic bacilli in different natural habitats (mainly in soils) and investigation of their biotechnological potential have appeared the basic directions of microbiological research in Armenia [6]. In this context, geothermal springs located in Armenia represent unique natural habitats of the thermophilic microbes. Recently, distribution of cultivable aerobic thermophilic endospore-forming bacteria in some geothermal springs of Armenia was studied and characterized tentatively based on the phenotypic features [3]. The present study reports on the phylogenetic diversity of cultivable thermophilic aerobic chemoorganotrophic endospore-forming bacteria isolated from earlier uninvestigated warm (Akhourik) and hot (Arzakan) geothermal springs of Armenia based on the analysis of their 16S rRNA genes.

Materials and Methods. *Sampling and physical-chemical measurements*. The location of warm (Akhourik) and hot (Arzakan) geothermal mineral springs of Armenia was determined using GPS technology. Water temperature and pH were measured *in situ* using a portative combined pH/EC/TDS/Temperature tester (HANNA HI98129/HI98130). Water and sediment samples of the studied springs were collected in sterile bottles and maintained on ice until processed. Sampling and all physical-chemical measurements were made in November 2008.

Enrichment experiments and microscopy. To enrich aerobic thermophilic bacteria, filtrate of water (1l filtered through 0.4 µm membrane filters) and sediment (1g) samples were inoculated in Nutrient Broth (Difco) and incubated overnight at 50, 60 and 65ºC with shaking at 240 rpm. Before inoculation, all samples were treated at 80ºC for 10 min to isolate the endospore-forming microorganisms only [4]. Cultures were 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 purity, cell morphology, sporulation and motility were determined by phase-contrast microscopy (Nikon, Eclipse E400 light

microscope) of freshly prepared wet mounts. Colony morphology, Gram reaction, thermophilic growth and catalase activity of all isolates were tested using the commonly accepted methods [4].

Nucleic acid extraction and polymerase chain reaction (PCR). 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) [18].

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, 2 µl 0.1% bovine serum albumin, and sterile water up to the final volume of 50 µl. PCR amplification was completed using a DNAEngine thermocycler (BIO RAD). First, the templates were denaturized for 3 min at 96ºC, then 30 cycles of the following steps were completed: denaturation for 30 s at 96ºC for, annealing for 30 s at 55ºC, and extension at 2.5 min at 72ºC. The 30 cycles were followed by a final 10 min extension at 72ºC. PCR products were viewed under UV light after standard ethidium bromide gel electrophoresis.

Sequencing and phylogenetic analysis. PCR products were purified using GenEluteTM PCR Clean-up Kit (Sigma) according to the manufacturer's recommendations, and were sequenced with 27f primer. Sequencing was performed on a ABI PRISM capillary sequencer according to the protocol of the ABI Prism BigDye Terminator kit (Perkin Elmer) [11].

A nucleotide BLAST search was performed in order to obtain information on the phylogenetically closest relative [7, 29]. The assembled 16S rRNA gene sequence was aligned with a representative set of 16S rRNA gene sequences obtained from the GenBank database. The sequences were edited and aligned with EditSeq and MegAlign of Lasergene software package (DNASTAR program). The alignments were initially done with the CLUSTAL W program option in MegAlign and were manually adjusted. Phylogenetic trees were generated using a neighbor-joining tree-building algorithm. Confidence in the branching points was determined by bootstrap analysis (1000 replicates) [15].

Results and Discussion. *Study sites and physical-ochemical analysis of geothermal waters.* The warm geothermal spring (Akhourik) is located at the coordinates of 40º 44' 34.04″ N, 43º 46' 53.95″ E, up to 1469 m high above the sea level, and has temperature in the range of 28.4-32.0ºC, pH 6.2. The hot geothermal spring (Arzakan) is located at the coordinates of 40º 27' 36.10″ N, 44º 36' 17.76″ E, up to 1490 m high above the sea level, and has temperature of ≥ 44ºC, pH 7.0-7.2. Despite the varying physical-chemical properties of the thermal springs used for the sampling, they belong to the category of hot springs from low-temperature fields and are characterized by neutral to alkaline pH and high concentration of dissolved minerals and gases. The hot spring is related to the hydrocarbonate sodium class of mineral springs (>20% is HCO_3^- and >20% is Na^+), while the warm spring is related to the hydrocarbonate-sulphate sodium-magnesium class (>20% are HCO_3^- and SO_4^{2-}, >20% are Na^+ and Mg^{2+}) [1].

Taxonomic affiliation and phylogenetic relationships of isolates. Collected water and adjacent sediment samples were analyzed to evaluate the total thermophilic aerobic endospore-forming bacterial abundance. In total, 14 aerobic thermophilic strains from the hot spring and 5 aerobic thermophilic strains from the warm one were isolated and identified. These strains were all rod-shaped (with exception of one, which was spherical), Gram-positive, endospore-forming and catalase-positive bacteria related to the phylum *Firmicutes*.

According to the Bergey's Manual of Systematic Bacteriology [13], the phylogenetic classification schemes placed the two most prominent types of endospore-forming bacteria, clostridia and bacilli, in two different classes of the phylum *Firmicutes* - *Clostridia* (mainly anaerobes) and *Bacilli* (mainly aerobes). Class *Bacilli* includes the order *Bacillales* and 10 families (such as *Alicyclobacillaceae, Bacillaceae, Paenibacillaceae, Planococcaceae, Sporolactobacillaceae, Thermoactinomycetaceae*

and others). The family *Bacillaceae* is systematically and phylogenetically diverse taxon that is presently being re-evaluated. Molecular taxonomic methods, based on comparative analysis of small-subunit-ribosomal RNA gene sequences, have had a huge impact on the classification of these organisms, and the number of taxa, including thermophiles, has increased greatly [8, 16]. Currently, the *Bacillaceae* family has more than 40 new genera of the same level as *Bacillus* [13]. Considering this large number of novel endospore forming bacteria, the new term of *Bacillus*-like genera was introduced [12, 27]. Aerobic spore-forming thermophilic Gram-positive rods isolated from hot springs and related habitats are also taxonomically diverse. Thermophilic aerobic spore-forming bacteria having growth optima in the temperature range of 45 to >70ºC are classified into the several genera, including *Brevibacillus, Alicyclobacillus, Anoxybacillus, Ureibacillus, Geobacillus, Bacillus*, and *Thermoactinomyces* [20].

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 web site [28].

BLAST results for the isolates, based on 16S rRNA gene sequences for identification of the closest relatives in the GenBank database, are reported in Table 1. The correlation of the generated 16S rRNA gene sequences of the isolates with the ones stored in the GenBank database indicates that they all belong to *Clostridium-Bacillus* subphylum, group of *Bacillus*-like genera distributed in four families: *Bacillaceae* (genera *Bacillus* and *Geobacillus*), *Paenibacillaceae* (genus *Paenibacillus*), *Planococcaceae* (genus *Sporosarcina*) and *Thermoactinomycetaceae* (genus *Thermoactinomyces*). They are closely related with the members of eleven species of five genera: *Bacillus* (*B. pumilus, B. murimartini, B. licheniformis and B. simplex*), *Geobacillus* (*G. stearothermophilus, G. caldoxylosilyticus G. thermodenitrificans* and *G. toebii*), *Sporosarcina sp.,Paenibacillus sp.* and *Thermoactinomyces sp.*

Among the described species, the closest relatives of isolates ArzA-8, ArzA-33 and ArzA-33a were *G. toebii* with the sequence homology rates of 97, 99 and 99%, respectively. Successive analysis of the amplified 16S rRNA gene revealed phylogenetic relationship of isolate ArzA-7 to *Geobacillus sp.* (99%, 810 bp), ArzA-6 to *G. thermodenitrificans* (98%), ArzA-11 to *G. stearothermophilus* (99%) and ArzA-3 to *G. caldoxylosilyticus* (96%, 500 bp). Isolates ArzA-4 had identical 16S rDNA sequences with 99% identity to the 16S rDNA from *B. licheniformis*, while other isolates (ArzA-2, ArzA-10 and ArzA-13a) shared a significant similarity (96-98%) to *B. simplex*. The isolate ArzA-5 exhibited 98% similarity by 16S rRNA gene (955 bp) to *Paenibacillus sp.* Another one, ArzA-13, had the 16S rRNA gene (910 bp) highly similar to *Sporosarcina sp*. (98%).

The phylogenetic tree based on comparative studies between the 16S rDNA sequences (>700 bp) of isolates obtained from water and sediments of the Arzakan geothermal mineral spring, on one hand, and a selected number of members *Bacillus, Geobacillus, Sporosarcina* and *Paenibacillus* available in GenBank, on the other hand, is shown in Figure 1 (a). The phylogenetic tree confirms that isolates ArzA-6, ArzA-7, ArzA-8, ArzA-11, ArzA-33 and ArzA-33a constitute a part of the cluster within the thermophilic group of bacilli (genus *Geobacillus*). The resulting tree reveals two groups containing *Bacillus* species. The isolate ArzaA-9 is related to the group, the members of which were closely similar to *B. simplex*, in spite of the fact that the sequences the isolate produced had the closest match with *Firmicutes* bacterium EU810844 (98%, 955 bp). Additional phenotypic and genotypic analyses are necessary to confirm taxonomic affiliation of this isolate. Each of the other two revealed clusters contained species from two different genera (*Paenibacillus* and *Sporosarcina*).

Table 1. BLAST results on bacterial 16S rRNA gene sequences for identification of the closest relatives in the GenBank database

Origin of isolates	Isolates (temperature of isolation, T°C)	Closest match Taxonomic affiliation, Phylotype accession no.	% of identity
Arzakan	ArzA-2 (50)	*B. simplex* AY833099	99
	ArzA-3 (65)	*G. caldoxylosilyticus* FJ823099	96
	ArzA-4 (50)	*B. licheniformis* FJ435674	99
	ArzA-5 (50)	*Paenibacillus sp*. DQ497239	97
	ArzA-6 (65)	*G. thermodenitrificans* FJ823098	98
	ArzA-7 (65)	*Geobacillus sp*. EU093964	99
	ArzA-8 (65)	*G. toebii* GQ487459	97
	ArzA-9 (60)	*Firmicutes* bacterium EU810844	98
	ArzA-10 (60)	*B. simplex* GU048877	98
	ArzA-11 (60)	*G. stearothermophilus* AY608948	99
	ArzA-13 (50)	*Sporosarcina sp*. DQ227775	98
	ArzA-13a* (50)	*B. simplex* AY833099	96
	ArzA-33 (65)	*G. toebii* AB116120	99
	ArzA-33a* (65)	*G. toebii* AB116120	99
Akhourik	AkhA-1 (50)	*B. pumilus* FJ237277	99
	AkhA-12 (60)	*Thermoactinomyces sp*. AB362275	97
	AkhA-14 (50)	*B. licheniformis* EF427891	97
	AkhA-14a* (50)	Uncultured bacterium EU773370	97
	AkhA-15 (50)	*B. murimartini* AJ316316	99

*Isolates designated as Arz13 and Arz13a, Arz33 and Arz33a, Arz14 and Arz14a were obtained from the same enrichments, but from different plates.

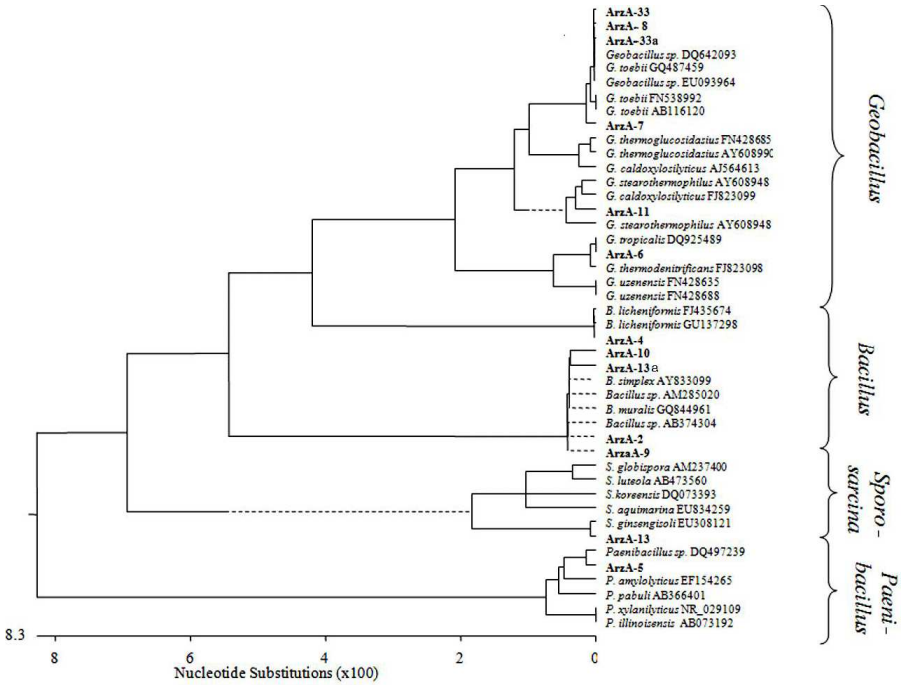
As shown in Table 1, the majority of isolates from the warm spring shared significant similarity (97-99%) to *Bacillus* by their 16S rDNA sequences. The 16S rDNA sequence of isolate ArzA-12 was identical (97%, 815 bp) to the sequences of *Thermoactinomyces sp*.

The phylogenetic tree built to demonstrate the relationship between the retrieved 16S rDNA sequences for the Akhourik spring and their closest relatives from GenBank is shown in Figure 1 (b). According to the presented dendrogram, the genus *Bacillus* appears heterogeneous. The four bacilli isolates are distributed among the three distinct groups (with the closest relatives of *B. licheniformis*, *B. pumilus*, and *B. murimartini*, respectively). A separate group contains AkhA-14a. The 16S rDNA sequence of isolate AkhA-14a was affiliated (97%, 725 bp) with uncultured representatives of the Domain *Bacteria*, but showed less than 95% similarity with *Bacillus sp*. This suggests that isolate AkhA-14a may appear to be a novel bacterial species.

As shown on the dendrogramm in Figure 1 (b), there is a separate cluster that contains representatives of thermoactinomyces. According to earlier reports based on 16S rRNA gene sequence analysis [26], *Thermoactinomyces* is more closely related to *Bacillus* species than to actinomycetes. *Thermoactinomyces* species produce endospores like bacilli, are aerobic, Gram-positive and thermotolerant. Yoon and Park [26] suggest that genus *Thermoactinomyces* should be placed within the family *Bacillaceae*.

Therefore, thermophilic microflora in the Akhourik mineral spring is taxonomically less diverse than in the Arzakan spring. All isolates detected in the warm spring were thermotolerant ones and included representatives of only two genera - *Bacillus* and *Thermoactinomyces* – with prevailing content of *Bacillus* composing 80% of the isolates in total. *Bacillus* and *Geobacillus* with their 5 and 7 isolates, respectively, were the predominant genera in the hot spring.

a.



b.

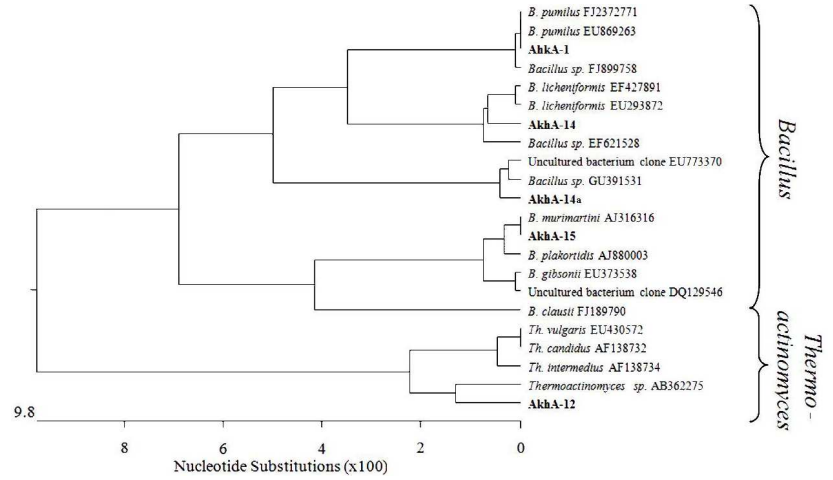


Fig. 1. Phylogenetic tree based on the comparative studies between 16S rDNA sequences of the isolates obtained from Arzakan (a) and Akhourik (b) geothermal mineral springs, on one hand, and selected members of *Bacillus*, *Geobacillus*, *Sporosarcina*, *Paenibacillus* and *Thermoactinomyces* available in GenBank, on the other hand. Scale bar corresponds to one nucleotide substitution per 100 nucleotides.

From the metabolic point of view, genus *Geobacillus* includes chemoorganotrophic, aerobic or facultative aerobic (oxygen as the electron acceptor is in some species replaceable by nitrate) obligatory thermophiles [20, 27]. In total, 50% of the isolates detected in the hot spring samples are most closely related to members of the genus *Geobacillus*, which are known to thrive in similar habitats [10, 27]. Representatives of *G. toebii* are the most distributed obligate thermophiles in the studied hot spring. Abundance of geobacilli is in agreement with temperature regime of the studied thermal spring (≥ 44ºC). All isolates from the hot spring that belonged to the genus *Bacillus* were thermotolerant microorganisms among which *B. simplex* appeared as the dominating species.

Although representatives of the genera *Paenibacillus*, *Thermoactinomyces* and *Sporosarcina* are commonly considered the species most frequently isolated from similar habitats [12, 23, 25, 26], both springs sampled in this study demonstrated significantly lower content of these species.

As part of microbial communities, thermophilic endospore-forming bacteria presumably have significant contribution in forming the composition of mineral waters, and promote normal course of geochemical circulations under extreme temperature conditions. Moreover, apart from the thermal conditions, abiotic factors such as pH, dissolved gases (H_2, CO_2, H_2S, CH_4) and high mineralization could act as limiting factors for microbial diversity and biomass [22]. 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 [24].

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

Acknowledgements

The part of this research was carried out at the Bergen University (Bergen, Norway) in framework of FEMS Research Fellowship-2008-2. Invaluable assistance of Prof. N.K. Birkeland and Prof. Yu.G. Popov and technical support of Ms. M. Madsen is to be highly emphasized.

REFERENCES

1. Геология Армянской ССР, IX. Минеральные воды. Ереван, Изд. АН Арм. ССР, 521, 1969.
2. Жизнь микробов в экстремальных условиях. Под ред. Д. Кашнера. М.: Мир, 519, 1981.
3. *Паносян О.А*. Термофильные бациллы термальных источников Армении. Биолог. журн. Армении, *60*, 3, 19-24, 2008.
4. Практикум по микробиологии. *Под ред. Нетрусова А.И.* М., Изд-во АН РФ, 603, 2005.
5. Access to Biodiversity and New Genes from Thermophiles by Special Enrichment Methods. PhD thesis *Cedric F.V.* Hobel, University of Iceland, Peykjavik, 96, 2004.
6. *Afrikian E.G., Khachaturian A.A., Avakian Z.G.* Ecology of some groups of extremophilic bacilli in soils. 5th Int. Symp. Microbiol. Ecol. (ISMES), *54*, 1990.
7. *Altschul S.F., Madden T.L., Schaffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl. Acids Res., *25*, 3389-3402, 1997.
8. *Ash C., Farrow A.E., Wallbanks S., Collins M.D.* Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal DNA sequences. Lett. Appl. Microbiol., *13*, 202-206, 1991.
9. *Charlier D., Droogmans L.* Microbial life at high temperature, the challenges, the strategies. Cell Mol. Life Sci., *62*, 2974-2984, 2005.

10. *Coleri A., Cokmus C., Ozcan B., Akkoc N., Akcelik M.* Isolation of α-glucosidase-producing thermophilic bacilli from hot springs of Turkey. Microbiology, *78*, 1, 56-66, 2009.
11. *Dahle H., Garshol F., Madsen M., Birkeland N.-K.* Microbial community structure analysis of produced water from a high-temperature North Sea oil-field. Antonie van Leeuwenhoek, *93*, 37-49, 2008.
12. *Derekova A., Mandeva R., Kambourova M.* Phylogenetic diversity of thermophilic carbohydrate degrading bacilli from Bulgarian hot springs. World J. Microbiol. Biothechnol., *24*, 1697-1702, 2008.
13. *Garrity G.M., Lilburn T.G., Cole J.T., Harrison S.H., Euzeby J., Tindall B.J.* Taxonomic Outline of the Bacteria and Archaea. Release 7.7 Part 9-The Bacteria: Phylum Firmicutes. Class *Bacilli*. http//www.bergeysoutline, March 2007.
14. *Gordon R.E., Haynes W.C., Pang C.H.W.* The Genus Bacillus. Agricult. Handbook. Washington: D.C., 1973.
15. *Hall B.G.* Phylogenetic trees made easy: A how-to manual, second edition. Sinauer associates, Inc.Publishers Sunderland, Massachusetts, USA, *221*, 2004.
16. *Joung K.B., Cote J.C.* Evaluation of ribosomal RNA gene restriction patterns for the classification of *Bacillus* species and related genera. J. Appl. Microbial., *92*, 97-108, 2002.
17. *Kristjansson J.K, Stetter K.* Thermophilic bacteria. In: Kristjansson J.K. (ed.) Thermophilic bacteria. CRC Press. Inc., Boca Raton, 1992.
18. *Lane D.J.* 16S/23S rRNA sequencing. In: Stackebrandt E., Goodfellow M. (eds) Nucleic acid techniques in bacterial systematics. John Wiley & Sons Ltd., Chichester, 115-148, 1991.
19. *Lau M.C.Y., Aitchison J.C., Pointing S.B.* Bacterial community composition in thermophilic microbial mats from five hot springs in central Tibet. Extremophiles, *13*, 139-149, 2009.
20. *Nazina T.N., Tourova T.P., Poltaraus A.B., Novikova E.V., Grigoryan A.A., Ivanova A.E., Lysenko A.M., Petrunyaka V.V., Osipov G.A., Belyaev S.S., Ivanov M.V.* Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. kaustophilus*, *G. thermoglucosidasius* and *G. thermodenitrificans*. Int. J. Syst. Evol. Microbiol., *51*, 433-446, 2001.
21. *Priest T.G., Goodfellow M., Todd C.* A numerical classification of the genus *Bacillus*. J. Gen. Microbiol., *134*, 1847-1882, 1988.
22. *Purcell D., Sompong U., Lau C.Y., Barraclough T.G., Peerapornpisal Y., Pointing S.B.* The effects of temperature, pH and sulphide on community structure of hyperthermophilic streamers in hot springs of northern Thailand. FEMS Microbiol. Ecol. *60*, 456-466, 2007.
23. *Sharp R.J., Riley P.W. White D.* Heterotrophic thermophilic bacilli. In: Kristjanssono J.K. (ed.) Thermophilic bacteria. CRC Press. Inc., Boca Raton 1992.
24. *Takacs-Vesbach C., Mitchell K., Jakson-Weaver O., Reysenbach A-L.* Volcanic calderas delineate biogeographic provinces among Yellowstone thermophiles. Environ. Microbiol., 10, 1681-1689, 2008.
25. *Xu D., Cote J.Ch.* Phylogenetic relationships between *Bacillus* species and related genera inferred from comparison of 3' end 16S rDNA and 5' end 16S-23S ITS nucleotide sequences. Int. J. Syst. Evol. Microbiol., 53, 695-704, 2003.
26. *Yoon J.H. and Park Y.H.* Phylogenetic analysis of the genus *Thermoactinomyces* based on 16S rDNA sequences. Int. J. Syst. Evol. Microbiol., 50, 1081-1086, 2000.
27. *Zeigler D.R.* The Genus Geobacillus. Introduction and strain catalog. In: Zeigler D.R. (ed.) Catalog of strains, vol. 3, 7th edn. *Bacillus* genetic Stock Center, The Ohio State University, USA, 2001.
28. http://www.ncbi.nlm.nih.gov/Blast.cgi (July 17, 2010).

Received 26.07.2010