DISTRIBUTION OF *GEOBACILLUS* SPECIES IN ARZAKAN (ARMENIA) GEOTHERMAL MINERAL SPRING

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Introduction

16S rRNA gene sequencing is a widely used standard technique in bacterial taxonomy and it is also routinely used in polyphasic approach when new descriptions of bacterial species or higher taxa are made [1, 2]. The phylogenetic approach to *Bacillus* and related genera taxonomy is accomplished mainly by analysis of 16S rRNA genes (16S rDNA) [3-5]. Particularly, 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* [6]. 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 [7, 8]. The members of genus *Geobacillus*can exhibit growth at temperatures ranging from 35-78 ºC and are widespread in various geographical locations. It was shown their presence in terrestrial hot springs, petroleum reservoirs, deep-sea hydrothermal vents and deep ocean-basin cores [7-9].

Interest in thermophilic bacteria from the genus *Geobacillus* has clearly emerged during the past two decades due to their significant potential for biotechnological applications including their importance as sources of thermostable enzymes and other products of industrial interest [10].

Numerous geothermal springs of different geotectonic origin and with different physicochemical properties are found in Armenia [11]. Recently microbiological investigations of some Armenian geothermal springs were carry out [12]. However, distribution of geobacilli in geothermal springs located in Armenia is still poor investigated. The study of distribution of geobacilli in Armenian geothermal spring is not only a taxonomical concern, but also a necessity in order to exploit its biotechnological potential.

The aim of the current study was to isolate and identify representatives of the genus *Geobacillus* habited in Arzakan hot spring using genotypic methods (16S rRNAgene sequences).

Material and Methods

The location of geothermal mineral spring in Arzakan was determined using GPS technology. Water temperature, pH and conductivitywere measured *in situ* using a portative combined pH/EC/TDS/Temperature tester (HANNA HI98129/HI98130). Sediment samples of the studied springs were collected in sterile bottles and maintained on ice until processed.

To enrich aerobic thermophilic bacilli the sediment (1g) samples were treated at 80ºC for 10 min, then inoculated in Nutrient Broth (Difco) and incubated overnight at 60 and 65ºC with shaking at 240 rpm. 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.

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) [13]. 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), 1,5mM $MgCl_2$, 0,2 µl *Taq* DNA polymerase, 2 µl0.1% bovine serum albumin, and sterile water up to the final volume of 50 µl. PCR amplification was completed using an DNAEnginethermocycler (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.

PCR products were purified using GenEluteT 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) [14].

16S rRNA gene sequences were compared with those contained in the GenBank by using BLASTn search at the NCBI web site (http://www.ncbi.nlm.nih.gov/Blast.cgi) [15].

Results and Discussion

The Arzakan geothermal spring is located at 40º 27' 36.10'' N, 44º 36' 17.76'' E, at 1490 m above sea level with a temperature of ~44^{O}C, pH 7.0-7.2, and a conductivity of 4378.3 µS/sm. Arzakan spring belongs to the category of hot springs from low-temperature fields and is characterized by neutral to alkaline pH and high concentration of dissolved minerals and gases. The studied spring is related to the hydrocarbonate sodium class of mineral springs (>20% is HCO_3^- and >20% is Na^+) [11].

Sediment samples were analyzed to evaluate the geobacilli abundance. In total, seventhermophilicgeobacilli strains designated as ArzA-3,ArzA-6, ArzA-7, ArzA-8, ArzA-11, ArzA-33 and ArzA-33a with growth temperature 60 and 65ºC were isolated and further identified.

16S rRNA genes from the extracted DNA of each isolate were successfully amplified by PCR and further sequenced. BLAST results based on 16S rRNA gene sequences for identification of the closest relatives in the GenBank database, are reported in Table 1.

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%), ArzA-6 to *G. thermodenitrificans* (98%), ArzA-11 to *G.stearothermophilus* (99%) and ArzA-3 to *G. caldoxylosilyticus* (96%).

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

Isolates (temperature of isolation, T°C)	The lengths of the DNA fragments, (bp)	Closest match Taxonomic affiliation, Phylotype accession no.	% of Identity
ArzA-3 (65)	680	*G. caldoxylosilyticus* FJ823099	96
ArzA-6 (65)	909	*G. thermodenitrificans*FJ823098	98
ArzA-7 (65)	803	*Geobacillus sp.*EU093964	99
ArzA-8 (65)	790	*G. toebii* GQ487459	97
ArzA-11 (60)	917	*G. stearothermophilus* AY608948	99
ArzA-33 (65)	987	*G. toebii* AB116120	99
ArzA-33a (65)	836	*G. toebii* AB116120	99

Representatives of *G. toebii* are the most distributed obligate thermophiles in the studied hot spring. 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.

Apart from the thermal conditions, low nutrient status, dissolved gases and high mineralization, as well as geological history of studied spring allowed the development of unique population and natural selection of a few species of geobacilli. As part of microbial community geobacilli presumably have significant contribution in forming the composition of mineral water and promote normal course of geochemical circulations under extreme temperature conditions.

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.

REFERENCES

1. ***Lane D. J.*** (1991) 16S/23S rRNA sequencing. In: Stackebrandt E., Goodfellow M. (eds) Nucleic acid techniques in bacterial systematics. John Wiley & Sons Ltd., Chichester, 115-148.

2. ***Rossello-Mora R.*** (2005) Updating prokaryotic taxonomy. J Bacteriol, 187, 6255-6257.

3. ***Ash C., Farrow A. E., Wallbanks S., Collins M. D.*** (1991) Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal DNA sequences. Lett. Appl. Microbiol., *13*, 202-206.

4. ***Joung K. B., Cote J. C.*** (2002) Evaluation of ribosomal RNA gene restriction patterns for the classification of *Bacillus* species and related genera. J. Appl. Microbial., *92*, 97-108.

5. ***Xu D., Cote J. Ch.***(2003) 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.

6. ***Logan NA, Berge O., Bishop A. H., Busse H. J., de Vos P., Fritze D., Heyndrickx M.R.A, Kampfer P., Rabinovitch L., Salkinoje-Salonen M. S., Seldin L., Ventosa A.*** (2009) Proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria. Int. J. Syst., Evol. Microbiol., 59, 2114-

2121.

7. ***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.***(2001) Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillussubterraneus* gen. nov., sp. nov and *Geobacillusuzenensis* 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.

8. ***Zeigler D. R.*** (2001) 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.

9. ***Charlier D., Droogmans L.*** (2005) Microbial life at high temperature, the challenges, the strategies. Cell Mol. Life Sci., *62*, 2974-2984.

10. ***Antranikian G., Egorova K.*** (2007) Extremophiles, a unique resource of biocatalysts for industry. In: Gerday, C., Glansdorff, N. (Ed.), Physiology and Biochemistry of Extremophiles. ASM Press, Washington D. C., 361-406.

11. Геология Армянской ССР, IX. (1969) Минеральные воды. Ереван, Изд. АН Арм. ССР, 521.

12. ***Паносян О. А.*** (2008) Термофильные бациллы термальных источников Армении. Биолог. журн. Армении, 60, 3, 19-24.

13. ***Lane D. J.*** (1991) 16S/23S rRNA sequencing. In: Stackebrandt E., Goodfellow M. (eds) Nucleic acid techniques in bacterial systematics. John Wiley &Sons Ltd., Chichester, 115-148.

14. ***Dahle H., Garshol F., Madsen M., Birkeland N.-K.*** (2008) Microbial community structure analysis of produced water from a high-temperature North Sea oil-field. Antonie van Leeuwenhoek, 93, 37-49.

15. ***Altschul S. F., Madden T. L., Schaffer A. A., Zhang J., Zhang Z., Miller W., Lipman D. J.*** (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl. Acids Res., *25*, 3389-3402.

ԱՐԶԱԿԱՆԻ (ՀԱՅԱՍՏԱՆ) ԵՐԿՐԱՋԵՐՄԱՅԻՆ ՀԱՆՔԱՅԻՆ ԱՂԲՅՈՒՐՈՒՄ *GEOBACILLUS* ՑԵՂԻ ՏԵՍԱԿՆԵՐԻ ՏԱՐԱՁՎԱԾՈՒԹՅՈՒՆԸ

Հ. Հ. ՓԱՆՈՍՅԱՆ

Արզականի երկրաջերմային հանքային աղբյուրից մեկուսացվել են գեոբացիլների յոթ շտամներ: Ըստ 16S ռՌՆԹ-ի գենի նուկլեոտիդային հաջորդականությունների վերլուծության մեկուսացված շտամները նույնականացվել են որպես *Geobacillustoebii* (3),*G. thermodenitrificans* (1), *G. caldoxylosilyticus* (1) և *Geobacillus sp.* (1) տեսակներ:*G. toebii* տեսակի ներկայացուցիչները հետազոտված աղբյուրում առավել տարածված գեոբացիլներն են:

РАСПРОСТРАНЕННОСТЬ ВИДОВ GEOBACILLUS В ГЕОТЕРМАЛЬНОМ МИНЕРАЛЬНОМ ИСТОЧНИКЕ АРЗАКАН (АРМЕНИЯ)

О. А. ПАНОСЯН

Из геотермального источника Арзакан изолированы семь штаммов геобацилл. На основании анализа гена 16S рРНК штаммы идентифицированы как *Geobacillustoebii* (3), *G. thermodenitrificans* (1), *G. caldoxylosilyticus* (1) и *Geobacillussp* (1). Представители вида *G. toebii* доминируют в изученном горячем источнике.