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## BACTERIAL COMMUNITY STRUCTURE OF ARMENIAN MINING TERRITORIES REVEALED BY PCR-DGGE FINGERPRINTING

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The bacterial community structure in the stone rock samples collected from Zangezur copper and molybdenum mine, Sotck gold mine, Shamlugh copper mine, as well as in the sludge sample collected from Alaverdi copper mine were investigated by PCR-DGGE fingerprinting. The presence of the phylum Actinobacteria, Bacteroidetes and Proteobacteria in the studied samples has been detected. The results give a snapshot of the microbial community structure and diversity in the Armenian mining areas.

### *Bacterial community – copper and gold mine – PCR-DGGE*

Դեռևստուրացևող գրադիենտային ժել էլեկտրաֆորեզի (ԴԳԺԷ) մեթոդի կիրառմամբ ուսումնասիրվել է բակտերիաների համակեցության կազմը Չանգեզուրի պղնձամոլիբդենային, Սոթքի ոսկու և Շամլուղի պղնձի մանրախճային հանքապարի հողային, ինչպես նաև Ալավերդու պղնձի հանքավայրի թափոնային նմուշներում: Բացահայտվել է, որ ուսումնասիրված հանքավայրերը հարուստ են Actinobacteria, Bacteroidetes և Proteobacteria ֆիլումների ներկայացուցիչներով: Տվյալները տեղեկատվություն են տալիս Հայաստանի հանքավայրերում բակտերիալ համակեցությունների կազմի և բազմազանության մասին:

### *Բակտերիական համակեցություն – պղնձի և ոսկու հանքապարներ – ՊՇՆ-ԴԳԺԷ*

Использованием метода денатурирующего градиентного геля электрофореза (ДГГЭ) показана структура бактериального сообщества в образцах почв с гравием рудных пород Зангезурского медно-молибденового комбината, Сотском месторождении золота, Шамлугском медном руднике и шлама Алавердского медного рудника. В исследуемых образцах обнаружено присутствие бактериальных фил Actinobacteria, Bacteroidetes и Proteobacteria. Результаты дают информацию о структуре и разнообразии бактериальных сообществ в месторождениях цветных металлов Армении.

### *Бактериальное сообщество – рудные породы меди и золота – ПЦР-ДГГЭ*

The development of industrial processes such as smelting, mining, metal forging and etc. results in release of heavy metals to the environment and disruption of ecological balance in most ecosystems [8]. Metal-rich natural habitats have become an extreme environment for the development and evolution of unique microbial communities [6]. The diverse bacterial groups have developed abilities to deal with the toxic levels of the metals by detoxification, metal absorption, uptake and accumulation, extracellular precipitation, efflux of heavy metals from the cells [11, 12].

Study of microbial diversity in different environments based on cultivable approach can provide quantitative yields and specific information on diversity of microbial communities [2, 23].

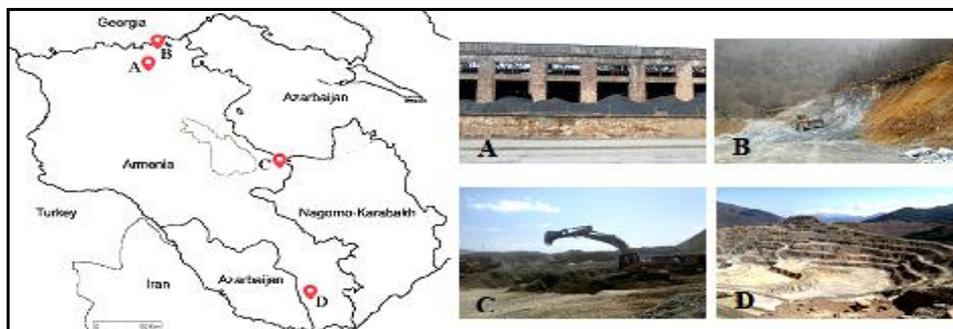
However, different studies in environment demonstrated that less than 1% of the total microbial community members are cultivable and applying culture-dependent methods will only reveal information about the very small fraction of microorganism able to grow under the given condition [5, 21]. Different fingerprint methods using 16S rRNA gene have been developed to study total microbial diversity [6, 14]. The use of molecular methods gives an opportunity to collect information on the composition of microbial communities [6, 14, 15].

Armenia has significant reserves of copper, molybdenum and gold, as well as lead, silver and zinc [9, 18]. Development of mining and smelting industry leads to increasing emissions of large amounts of waste into the environment which is the primary pollutants of heavy metals in Armenia.

The objective of this study was to determine the bacterial community structure in the stone rock samples collected from Zangezur copper and molybdenum mine (Kajaran), Sotk gold mine, Shamlugh copper mine, as well as in the sludge sample collected from Alaverdi copper smelter based on PCR-DGGE fingerprinting.

**Materials and methods. Sampling sites.** The stone rock and sludge samples were taken from Zangezur copper and molybdenum mine, Sotck gold mine, Alaverdi copper smelter and Shamlugh copper mine (fig. 1). The pH was measured using a portable potentiometer (HANNA, HI98129 pH/Conductivity/TDS Tester). Coordinates of studied sites was determined by GPS (Garmin GPSMAP 64s). Zangezur copper and molybdenum mine is located in Armenia's southern Syunik region and the coordinate is E46°08'54.28" N39°09'13.15" (elevation is 1865 m). The Sotck gold mine is one of the largest deposits in Armenia and located 20 km east of the city Yardenis (E45°58'00.76" N40°13'52.20", elevation is 2477 m). Alaverdi copper smelter and Shamlugh copper mine are located at Armenia's northern Lori province (E44°39'41.96" N41°5'58.79" and E44°44.470' N41°09.121' correspondingly, elevation 778 m).

Collected samples were stored in the cooling bag and transported to the laboratory for nucleotide acid extraction.



**Fig. 1.** Location of study sites. Maps of Armenia showing the locations of studied mining territories with red marks. Close up photograph of A. Sludge of Alaverdi copper smelter, B. Shamlugh copper mine, C. Sotk gold mine and D. Zangezur copper and molybdenum mine. The source of the map: <http://map-caucasus.com/>

#### **DNA extraction and PCR-DGGE**

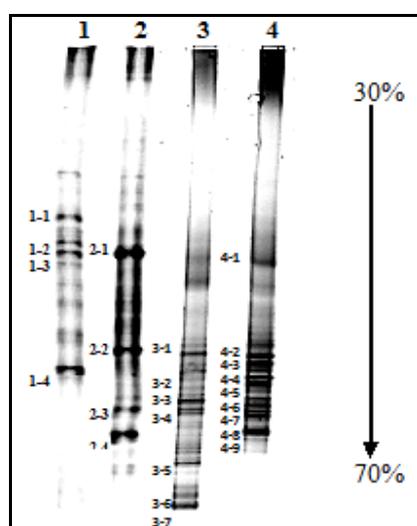
DNA was extracted from 0.5g soil/sediments using Fast DNA SPIN Kit for Soil (MP Biomedicals, USA) according to the manual. The bacterial community structure in the soil and sludge samples were studied using PCR Denaturing Gradient Gel Electrophoresis (DGGE) as described by [10]. The extracted DNA was used as template for amplification of the V3 region of 16S rRNA gene sequence by using L340F and K517R primer sets [1, 4].

The DGGE analysis of PCR products were performed using TV-400-DGGE System (Topac Inc., USA) with 8% (w/v) polyacrylamide gel (37.5:1 acrylamide/bisacrylamide) in 0.5x TAE (20 mM Tris-HCl, 10 mM Acetat, 0.5 mM EDTA) buffer and denaturants (100 % denaturant contain 7 M urea an 40 % deionized formamide). A denaturant gradient was 30-70 %.

### Sequencing and Phylogeny

Sequencing for DGGE band amplicons were performed on ABI PRISM capillary sequencer according to the protocol of the ABI Prism BigDye Terminator kit (Perkin Elmer). Raw data of DNA sequences were analyzed with program Chromas and BioEdit. Closest matches for the partial 16S rDNA sequences were identified by basic local alignment search tool (BLAST) with nucleotide database in NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Alignment for phylogenetic analysis was made by using ClustalW [20]. Phylogenetic tree was constructed using the neighbor joining method with MEGA 6.10 [19]. Bootstrapping analysis for 1000 replicates was performed to estimate the confidence of tree topologies [3].

**Results and Discussion.** PCR-DGGE based analysis of the extracted environmental DNA was done to provide a snapshot of the microbial communities' structure in the studied samples. DGGE patterns showed the occurrence of complex bacterial communities in the analyzed samples. Difference of microbial communities was obtained from different samples of Armenian mines (fig. 2).



**Fig. 2.** Bacterial community profile determined with PCR-DGGE of partial 16S rRNA genes fragments from environmental DNA (1- Sotck gold mine, 2- Zangezur copper and molybdenum mine, 3- Alaverdi copper mine, 4- Shamlugh copper mine).

Aiming to identify the dominant taxa associated with the PCR-DGGE profiles several DGGE bands were excised from the gel. The successful sequencing of partial 16S rRNA gene could be obtained only for 24 bands of the DGGE gel. Most of the bacteria detected by DGGE fingerprinting was mainly closely related to uncultivated organisms (tab. 1) and shared less than 95 % identity with their closest match in GenBank indicating a unique community of studied samples.

The stone rock samples of Sotck Gold mine rich with the members of the phylum Proteobacteria (50%) and Actinobacteria (50%). The bacterial groups belonged to phylum Bacteroidetes (10%) and Actinobacteria (90%) were found in the samples of Zangezur copper and molybdenum mine.

The sequences derived from the extracted DNA of the bands from line 3-1 to line 3-7 (the sludge sample of Alaverdi copper mine) belonging to the phylum Actinobacteria, which includes bacterial genus of *Micromonospora*, *Geodermatophilus*, *Cryptosporangium*, *Rhodococcus*, *Saccharomonospora* and *Blastococcus* usually founding in the soil (tab. 1). The members of the phylum Actinobacteria are widely distributed in the soil and have found in the different mining areas. They were characterized with heavy metals resistance and bioaccumulation potential.

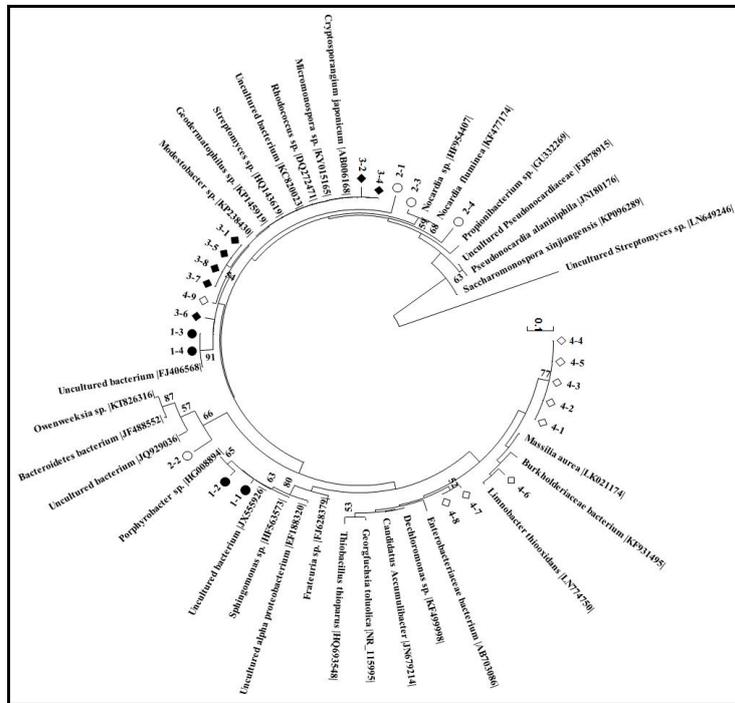
**Table 1.** Closest phylogenetic affiliation of the bacterial 16S rRNA gene based on BLAST comparison to the GenBank database

Sampling Site	Band ID	Sequence length (bp)	Closest phylogenetic match [Accession]	Closest culture phylogenetic match [Accession]	Identity %
Sotck	1-1	174	Uncultured alpha proteobacterium DGGE 16-2 [LN681342]	<i>Pseudomonas</i> sp. TF7(2013) [KC906191]	99/95
	1-2	178	Uncultured alpha proteobacterium DGGE 16-2 [LN681342]	<i>Pseudomonas</i> sp. TF7(2013) [KC906191]	97/96
	1-3	163	Uncultured bacterium DGGE 16-8 [LN681348]	<i>Nocardioides</i> sp. strain HBUM200119 [KY945743]	98/99
	1-4	164	Uncultured bacterium DGGE 16-8 [LN681348]	<i>Nocardioides</i> sp. HCA3-17_Pa   KX419031]	99/100
Zangezur	2-1	159	Uncultured bacterium DGGE gel 1.2 [LN649247]	<i>Propionibacterium</i> sp. Taian [GU332269]	96/95
	2-2	180	Uncultured bacterium clone Arz-B-45   JQ929036]	<i>Owenweeksia</i> sp. TH136   KT826316]	99/95
	2-3	160	Uncultured Streptomyces sp. DGGE gel 10 [LN649246]	<i>Nocardia</i> sp. strain AC376_FSS578   KX928418]	95/99
	2-4	171	Uncultured Streptomyces sp. DGGE gel 10 [LN649246]	<i>Propionibacterium</i> sp. Taian   GU332269]	95/92
Alaverdi	3-1	131	<i>Micromonospora</i> sp. WMMA2015 [KY015165]	Same as CPM	98
	3-2	139	<i>Cryptosporangium japonicum</i> [AB006168]	Same as CPM	92
	3-3	156	<i>Rhodococcus</i> sp. ADC4[DQ272471]	Same as CPM	98
	3-4	159	<i>Geodermatophilus</i> sp. RKEM 688 [KU198754]	Same as CPM	98
	3-5	140	<i>Saccharomonospora xinjiangensis</i> ST187 [KM588177]	Same as CPM	96
	3-6	137	Uncultured bacterium clone OTU233 [KY466264]	<i>Blastococcus</i> sp. URHD0003 [LN876424]	98/98
	3-7	179	<i>Geodermatophilus</i> sp. RKEM 688 [KU198754]	Same as CPM	99
Shamlugh	4-1	160	Uncultured beta proteobacterium clone beta3 [KP714254]	<i>Georgfuchsia toluolica</i> strain G5G6 [NR_115995]	89/89
	4-2	164	Uncultured bacterium clone F5K2Q4C04IVUVQ [GU912975]	<i>Klebsiella oxytoca</i> strain J7 [KT767956]	85/85
	4-3	163	Uncultured bacterium clone 1A247 [GQ164499]	<i>Enterobacteriaceae bacterium</i> KRT2 [AB703086]	89/87
	4-4	169	Uncultured bacterium clone CarbonSeq02a_030410_A10 [KC605441]	<i>Burkholderiaceae bacterium</i> FMAC7j [KF931495]	87/84
	4-5	161	Uncultured bacterium clone QKAB3ZF121 [KJ707428]	<i>Georgfuchsia toluolica</i> strain G5G6 [NR_115995]	89/89
	4-6	165	Uncultured beta proteobacterium clone J45-69 16S [KC603355]	<i>Limnobacter thiooxidans</i> Nb15RA-1 [KP296193]	93/93
	4-7	157	Uncultured bacterium isolate DGGE gel band S27 [KU497528]	<i>Thiobacillus thioparus</i> [HQ693548]	95/93
	4-8	159	Uncultured <i>Thiobacillus</i> sp. clone PS107 [KF517403]	<i>Rhodocyclaceae bacterium</i> KABU2 [LC094733]	90/88
	4-9	166	Uncultured bacterium clone dldaqu9 [KC820023]	<i>Rhodococcus</i> sp. ADC4 [DQ272471]	92/91

CPM- Closest phylogenetic match

The bacterial community in the stone rock samples of Shamlogh copper mine contains bacteria from the phylum Proteobacteria (90%) and Actinobacteria (10%). The phylum Proteobacteria is represented with anaerobic bacterium of the genus *Georgfuchsia*, aerobic thiosulfate-oxidizing bacteria belonging to the genus *Limnobacter* and *Thiobacillus* and soil bacterium of the genus *Klebsiella* (tabl. 1). The phylum Actinobacteria represents only with bacterium from the genus *Rhodococcus*.

Phylogenetic analyses of the 16S rDNA sequences derived from DGGE profiles and their closes matches in the GenBank has showed that the bacterial community representatives of Sotck gold mine involved in the cluster with *Propionibacterium* sp. and uncultured bacterium. The DGGE gel bands derived from Zangezure copper and molybdenum stone rock samples involved in the clusters with *Nocardia fluminea* and some uncultured bacterial groups (fig. 3). The bacterial phylum of Actinobacteria is more frequently found in the soil samples, as well as in the contaminated environments. It is therefore possible that these bacteria were widely present in the soil of the different mining regions and resisted to the impact of the heavy metals [13].



**Fig. 3.** Circular phylogram showing the phylogenetic affiliation of 16S rDNA sequences derived from DGGE profiles (●-Sotck gold mine, ○- Zangezur copper and molybdenum mine, ◆- Alaverdi copper mine, ◇- Shamlogh copper mine). The bootstrap values per 100 bootstrap analyses are presented on the tree. Scale bar represents 0.1 substitutions per site.

The bacterial sequences derived from the sludge of Alaverdi copper smelter and stone rock of Shamloghe copper mine is mostly involved in separate clusters which are indicated on the uniqueness of the studied samples.

Aerobic thiosulfate-oxidizing bacteria belonging to the genus *Limnobacter* and *Thiobacillus* have been found in moderated acidic sample of the Shamlogh copper mine stone rock. Only two species of the genus *Limnobacter* (*L. thiooxidans* and *L. litoralis*) are known and firstly were isolated from sediment of the littoral zone of a freshwater

lake (Lake Chiemsee, Bavaria, Germany) [17], while *L. litoralis* was isolated from a 22-year-old volcanic deposit at a site lacking vegetation on the island of Miyake, Japan [7].

The presence of anaerobic bacterium *Georgfuchsia toluolica* belonged to the phylum *Betaproteobacteria* in the stone rock sample of Shamlugh copper mine is also interesting. A bacterium (strain G5G6) that grow with toluene and utilizes Fe(III), Mn(IV), while using nitrate as terminal electron acceptors for growth on aromatic compounds, does not grow on sugars, lactate or acetate, was isolated from a polluted aquifer (Banisveld, the Netherlands) [22]. The strain G5G6 (DSMZ 19032(T)=JCM 14632(T)) is a novel taxon of the *Betaproteobacteria*.

The low similarity (89-93%) of the DNA sequences extracted from DGGE gel bands with *L. thiooxidans* Nb15RA-1 and *G. toluolica* G5G6 strains (see table 2) could be promising for isolation of a new species or sub-species of newly described genera of the *Betaproteobacteria* from Shamlugh copper mine.

Although diversity of dominant bacterial groups was detected, the active microbial population in the studied metal-rich niche is not identified. Generally, the modern techniques of microbial community analysis, including all their limitations, seem to be suitable for determination of changes in the community structure exposed to the impact of pollutants.

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