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Microbial diversity in an Armenian geothermal spring assessed by molecular and culture-based methods

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The phylogenetic diversity of the prokaryotic community thriving in the Arzakan hot spring in Armenia was studied using molecular and culture-based methods. A sequence analysis of 16S rRNA gene clone libraries demonstrated the presence of a diversity of microorganisms belonging to the Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Epsilonproteobacteria, Firmicutes, Bacteroidetes phyla, and Cyanobacteria. Proteobacteria was the dominant group, representing 52% of the bacterial clones. Denaturing gradient gel electrophoresis profiles of the bacterial 16S rRNA gene fragments also indicated the abundance of Proteobacteria, Bacteroidetes, and Cyanobacteria populations. Most of the sequences were most closely related to uncultivated microorganisms and shared less than 96% similarity with their closest matches in GenBank, indicating that this spring harbors a unique community of novel microbial species or genera. The majority of the sequences of an archaeal 16S rRNA gene library, generated from a methanogenic enrichment, were close relatives of members of the genus *Methanoculleus*. Aerobic endospore-forming bacteria mainly belonging to *Bacillus* and *Geobacillus* were detected only by culture-dependent methods. Three isolates were successfully obtained having 99, 96, and 96% 16S rRNA gene sequence similarities to *Arcobacter* sp., *Methylocaldum* sp., and *Methanoculleus* sp., respectively.

Additional supporting information may be found in the online version of this article at the publisher's web-site.

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Introduction

The ability of microbes to thrive in high-temperature environments has prompted study by researchers to better understand the necessary physiological and molecular adaptations that may enable their utilization in various biotechnological applications [1]. The most common thermal biotypes are deep-sea and coastal marine hydrothermal vents, deep oil-field reservoirs and other subsurface habitats, and terrestrial hot springs. Natural geothermal springs, including terrestrial hot springs, are widely distributed in various regions of our planet, and are primarily associated with tectonically active zones in areas where the Earth's crust is relatively thin. Phylogenetic characterizations of microbiota have been performed for many geothermal springs in various parts of world, and many novel extremophiles have been isolated and described [2]. In Armenia, where traces of recently active volcanic processes are still noticeable, many geothermal springs with different geotectonic origins and physicochemical properties are found [3]. These thermal springs represent a source for discovery of new extremophilic microorganisms and biotechnological resources.

Only a small fraction of the microorganisms found in a natural habitat can be cultivated under laboratory conditions and subsequently isolated. The knowledge of environmental microbial diversity has been largely aided by the development of culture-independent molecular phylogenetic techniques [4–7]. Using a combination of several approaches of traditional microbiology with state-of-the-art molecular biology techniques has

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substantially increased our understanding of the structural and functional diversity of microbial communities. It has been reported that hot springs are inhabited by a variety of microbes belonging to the Bacteria and Archaea domains that tolerate environmental extremes and could have some yet undescribed industrial potential [8].

In this investigation, we applied culture-dependent and -independent approaches to describe the microbial diversity in the Arzakan hot spring. The culture-independent studies involved denaturing gradient gel electrophoretic (DGGE) analysis of 16S rRNA genes and construction of clone libraries to reveal dominant microbial populations. Cultivation efforts included enrichment and isolation of various thermophilic physiological groups including endospore-forming heterotrophs, fermentative bacteria, iron and sulfate reducers, methanotrophs, and methanogens.

Materials and methods

Site description and physicochemical analyses of spring water

The Arzakan geothermal spring is located at 40° 27' 36.10" N, 44° 36' 17.76" E, at 1490 m above sea level, with a temperature of ~44 °C, pH 7.0–7.2, and a conductivity of 4378.3 μS sm^{-1} (Fig. 1). Sampling was performed in January 2009. The hot spring belongs to the hydrocarbonate sodium class of mineral springs and possesses a high concentration of dissolved minerals and gases (of which >20% is HCO_3^- and >20% is Na^+) [3]. Analyses of major and minor elements in the water revealed the following composition (in ppm): Na, 1183; Ca, 153; K, 108; Si, 47; Mg, 29; B, 15; Sr, 2.3; As, 1.6; Li, 1.3; Mn, 0.12; Fe, 0.72; Ba, 0.09; Cl^-, 297; SO_4^{2-}, 200. Nitrate was not detected (<2 ppm). For trace elements, the following concentrations were obtained (in ppb): Cr, 0.28; Co, 0.49; Cu, 0.82; Zn, 6.73.

Temperature, pH, and conductivity were determined *in situ* using a portable combined pH/EC/TDS temperature tester (HANNA; HI98129/HI98130). Water and sediment samples were collected from a shallow part in the outlet of the spring using sterile glass flasks and were maintained on ice for 6 h until processing. For chemical analyses, water was filtered through 0.2-μm syringe filters and stored cold in a plastic tube until transported back to the laboratory. Aliquots for analyses of major and minor elements (Na, K, Mg, Ca, Si, B, Sr, Li, Mn, Fe, Ba, As) by ionic coupled plasma optical emission spectrometry (ICP-OES; Thermo Iris) and trace elements (Cr, Co, Cu, Zn) by mass spectrometry (ICP-MS; Thermo Element 2) were acidified by addition of HNO_3 to 2% and stored in acid-cleaned high-density polyethylene (HDPE) bottles. Aliquots for analyses of anions (Cl^-, SO_4^{2-}, NO_3^-) by ion chromatography (IC; Metrohm) were stored in HDPE bottles. The samples were stored at 4 °C until analysis.

Growth experiments

For enrichment of aerobic endospore-forming thermophilic bacteria, nutrient broth (Difco) was inoculated with slurry water and sediment samples (1 g) and incubated overnight at 50, 60, and 65 °C with shaking at 240 rpm. Before inoculation, all samples were incubated at 80 °C for 10 min so that only spore-forming microorganisms were isolated [9]. Cultures were further purified by streaking samples on the same medium supplemented with agar (2% w/v). All colonies obtained

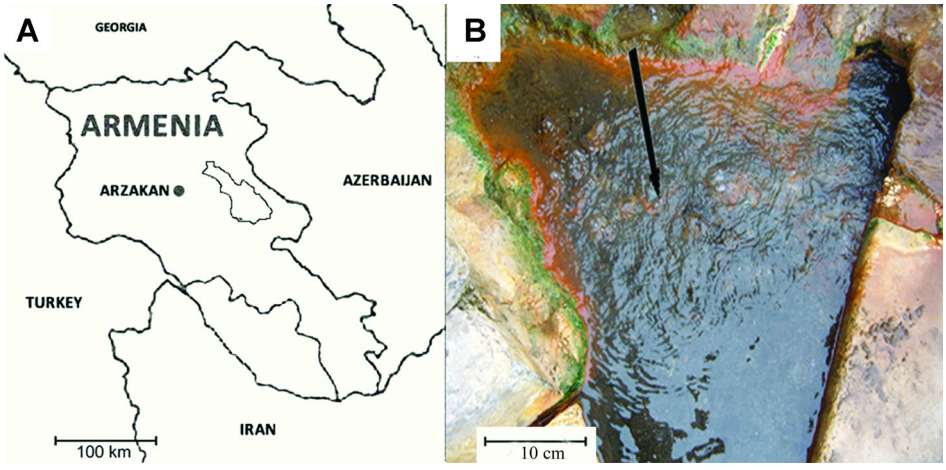

Figure 1. Location of study site. (A) Map of Armenia showing the location of Arzakan. (B) Close-up photograph of the source pool of the Arzakan hot spring with the sampling site indicated by an arrow.

on plates were selected 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, sporulation ability, and motility were determined by phase-contrast microscopy (Nikon; Eclipse E400 microscope) of freshly prepared wet mounts. All isolates were tested for Gram reaction, thermophilic growth, and catalase activity [9].

For enrichment of aerobic methane-oxidizing bacteria, a mineral medium was used containing the following (in $g\,L^{-1}$): KNO_3, 1; $MgSO_4 \cdot 7H_2O$, 1; $CaCl_2 \cdot 2H_2O$, 0.2. To this medium, $1\,ml\,L^{-1}$ of a stock solution was added containing the following trace elements (in $g\,L^{-1}$): $ZnSO_4 \cdot 7H_2O$, 0.4; disodium EDTA, 0.25; $MnCl_2 \cdot 2H_2O$, 0.02; H_3BO_3, 0.015; $Na_2MoO_4 \cdot 2H_2O$, 0.04; $NiCl_2 \cdot 6H_2O$, 0.01; $CuSO_4 \cdot 5H_2O$, 0.2; $CoCl_2 \cdot 6H_2O$, 0.05, and $1\,ml\,L^{-1}$ of iron stock solution (FeNaEDTA, $4.5\,g\,L^{-1}$). After autoclaving, $20\,ml\,L^{-1}$ of a phosphate stock solution containing $37.4\,g\,L^{-1}$ KH_2PO_4 and $49\,g\,L^{-1}$ $Na_2HPO_4 \cdot 2H_2O$ was added to the medium. Finally, the pH was adjusted to 6.7 with HCl or NaOH. Twenty milliliters of medium was aseptically transferred to a 100-ml sterile serum flask and inoculated with 1 ml of the sample. The flask was closed with a butyl rubber cap and an aluminum crimp seal. A mixture of methane and air was added aseptically through a syringe to achieve 80 and 20% concentration of methane and air, respectively, in the headspace. The flask was placed on a rotary shaker at 45 °C and 125 rpm in the dark, and the gas mixture was replaced once per week.

Enrichment of anaerobic microorganisms was performed using an anaerobically prepared basal medium containing the following components (in $g\,L^{-1}$ distilled water): NaCl, 1; $MgCl_2 \cdot 6H_2O$, 0.9; $MgSO_4 \cdot 7H_2O$, 1.4; KCl, 0.33; NH_4Cl, 0.25; $CaCl_2 \cdot 2H_2O$, 0.14; KH_2PO_4, 0.45 [10]. To this medium, $1\,ml\,L^{-1}$ of stock solution was added, containing the following trace elements: $10\,ml\,L^{-1}$ 25% HCl and (in $g\,L^{-1}$) $FeCl_2 \cdot 4H_2O$, 1.5; $CoCl_2 \cdot 6H_2O$, 0.19; $MnCl_2 \cdot 4H_2O$, 0.1; $ZnCl_2$, 0.07; $Na_2MoO_4 \cdot 4H_2O$, 0.036; $NiCl_2 \cdot 6H_2O$, 0.024; H_3BO_3, 0.006; $CuCl_2 \cdot 2H_2O$, 0.002, and 0.5 mL of a 0.02% resazurin stock solution [10]. After autoclaving in a dispenser, the medium was reduced with 4 ml 0.5 M Na_2S under a nitrogen atmosphere. Ten milliliters vitamin solution [11] was added and the pH was adjusted to 6.8 with 1 M NaOH or 1 M HCl. Twenty milliliters of medium was transferred into aliquots in 100-ml serum bottles, which were subsequently sealed with butyl rubber stoppers. Substrates were added from separate anaerobically prepared stock solutions. The medium to enrich anaerobic fermentative bacteria contained yeast extract (0.1% w/v), peptone (0.25% w/v), and maltodextrin (0.25% w/v). The same basal medium supplemented with $4\,g\,L^{-1}$ of $NaHCO_3$ was used for enrichment of Fe(III)- and sulfate-reducing bacteria and methanogens. Lactate (20 mM) and sodium acetate (0.5% w/v) were added as substrates for the enrichment of heterotrophic sulfate- and Fe(III)-reducing microorganisms. The head space contained N_2 and CO_2 (80:20 v/v). The medium for enrichment of Fe(III)-reducing microorganisms was supplemented with amorphic Fe(III) oxide (90 mmol of Fe(III) L^{-1}), which was prepared by neutralizing a solution of $FeCl_3$ with 10% w/v NaOH. To enrich chemolithotrophic (H_2-oxidizing) sulfate- and Fe(III)-reducing microorganisms and methanogens, the gas phase contained H_2 and CO_2 (80:20 v/v). Enrichments were incubated at 37 and 50 °C.

DNA extraction and polymerase chain reaction (PCR)

Cells were harvested by filtration of 1.5 L of a mixture of water and sandy sediment through 0.2-μm pore-size polycarbonate membrane filters. DNA was extracted directly from the filters carrying cells and sandy sediment using enzymatic digestion [12] and a sodium dodecyl sulfate (SDS) lysis procedure modified from the protocol of Dempster *et al.* [13]. The samples were suspended and incubated at 65 °C for 30 min in 9.5 ml TE buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA) containing 10 μl RNAase ($10\,mg\,ml^{-1}$) prior to addition of 0.5 ml 10% SDS and 50 μl proteinase K ($20\,mg\,ml^{-1}$) and incubation at 37 °C for 1 h. Subsequently, 0.1 times the volume of 3 M sodium acetate (pH 4.6) and cetyltrimethylammonium-bromide (CTAB) extraction buffer (10% CTAB in 0.7 M NaCl) were added, and the mixture was incubated at 65 °C for 20 min. DNA was extracted from the suspension with an equal volume of chloroform:isoamyl alcohol (24:1 v/v). To the aqueous phase, an equal volume of isopropanol was added. The tube was inverted a few times and centrifuged. The DNA pellets were washed twice with 70% ethanol, air-dried, and resuspended in TE buffer.

From enrichments, DNA was extracted using the SoilMaster™ DNA Extraction Kit (Epicentre) according to the manufacturer's recommendations. For the extraction of genomic DNA from aerobic endospore-forming isolates, a GenElute™ Bacterial Genomic DNA Kit (Sigma) was used according to the manufacturer's recommendations.

Extracted DNA was used as a template for amplification of 16S rRNA genes by PCR. The reaction mixtures contained 1 μl DNA (20–100 ng), 5 μl 10 × PCR buffer, 5 μl 10 mM dNTP (dATP, dGTP, dCTP, and dTTP each at the concentration of 10 mM), 1 μl of each primer ($25\,pmol\,\mu l^{-1}$), 2 μl $MgCl_2$ (25 mM), 0.2 μl *Taq* DNA polymerase (1.0 U; Invitrogen), 0.1% bovine serum albumin, and sterile water to a final volume of 50 μl. PCR amplification was performed using a DNA Engine thermocycler (BIO

Table 1. Target, position, specificity, and sequences of oligonucleotide primers used for PCR.

Target	Position[a]	Oligonucleotide primers sequences (5′–3′)
Bacterial 16S rRNA gene [14]	27F	GAGTTTGATCCTGGCTCA
	1525R	GAAAGGAGGAGATCCAGCC
Bacterial 16S rRNA gene (DGGE) [15]	8-28F	**GC**[b]-AGAGTTTGATCCTGGCT CAG
	518R	ATTACCGCGGCTGCTGG
Bacterial 16S rRNA gene (DGGE) [16]	341F	**GC**[b]-CCTACGGGAGGCAGCAG
	518R	ATTACCGCGGCTGCTGG
Archaeal 16S rRNA gene [17]	21F	TTCCGGTTGATCCYGCCGGA
	958R	YCCGGCGTTGAMTCCAATT

[a]Corresponding to 16S rRNA gene sequence position in *Escherichia coli*.
[b]GC-clamp: CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG.

RAD). The templates were first denatured for 3 min at 96 °C, followed by 30 cycles (35 cycles for archaeal DNA) of the following steps: denaturation for 30 s at 96 °C, annealing for 30 s at 55 °C, and extension for 2.5 min at 72 °C. The 30 cycles were followed by a final 10 min extension at 72 °C. Universal archaeal and bacterial 16S rRNA gene oligonucleotide primer sets, as well as primers for amplification of ~200 and ~500 bp fragments of the bacterial 16S rRNA gene were used (Table 1). PCR products were viewed under UV light after standard gel electrophoresis and ethidium bromide staining.

Clone library construction

PCR products obtained from environmental DNA or DNA extracted from enrichments were purified with the GenElute™ PCR Clean-up Kit (Sigma) and cloned with the TOPO TA cloning kit version O, using chemical transformation according to the manufacturer's instructions (Invitrogen). Plasmid DNA from selected clones was purified using the GenElute™ Plasmid Mini-Prep Kit (Sigma) according to the manufacturer's recommendations.

DGGE

A PCR–DGGE fingerprinting method was applied to obtain information about the occurrence of the dominant bacterial populations. DGGE–PCR conditions were carried out as described by Muyzer *et al.* [18]. Bacterial DNA extracted directly from environmental samples or enrichments were used as templates for PCR. The PCR products were analyzed by electrophoresis through a 0.75-mm thick vertical denaturing gel made using stock solutions A (8% acrylamide in 0.5 × Tris–acetate–EDTA buffer (TAE)) and B (8% acrylamide, 7 M urea, and 40% formamide in 0.5 × TAE buffer). The denaturing gradient ranged from 15 to 65% (where 100% denaturant corresponds to stock solution B). Electrophoresis was performed at 60 °C, first for 10 min at 10 V and subsequently for 16 h at 70 V in a tank containing 5 L 0.5 × TAE buffer. After electrophoresis, the gel was soaked for 1 h with 10 ml Sybr Gold (Molecular Probes) solution (1 μl of 10,000 × Sybr Gold in 10 ml 1 × TAE buffer), rinsed for 10 min in water, and photographed on a UV transilluminator. Bands were excised and the small blocks of acrylamide were placed into 20 μl sterile distilled water to allow passive diffusion of DNA at 4 °C overnight. A suitable volume (1–5 μl) of the eluate was used as template DNA in a reamplification PCR using the same primers and reaction conditions as before. Before being sequenced, the reamplified PCR products were purified with the GenElute™ PCR Clean-up Kit (Sigma) as specified by the manufacturer.

Sequencing and phylogenetic analysis

Sequencing was performed on an ABI PRISM capillary sequencer according to the protocol of the ABI Prism BigDye Terminator kit (Perkin Elmer). Archaeal and bacterial library clones were sequenced with primers 21F and 27F, respectively. Reamplified DGGE–PCR products were directly sequenced using the same primers used for amplification. Sequences were screened for chimeras using the Mallard version 1.02 package (http://www.bioinformatics-toolkit.org/index.html) chimera check tool. Nonchimeric sequences were grouped into operational taxonomic units (OTUs) comprising sequences with more than 98% similarity. A nucleotide BLAST search was performed in order to obtain information on the phylogenetically closest relative (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/Blast) [19]. The assembled 16S rRNA gene sequences were 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 5.1. The alignments were initially performed with the ClustalW option in MegAlign and were manually adjusted. Phylogenetic reconstructions were produced using the neighbor-joining tree-building

algorithm. Confidence in branching points was determined by bootstrap analysis (1000 replicates) [20].

Accession numbers

The 16S rRNA gene sequences reported here have been deposited in GenBank under accession numbers JQ929010–JQ929040 and JX456536–JX456538. Reference sequences used are noted in the tree (Figs. 3 and 4).

Results

Direct microscopy

Direct microscopic observations of water and sediment samples using a phase-contrast microscope revealed several morphotypes of microorganisms. Sheathed and unsheathed rods seemed to be dominant, but coccoid, spirochete-like, and filamentous cells were also frequently observed.

Bacterial clone library and DGGE analysis

A bacterial 16S rRNA gene library was constructed using DNA extracted directly from the samples as a template for PCR. A total of 30 clones were initially sequenced in the forward direction (700–900 bp). Among these, seven sequences were found to be chimeric and were excluded from further analysis. Sequence analysis of the remaining 23 clones indicated that they originated from a wide range of genera, which clustered among Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and Epsilonproteobacteria (a total of 12 clones), Cyanobacteria (eight clones), Firmicutes (two clones), and Bacteroidetes (one clone). BLAST results of the closest relatives of the clones are shown in Table 2. A total of 12 OTUs were identified. One representative of each OTU was selected for complete sequencing.

The high presence of microbes from the proteobacteria lineage (12 of 23 clones) indicated a large diversity of Gram-negative microorganisms. Two clones of

Table 2. Blast results of closest relatives of clones obtained from the bacterial 16S rRNA genes.

Phylogenetic affiliation	Sequence representing OTU accession no.	No. of clones in OTU	Closest match accession no.	Closest species accession no.	% Similarity to closest match (closest species)
Alphaproteobacteria	Arz-B-38	1	Uncultured *Rhodobacteraceae* bacterium	*Rubribacterium polymorphum*	99 (95)
	JQ929034		FJ516790	EU857676	
	Arz-B-27	1	Uncultured bacterium clone	*Azospirillum palatum*	94 (93)
	JQ929030		EF507986	EU747318	
Betaproteobacteria	Arz-B-8	1	*Azoarcus buckelii*	*Azoarcus buckelii*	97
	JQ929037		NR027190	NR027190	
	Arz-B-35	1	Uncultured Betaproteobacteria	*Rubrivivax gelatinosus*	98 (96)
	JQ929033		JN825468	JX049412	
	Arz-B-13	2	Uncultured bacterium clone	*Rhodoferax antarcticus*	98 (97)
	JQ929027		AF407413	GU233447	
	Arz-B-28	1	Uncultured Betaproteobacteria	*Methylobacillus glycogenes*	94 (90)
	JQ929031		DQ837269	FR733701	
Gammaproteobacteria	Arz-B-2	2	Uncultured Gammaproteobacteria	*Lysobacter taiwanensis*	96 (95)
	JQ929032		AJ534675	DQ314555	
	Arz-B-25	1	*Methylocaldum* sp.	*Methylocaldum gracile*	99 (98)
	JQ929029		EU275146	NR026063	
Epsilonproteobacteria	Arz-B-43	2	Uncultured Epsilonproteobacteria	*Arcobacter suis*	99 (99)
	JQ929035		AF246706	FJ573216	
Bacteroidetes	Arz-B-45	1	Uncultured bacterium	*Balneola alkaliphila*	91 (87)
	JQ929036		AJ318137	EU008564	
Firmicutes	Arz-B-22	2	Uncultured bacterium	*Fusibacter paucivorans*	98 (95)
	JQ929028		AB294297	NR024886	
Cyanobacteria	Arz-B-11	8	*Spirulina subsalsa*	*Spirulina subsalsa*	98
	JQ929026		AY575935	AY575935	

Alphaproteobacteria had closest matches with uncultured bacteria. One clone sequence was closely related (95% similarity) to *Rubribacterium polymorphum*, while another was closely related (93% similarity) to *Azospirillum palatum*. The clone sequences affiliated with Betaproteobacteria were predominant within the proteobacteria lineage and resulted in 90–97% matches to cultivated species. Clone Arz-B-28 was particularly divergent, as it shared only 90% similarity with *Methylobacillus glycogenes*. One of the two Gammaproteobacteria clones showed a 95% match to *Lysobacter taiwanensis* isolated from a similar habitat, and the other corresponded to the methanotrophic genus, *Methylocaldum*. The two Epsilonproteobacteria clones showed a strong affiliation with the genus *Arcobacter*.

One clone sequence showed 91% similarity to uncultured Bacteroidetes and had a maximum match of only 87% with *Balneola alkaliphila* as its closest cultivated relative.

Eight clones were affiliated with Cyanobacteria, sharing 98% similarity with *Spirulina subsalsa*.

Gram-positive microorganisms were less diverse, with only one OTU with an affiliation to uncultured members of the phylum Firmicutes and with a closest cultivated match (95%) to the genus *Fusibacter*.

The relative abundance of the phylogenetic groups is summarized in Fig. 2. Representatives of the bacterial phyla Alphaproteobacteria (8%), Betaproteobacteria (22%), Gammaproteobacteria (13%), Epsilonproteobacteria (9%), Firmicutes (9%), and Bacteroidetes (48%), and Cyanobacteria (35%) were found. The Proteobacteria was the dominant group, representing 52% of the total bacterial clone library.

Heterotrophic microorganisms detected in the bacterial library were clustered among the fermentative microorganisms. Although most of the retrieved sequences were similar to uncultured Bacteria, some of them were phylogenetically associated with environmental clones obtained from similar habitats. The Arzakan spring appears to harbor unique microbial communities as a large fraction of sequences have $\leq$97% similarity to their closest relatives and, thus, likely represent novel genera or species.

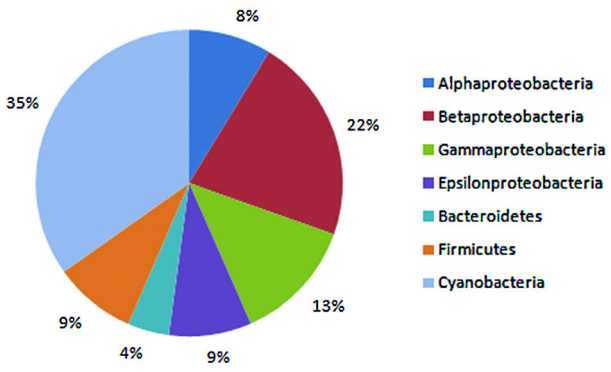

Figure 2. Pie chart showing the phylum-level distribution of bacterial phylotypes in the Arzakan hot spring.

The DGGE analysis demonstrated that the dominant bacterial populations are related to Proteobacteria, Bacteroidetes, and Cyanobacteria, which is in good agreement with the clone library results. Both cloning and DNA-based PCR–DGGE analysis of the bacterial communities confirmed the high presence of representatives of the phylum Proteobacteria and the genus *Spirulina* (Supporting Information Fig. S1, Table S1).

Growth experiments

Samples were also used as inocula in attempts to enrich fermentative bacteria, methanotrophs, and iron and sulfate reducers. Enrichment attempts to cultivate sulfate- and Fe^{3+}-reducing bacteria under chemolithotrophic and heterotrophic conditions were not successful. Enrichments in anaerobically prepared media, containing a mixture of yeast extract, dextrin, and peptone, yielded appreciable growth at 45–50 °C after 2 days. A bacterial strain designated as Arz-ANA-2 was successfully isolated after several dilutions to extinction. Analysis of the amplified 16S rRNA gene revealed a 99% phylogenic relationship to *Arcobacter* sp. (HM584709), an Epsilonproteobacteria. The 16S rRNA gene sequence of the isolate was submitted to the GenBank database under accession number JQ929025.

As one methanotrophic phylotype was detected by using molecular methods, a methanotrophic enrichment medium was also prepared. After inoculation with spring samples, microbial growth was observed by changes in turbidity within 3 weeks, and bacterial growth was confirmed by microscopy. After subculturing in fresh medium several times, only a single morphotype, designated as Arz-AM-1, was observed. The 16S rRNA gene sequences identified the isolate as *Methylocaldum* sp. (96% similarity). The 16S rRNA gene sequences of the methanotrophic isolate were submitted to the GenBank database under accession number JQ929024.

A total of 14 aerobic thermophilic bacilli strains (ArzA-2 to ArzA-11, ArzA-13, ArzA-13A, ArzA-33, and ArzA-33A) were isolated from nutrient broth plates incubated at 50, 60, and 65 °C. All strains were Gram-positive, endospore-forming, and catalase-positive bacteria belonging to Firmicutes. Seven strains with a growth range of 37–70 °C (optimum 55 °C) were considered as thermophiles. Most of the isolates were able to grow in the pH

Table 3. BLAST results of 16S rRNA gene sequences of aerobic endospore-forming isolates and accession numbers.

Isolates (temperature of isolation, *T* °C)	Closest match Taxonomic affilation, phylotype accession no.	% Similarity to closest match	Accession no.
ArzA-2 (50)	*B. simplex* AY833099	99	JQ929014
ArzA-3 (65)	*G. caldoxylosilyticus* FJ823099	96	JQ929017
ArzA-4 (50)	*B. licheniformis* FJ435674	99	JQ929018
ArzA-5 (50)	*Paenibacillus* sp. DQ497239	97	JQ929019
ArzA-6 (65)	*G. thermodenitrificans* FJ823098	98	JQ929020
ArzA-7 (65)	*Geobacillus* sp. EU093964	99	JQ929021
ArzA-8 (65)	*G. toebii* GQ487459	97	JQ929022
ArzA-9 (60)	Firmicutes bacterium EU810844	98	JQ929023
ArzA-10 (60)	*B. simplex* GU048877	98	JQ929010
ArzA-11 (60)	*G. stearothermophilus* AY608948	99	JQ929011
ArzA-13 (50)	*Sporosarcina* sp. DQ227775	98	JQ929013
ArzA-13A (50)	*B. simplex* AY833099	96	JQ929012
ArzA-33 (65)	*G. toebii* AB116120	99	JQ929016
ArzA-33a (65)	*G. toebii* AB116120	99	JQ929015

range 6.5–8.5 and 0–5% NaCl. 16S rRNA gene sequence analyses revealed affiliations with the families Bacillaceae, Paenibacillaceae, and Planococcaceae (Table 3). *Geobacillus* (seven isolates) and *Bacillus* (five isolates) were the most predominant recovered genera.

A hydrogenotrophic methanogen isolate designated as Arz-ArchMG-1 was successfully obtained having a 16S rRNA gene sequence with a similarity of 97% to *Methanoculleus* sp. The 16S rRNA gene sequence of this isolate was submitted to the GenBank database under accession number JQ929040.

Archaeal clone library

An attempt to construct an archaeal 16S rRNA gene clone library using environmental DNA as a template was not successful because a PCR product using archaeal primers could not be obtained. A library was, however, generated from a methanogenic enrichment culture. A total of 10 clones were sequenced. Among these, three sequences were found to be chimeric and were excluded from further analyses. A total of two OTUs were identified from the remaining seven clones. Four phylotypes were moderately (96%) related to *Methanoculleus* sp., while three sequences were most closely related (97%) to uncultured Archaea (Table 4). Sequences were only moderately related to database entries, and could, therefore, represent new archaeal taxa.

Phylogenetic trees

Neighbor-joining evolutionary distance trees for all retrieved bacterial and archaeal 16S rRNA gene sequences were constructed (Figs. 3 and 4). Representatives from seven different bacterial phyla were identified. The branch lengths from the nodes separating the OTUs from their closest cultivated species indicate, in many cases, novel genera or species. This is particularly true for the *Balneola*-like sequence, Arz-B-11, and the betaproteobacterial sequence, Arz-B-28, which are both widely separated from their closest relatives, and might represent novel genera belonging to Bacteroidetes and Betaproteobacteria, respectively. As the rest of the clone library sequences also form quite distinct lineages that are well-separated from their closest relatives, they thus, possibly represent novel species. The 16S rRNA gene sequence of the cultivated endospore formers are, however, more similar to previously isolated organisms. The archaeal OTUs Arz-Arch-1 and 2 branch from within the *Methanoculleus* genus but are well-separated from

Table 4. Blast results of closest relatives of clones obtained from the archaeal 16S rRNA gene library.

Sequence representing OTU, accession no.	No. of clones in OTU	Closest match accession no.	Closest species accession no.	% Similarity to closest match (closest species)
Arz-Arch-1 JQ929038	4	*Methanoculleus* sp. LH2 DQ987521	*Methanoculleus thermophilus* AJ862839	96 (96)
Arz-Arch-2 JQ929039	3	Uncultured archaeon AB288249	*Methanoculleus marisnigri* NR074174	97 (94)

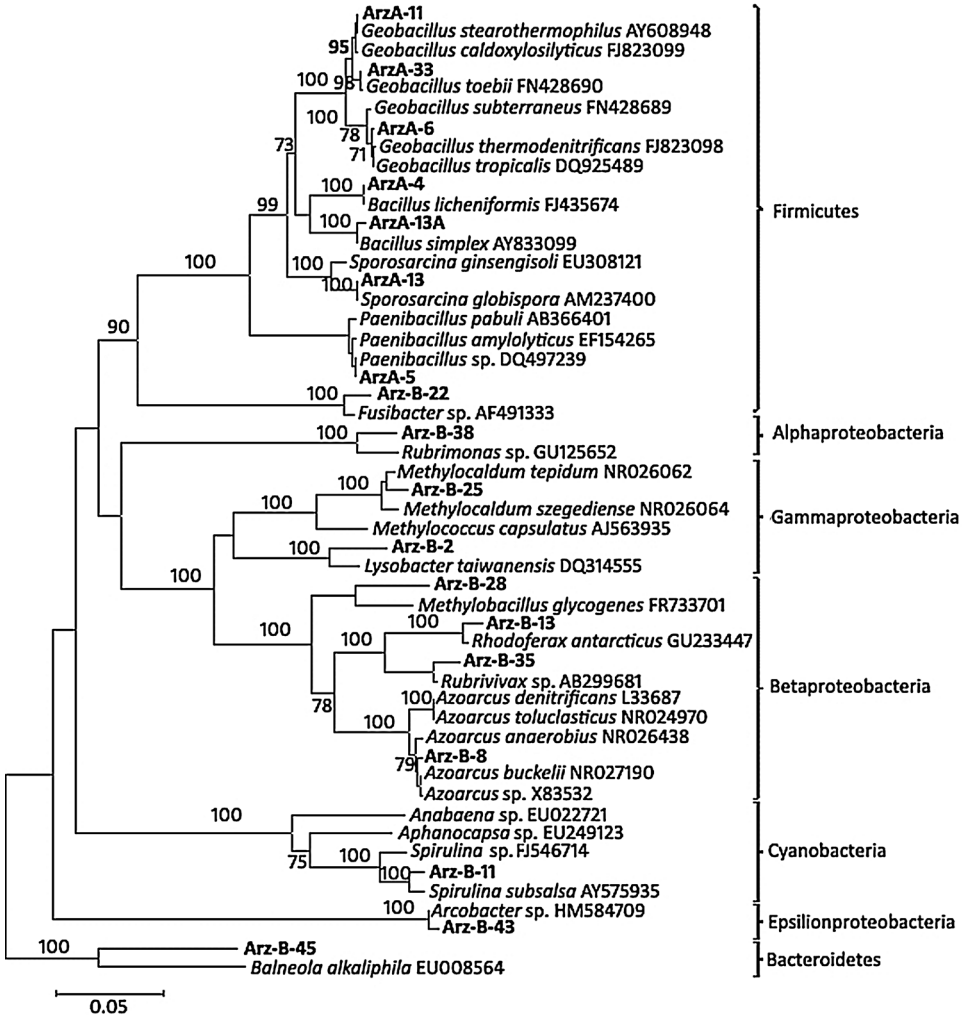

Figure 3. Neighbor-joining evolutionary distance phylogenetic tree based on bacterial 16S rRNA gene sequences. The positions of the bacterial clones (Arz-B) and bacilli isolates (ArzA) obtained from the Arzakan hot spring are indicated in bold. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more than 85% bootstrap support. Bar indicates 0.05 substitutions per nucleotide position.

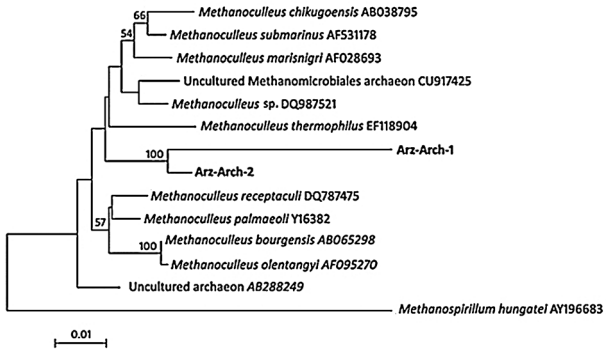

Figure 4. Neighbor-joining evolutionary distance phylogenetic tree based on archaeal 16S rRNA gene sequences obtained from methanogenic enrichment and representative members of the genus *Methanoculleus*. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more than 50% bootstrap support. Bar indicates 0.01 substitutions per nucleotide position.

other *Methanoculleus* spp. (Fig. 4) and clearly represent novel methanogenic species.

Discussion

The objective of this study was to assess the microbial diversity of a terrestrial hot spring in Armenia. Cultivation-dependent and molecular techniques were combined to analyze both bacterial and archaeal populations.

Our results revealed that the studied hot spring harbors unique microbial assemblages, as most of the detected microorganisms appear to represent novel species. Most of the detected bacteria seem to be mesophilic or moderately thermophilic. A comparison of the optimum growth temperature of the closest

cultivated relatives to the microorganisms detected in the clone libraries suggested that most of the detected microorganisms, including microorganisms representing some of the most dominant groups, are likely able to grow at reservoir temperature and, therefore, should not be regarded as contaminants. The bacterial clone library is composed of 16S rRNA gene sequences from taxa that are not described in the literature as being associated with geothermal environments. The explanation for this observation may be the presence of contamination from surrounding soils. Another possible explanation may be that microorganisms with a significant level of 16S rRNA gene sequence similarity may have distinct physiological properties, especially when features other than the temperature (e.g., alkalinity and salinity) create a microenvironment suitable for colonization.

The majority of the microorganisms detected in the gene libraries were most closely related to uncultivated microorganisms that are only detectable by using molecular methods. Some of the microorganisms are phylogenetically associated with environmental clones obtained from terrestrial thermal springs. The relatively low similarity (<97%) between most of the sequences in the bacterial 16S rRNA gene library and PCR–DGGE fingerprinting results indicates that the hot spring harbors a unique microbial community.

The molecular phylogenetic approach applied in this study has several potential biases (e.g., preferential PCR amplification, different susceptibility to the cell lysis procedures, contamination by non-indigenous strains), which need to be acknowledged during data interpretation. In addition, the number of clones is not large enough to fully represent the microbial community. However, although the results do not provide a complete quantitative picture of microbial diversity, they do provide a reliable first estimate of the microbial community structure.

The phylogenetic analysis of Bacteria identified the dominant phylotypes as members of Proteobacteria. The phylogeny for Proteobacteria revealed considerable diversity. Despite some ambiguities related to the lack of similar sequences in GenBank, the phylotypes shared a close affiliation to those obtained from other thermal environments. While it is not possible to predict their metabolism from environmental sequences alone, the closest phylogenetic affiliations were found in aerobic and anaerobic heterotrophs and methanotrophs (within the Proteobacteria lineage).

The primary production of the Arzakan geothermal system can be supported by a complex microbial community composed of chemolithotrophs and phototrophs. Two sequences related to an *Arcobacter* sp., a sulfide-oxidizing Epsilonproteobacteria, were recovered, which presents a clear example of a close connection between the geosphere and biosphere. The primary production of the ecosystem can be supported by methanotrophs, belonging to the phylum Gammaproteobacteria, as a group of chemoorgano-autotrophic bacteria. Methanotrophs mainly belonging to the genera *Methylocaldum* were detected by both culture-dependent and -independent methods.

There is an obvious light effect on the bacterial composition as the sample was collected from a shallow part in the outlet of the spring. Amongst the Cyanobacteria, eight clone sequences were related to *Spirulina subsalsa*. Cyanobacteria are the most commonly reported microbial group in these types of environments and are considered to be the major primary producers in these habitats [21]. Representatives of Rhodobacteraceae (purple non-sulfur anoxygenic phototrophs) were found to share these environments with the cyanobacteria.

A number of studies have described the microbial communities in the near neutral (pH ~7) and saline geothermal waters using cultivation and culture-independent techniques in different parts of the world [1]. These results indicate that microbial diversity is relatively low under extreme physicochemical conditions, suggesting that strong selective forces limit the diversity of the microbial communities in such environments, with the majority of the clones being closely related to photoautotrophic Cyanobacteria and heterotrophic Alphaproteobacteria.

The DGGE patterns also showed that the majority of the sequences were related to representatives of Gammaproteobacteria, Bacteroidetes, and Cyanobacteria. The DGGE profile was in good agreement with the clone library results, indicating that Proteobacteria is the most abundant bacterial phylum. Both cloning and PCR–DGGE analysis confirmed large amounts of *Spirulina* and Bacteroidetes species. Although most of the retrieved sequences are most similar to uncultured bacteria, some of them are phylogenetically related to environmental clones obtained from other geothermal springs.

It is known that only a small fraction of microorganisms occurring in a natural habitat are revealed using culture-dependent techniques. Efforts to isolate Bacteria and Archaea from thermal ecosystems have often been unsuccessful due to the difficulties in reproducing the complex geochemical composition of their environments. This method alone results in a limited knowledge of the actual microbial diversity [22]. It was shown previously that the microbial diversity

depicted by DGGE analysis is lower than the true diversity, as this technique detects only dominant populations constituting more than 1% of a complex microbial community [23]. We can thus assume that a certain fraction of the microbial populations in our study site remained undetected. In our cultivation experiments, several thermophilic and thermoresistant spore-forming strains were isolated and identified as *Bacillus*, *Geobacillus*, *Paenibacillus*, and *Sporosarcina* spp., which were not detected by the molecular analyses. Probably endospores were not opened by the applied DNA extraction method. A total of 50% of the isolates recovered from the hot spring samples are most closely related to members of the *Geobacillus* genus, which are known to thrive in similar habitats [24, 25]. Representatives of *Geobacillus toebii* were the most abundant obligate thermophiles. Having large metabolic potential, thermophilic, aerobic, endospore-forming bacteria represent the major components of bacterial saprotrophic complexes and are involved in the aerobic degradation of carbohydrates [24]. The growth of these heterotrophs can be supported by both autochthonous and allochthonous organic matter [25].

An attempt to construct an archaeal 16S rRNA gene clone library using environmental DNA as a template was not successful. The reason for this difficulty may be inhibition effects of minerals from the sediments on DNA isolation or on the PCR amplification reaction. Dominance of bacterial DNA in the total environmental DNA preparations likely also inhibited amplification of archaeal 16S rRNA genes.

Conversely, a clone library was generated from a methanogenic enrichment culture. Ten sequences in the archaeal library from methanogenic enrichments were close relatives of members of the genus *Methanoculleus*, which includes hydrogenotrophic methanogens. Hydrogenotrophic methanogens are, by virtue of their specific physiological capacities, pioneer microorganisms that inhabit anoxic locations on Earth where only geochemical H_2 and CO_2 are available as primary substrates [26].

The detection of methanogens is in agreement with the conditions of the spring, where electron acceptors such as O_2, NO_3^-, Fe^{3+}, and SO_4^{2-} are limiting [3, 11]. Furthermore, sulfate- and iron-reducing microorganisms were not recovered, indicating a methanogenic type of anaerobic terminal respiration process. This is in agreement with the absence of anaerobically respiring bacteria in the libraries.

The Arzakan spring seems to be an extreme environment for life forms in terms of its thermal and unique chemical properties. Besides the thermal conditions, the limiting factor for microbial diversity and biomass may be a combination of abiotic factors including pH, dissolved gases (H_2, CO_2, H_2S, CH_4), and high mineralization [27]. The geothermal systems of Armenia are known to contain a high concentration of minerals [3], and thus, the mineralogy may also have a strong influence on the community composition. Recent studies have also highlighted that other factors, such as biogeography and geological history, can be important in determining the thermophilic diversity of geothermal springs [28, 29].

This characterization study only represents a "snapshot" in time and the microbial diversity may change, for example, with seasonal changes or rainfall. A recent study of the microbial diversity of the Arzakan hot spring based on tagged pyrosequencing and clone library construction using environmental DNA yielded similar results for the bacterial community composition, although sequences related to *Methanoculleus* were not obtained [30]. The latter discrepancy can be explained by a cultivation bias, as our analysis of the archaeal diversity, indicating a dominance of *Methanoculleus* spp., was based on DNA extracted from methanogenic enrichment.

Overall, this study provides valuable information regarding the diversity of microorganisms that inhabit the Arzakan geothermal spring.

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Conflict of interest statement

The authors declare no financial or commercial conflicts of interest.

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