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ORIGINAL PAPER

An integrated study reveals diverse methanogens, Thaumarchaeota, and yet-uncultivated archaeal lineages in Armenian hot springs

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Abstract Culture-independent and enrichment techniques, with an emphasis on members of the Archaea, were used to determine the composition and structure of microbial communities inhabiting microbial mats in the source pools of two geothermal springs near the towns of Arzakan and Jermuk in Armenia. Amplification of small-subunit rRNA genes using "universal" primers followed by pyrosequencing (pyrotags) revealed highly diverse microbial communities in both springs, with >99 % of pyrosequences corresponding to members of the domain Bacteria. The spring in Arzakan was colonized by a photosynthetic mat dominated by Cyanobacteria, in addition to Proteobacteria, Bacteroidetes, Chloroflexi, Spirochaeta and a diversity of other Bacteria. The spring in Jermuk was colonized by phylotypes related to sulfur, iron, and hydrogen chemolithotrophs in the Betaproteobacteria and Epsilonproteobacteria, along with a diversity of other Bacteria. Analysis of near full-length small subunit rRNA genes amplified using Archaea-specific primers showed that both springs are inhabited by a diversity of methanogens, including *Methanomicrobiales* and *Methanosarcinales* and relatives of *Methanomassiliicoccus luminyensis*, close relatives of the ammonia-oxidizing archaeon (AOA) "*Candidatus* Nitrososphaera gargensis", and the yet-uncultivated Miscellaneous Crenarchaeotal Group and Deep Hydrothermal Vent Crenarchaeota group 1. Methanogenic enrichments confirmed the predicted physiological diversity, revealing methylotrophic, acetoclastic, and hydrogenotrophic methanogenesis at 45 and 55 °C, but not 65 °C. This is one of only a few studies combining cultivation-independent and -dependent approaches to study archaea in moderate-temperature (37–73 °C) terrestrial geothermal environments and suggests important roles for methanogenic archaea and AOA in the carbon and nitrogen biogeochemical cycles in these environments.

Keywords Thermophiles · Methanogens · Ammonia-oxidizing archaea · Miscellaneous Crenarchaeotal Group · Armenia

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Introduction

The importance of archaea in extreme environments is widely acknowledged and the contribution of marine and soil archaea to global biogeochemical cycles is increasingly evident (Hatzenpichler 2012; Knittel and Boetius 2009). However, many major lineages of archaea have eluded cultivation and our knowledge of the phylogenetic and functional diversity of archaea is far from complete (Schleper et al. 2005). Terrestrial geothermal springs host diverse and often abundant populations of archaea. Studies have documented increases in the relative abundance of archaea at extremes of low pH and high temperature within terrestrial geothermal systems (Hou et al. 2013) or with increasing temperature along temperature gradients within individual circumneutral springs (Cole et al. 2013).

Moderate-temperature geothermal systems (herein defined as a thermal system (Neuendorf et al. 2005) cool enough to permit phototrophy at the source (Brock 1967), operationally 37–73 °C) with neutral or alkaline pH are often colonized by visible microbial growth that forms laminated mats or streamers dominated by Cyanobacteria or Chloroflexi (Miller et al. 2009; Klatt et al. 2011). Archaea are greatly outnumbered by bacteria in these habitats and as a result, microbial ecology studies of photosynthetic mats in geothermal areas focus overwhelmingly on the latter. However, a few studies have shown that archaea perform essential functions in these ecosystems, particularly methanogenesis (Zeikus et al. 1980) and chemolithotrophic ammonia oxidation (Hatzenpichler et al. 2008).

Studies in Yellowstone National Park have documented methanogenesis in photosynthetic mat communities, although methanogens are outnumbered by several orders of magnitude by bacteria (Zeikus et al. 1980). Methanogens also dominate planktonic communities of some thermal aquifers where they are proposed to be primary producers by using geogenic H_2 as an electron donor for primary production (Chapelle et al. 2002; Stevens and McKinley 1995). Interestingly, although marine methanogens have the highest known growth temperature of any organism on Earth, 122 °C (Takai et al. 2008), methanogenesis in terrestrial geothermal systems has not been measured above 72 °C (Zeikus et al. 1980) and methanogens are not generally detected in cultivation-independent studies above that temperature (Hou et al. 2013; Spear et al. 2005).

A comparatively recent discovery is ammonia-oxidizing archaea (AOA) in terrestrial geothermal environments (de la Torre et al. 2008; Hatzenpichler et al. 2008). AOA are widely distributed in terrestrial geothermal systems (Zhang et al. 2008). Cultivation studies have documented growth of AOA up to 74 °C (de la Torre et al. 2008) and in situ activity measurements and quantitative studies of AOA populations have revealed active and abundant AOA up to ~81 °C (Cole et al. 2013; Dodsworth et al. 2011). Studies focusing on the distribution and relative abundance of the isoprenoid glycerol dialkyl glycerol tetraether (iGDGT) crenarchaeol, a possible biomarker for AOA, suggest AOA are most abundant relative to other archaea at 45–50 °C (Zhang et al. 2006).

In this study we performed a comprehensive microbial community census in two spring sources in Armenia, followed by a more detailed study focusing on archaea. The towns of Arzakan and Jermuk mark the two regions of highest heat flow and steepest geothermal gradient in Armenia along the Eastern Volcanic Belt (Badalyan 2000). Correspondingly, thermal features in Arzakan and Jermuk reach 44 and 64 °C, respectively, temperatures higher than any other regions in Armenia (Mkrtchyan 1969). Our study of springs in Arzakan and Jermuk revealed diverse microbial communities, with abundant Cyanobacteria in the spring in Arzakan and putative chemolithotrophs in the spring in Jermuk. A focused census of archaea revealed diverse methanogens, close relatives of "*Candidatus* Nitrososphaera gargensis", and the yet-uncultivated Miscellaneous Crenarchaeotal Group (MCG) and Deep Hydrothermal Vent Crenarchaeota group 1 (DHVC1). Finally, microbial enrichments demonstrated methylotrophic, acetoclastic, and hydrogenotrophic methanogenesis at 45 and 55 °C, but not 65 °C. This study further supports the important roles of methanogenic archaea and AOA in moderate-temperature terrestrial geothermal environments and is, to our knowledge, one of the first studies of the microbiology of the abundant geothermal systems in the Minor Caucasus.

Materials and methods

Study sites, sample collection, and DNA extraction

Water temperature, pH, and conductivity were measured in situ during the sampling using a portable

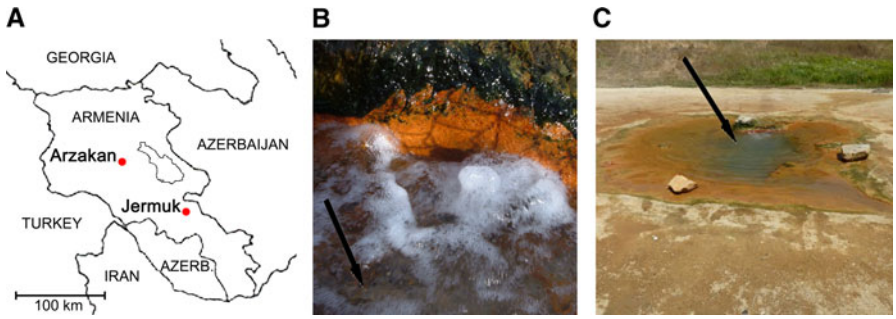

Fig. 1 Location of study sites. **a** Map of Armenia showing locations of Arkakan and Jermuk. **b** Close up photograph of the source pool of Arzakan hot spring with the sampling site shown (*arrow*). Vigorous degassing and cyanobacterial mats are visible. **c** Photo of the source pool of Jermuk hot spring with the sampling site shown (*arrow*)

combined pH/EC/TDS/Temperature tester (HANNA HI98129/HI98130). The spring in Arzakan is located in central Armenia at 40°26.902′N, 44°36.508′E, 1,490 m above sea level. The spring is circumneutral (pH 7.20) and has a relatively high dissolved mineral content (4378.3 µS/cm) and is classified as a HCO_3^-/Na^+-type spring (Fig. 1a, b; Mkrtchyan 1969). The mat–water interface (top ~1 cm) of an orange pigmented microbial mat was aseptically sampled within the 44 °C, vigorously degassing source pool of in early spring of 2012.

The spring in Jermuk is located in southeast Armenia within the Vayots Dzor region at 39°50.479′N, 45°40.067′E (Fig. 1a, c; Mkrtchyan 1969). The spring is circumneutral (pH 7.50), classified as a HCO_3^-/SO_4^{2-}/Na^+-type spring, and has a relatively high dissolved mineral content of 4,340 µS/cm (Mkrtchyan 1969). A sample of the sediment–water interface (top ~1 cm) within the 53 °C source pool was aseptically collected in early spring 2012.

Both samples were placed on ice during transport to the laboratory in Yerevan and DNA was extracted within 12 h of collection using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH) as described previously (Costa et al. 2009) and stored at −80 °C.

Pyrosequencing and pyrotag analysis

To obtain censuses of organisms in the samples, the V4–V8 variable regions of the small subunit rRNA gene were PCR amplified with a version of the "universal" forward primer 515F modified to improve coverage of archaea (5′-GTGYCAGCMGCCGCGGTAA-3′; Hou et al. 2013) in combination with the "universal" reverse primer 1391R (5′-GACGGGCG GTGWGTRCA-3′; Lane 1991) at Research and Testing Laboratory (Lubbock, TX). Pyrosequencing of the V4 region was carried out from the 515F-end of the amplicons with a GS FLX sequencer (454 Life Sciences, USA). Processing of the pyrosequences was performed with mothur v 1.28 (Schloss et al. 2009). Sff files were trimmed and denoised per the Schloss SOP for Titanium-generated data. Reads with homopolymers of greater than eight nucleotides or a length of fewer than 200 nucleotides were excluded from further processing. The mothur-provided bacterial and archaeal Silva (Pruesse et al. 2007) reference files were concatenated into a single file against which quality-filtered sequences were aligned using default Needleman–Wunsch algorithm parameters. The alignment was manually curated using BioEdit v7.0.5.3 (Hall 1999). OTUs at 97, 95, 92, 85, and 80 % minimum identity levels were identified and the most abundant representative sequence within each OTU was inspected for chimeras with chimera.uchime (Edgar et al. 2011). The sequences served as their own reference and potential chimeras were omitted from further processing. Each OTU was queried against the Silva database using the Wang (Bayesian) method (Wang et al. 2007) and assigned the most detailed taxonomy that matched ≥90 % of the sequences within a given OTU, with a confidence cutoff of 50 %. The datasets were used to calculate OTUs observed, Chao1, Simpson's Evenness, and Shannon diversity at each OTU identity level measured.

PCR, clone library construction, Sanger sequencing, and Sanger sequence processing

Near-complete archaeal small subunit rRNA genes were amplified by PCR using primers 8aF (5′-YCYG

GTTGATCCTGCC-3′) and 1512uR (5′-ACGGHTACCTTGTTACGACTT-3′; Eder et al. 1999). PCR mixtures (25 μL total volume) contained 1 μL of extracted DNA as template, 1× Taq reaction buffer, 200 nM of each primer, 200 μM each dNTP, and 0.65 U of GoTaq DNA polymerase (Promega, Madison, WI). Cycling conditions were: denaturation at 96 °C for 4 min followed by 35 cycles of denaturation (30 s at 94 °C), primer annealing (30 s at 55 °C), and elongation (1.5 min at 72 °C), with a final elongation step (10 min at 72 °C). PCR products were ligated into a TA TOPO cloning vector (Invitrogen, Carlsbad, CA) and sequenced using the M13 forward primer at Functional Biosciences (Madison, WI) using a Big Dye V3.1 chemistry on an ABI 3730xl sequencer (Applied Biosystems, Foster City, CA). Quality-trimmed, non-chimeric sequences were imported into mothur and aligned for OTU clustering at 97 % using the nearest neighbor algorithm. A representative of each OTU was sequenced with the M13 reverse primer and assembled into contigs in BioEdit. Contigs were imported into mothur and alpha diversity statistics were calculated as described above for pyrotags.

Phylogenetic analysis

Alignments were made by the Needleman–Wunsch algorithm against concatenated Silva reference files using mothur as described above for pyrotags. Alignments were curated manually using BioEdit and phylogenetic trees were constructed with Phylip (Felsenstein 1989) using three different methods: maximum likelihood (dnaml) with global rearrangements; distance matrix and neighbor-joining (F84 correction; dnadist and neighbor); and maximum parsimony (dnapars) with a heuristic search. Resulting trees were visualized using Dendroscope v2.7.4 (Huson et al. 2007).

Methanogenic enrichments and quantification of methane

Methanogen enrichment medium was based on Medium 1 (Whitman et al. 2006) and contained per liter: 5 g $NaHCO_3$, 1 g NH_4Cl, 1 g $MgCl_2$ hexahydrate, 0.4 g $CaCl_2$ dihydrate, 0.4 g K_2HPO_4 trihydrate, 1 mg resazurin, 2 g yeast extract, 2 g trypticase peptones, 0.5 g cysteine hydrochloride, 0.5 g Na_2S nonahydrate, 10 mL vitamin solution, 10 mL trace element solution. Vitamin and trace element solutions were prepared as described (Whitman et al. 1986). Medium 1 was prepared anaerobically as described (Whitman et al. 2006) in 30-mL serum vials with 20 mL of headspace (200 kPa total pressure of 80:20 N_2:CO_2). Initial enrichments additionally contained one of three substrates: H_2 (replacing N_2 in headspace); 10 mM sodium acetate; or 50 mM methanol. Approximately 0.1 g of hot spring sediment was added in an anaerobic chamber (headspace of 5 % H_2, 5 % CO_2, 90 % N_2; Coy Laboratory Products, Grass Lake, MI, USA) as inoculum. Cultures were incubated in the dark at 45, 55, and 65 °C for up to 7 days. In cultures where methane was detected in the headspace (see below), 0.1 mL was transferred to new medium. After two rounds of initial enrichment, identical medium without yeast extract and peptone was used. Methane in 1-mL samples of culture headspace was quantified using a GC-2014 (Shimadzu) equipped with a flame ionization detector. An 80/100 mesh Porapak N column (Supelco) with nitrogen as a carrier gas (30 ml/min) was used with injector, column and detector temperatures of 100, 55 and 150 °C, respectively.

SFF files containing the original unfiltered pyrosequences were submitted to the NCBI Sequence Read Archive (Arzakan universal, SRR747863; Jermuk universal, SRR747864) and associated with NCBI BioProject SRP018743. Near full-length 16S rRNA gene sequences were submitted to Genbank under accession numbers KC682067–KC682083 (Arzakan) and KC682084–KC682097 (Jermuk).

Results and discussion

Small subunit rRNA gene pyrotags

Analysis of small subunit rRNA gene pyrotags revealed diverse communities dominated by Bacteria in both springs (Table 1; Fig. 2; Tables S1, S2). In total, 321 and 208 species-level OTUs representing 64 and 38 phylum-level groups were detected in Arzakan and Jermuk, respectively (Table 1). In both springs, relatively few of the total pyrotags could be assigned to known genera (16 % at Arzakan and 51 % at Jermuk; Tables S1, S2), underscoring the novelty of these two ecosystems and the need for continued efforts to cultivate and describe microorganisms in geothermal systems. The Chao1 richness estimator suggested

Table 1 Alpha diversity calculations at OTU percent definitions of 80, 85, 92, 95, and 97 percent, approximating the phylum, class/order, family, genus, and species, respectively

Spring and data set	Arzakan total (pyrotags)[a]	Jermuk total (pyrotags)[a]	Arzakan archaea (clone lib.)[b]	Jermuk archaea (clone lib.)[b]
Total # sequences	1685	1631	57	49
No. of OTUs observed				
80 %	64	38	5	5
85 %	124	77	7	7
92 %	229	141	9	7
95 %	280	175	11	8
97 %	321	209	13	9
Chao1				
80 %	73	44	6	5
85 %	205	144	10	8
92 %	405	200	12	8
95 %	561	303	22	10
97 %	756	406	26	11
Simpson's evenness				
80 %	0.089	0.149	0.421	0.412
85 %	0.106	0.095	0.338	0.461
92 %	0.070	0.078	0.306	0.525
95 %	0.061	0.067	0.269	0.525
97 %	0.066	0.057	0.252	0.524
Shannon diversity				
80 %	2.316	2.138	0.940	1.099
85 %	3.245	2.586	1.126	1.470
92 %	3.840	3.172	1.354	1.470
95 %	4.041	3.366	1.527	1.503
97 %	4.269	3.454	1.594	1.536

[a] Obtained from pyrotags using universal primers 515F/1391uR

[b] Obtained from clone libraries (clone lib.) constructed using archaeal specific primers 8aF/1512uR

species richness of 756 and 406 species in Arzakan and Jermuk, respectively, and showed that the pyrotag sequencing effort was incomplete (Table 1). Evenness was low at all taxonomic levels (Table 1).

Several OTUs in Arzakan were confidently assigned to Cyanobacteria, which is consistent with the gross morphology of the mat at the spring source (Fig. 1b) and suggests a dominant role for photosynthetic primary production. The most abundant Cyanobacteria OTUs were confidently assigned to the genera *Spirulina*, *Stanieria*, *Leptolyngbia*, and *Rivularia/Calditrix* (Table 2). Other major groups included Betaproteobacteria, Spirochaeta, Bacteroidetes, and Chloroflexi; however, the most abundant OTUs in these groups could not be assigned taxonomy below the family level.

The most abundant phyla represented in the pyrotag dataset from Jermuk were the Proteobacteria, Bacteroidetes, and Synergistetes. Several abundant Proteobacteria OTUs were related to obligate or facultative chemolithoautotrophs capable of using sulfur compounds, Fe^{2+}, and/or H_2 as electron donors, including the genera *Thiobacillus*, *Sulfuricurvum*, *Siderooxydans*, and *Hydrogenophaga*, suggesting the importance of chemolithotrophy in primary productivity (Kämpfer et al. 2005; Kellermann and Griebler 2009; Kodama and Watanabe 2004; Liu et al. 2012). The gross morphology of the mat was consistent with iron precipitation at the spring source as ferrous iron supplied from the subsurface is oxidized as the spring water becomes oxygenated. The Bacteroidetes were diverse and many could not be assigned to known genera. An exception was an abundant OTU assigned to the genus *Lutibacter*, which contains chemoorganotrophs most commonly found in marine environments (Lee et al. 2006). Other Bacteroidetes and the

Synergistetes in Jermuk are likely involved in heterotrophic processing of mat exudates and biomass.

Archaeal sequences were present at low abundance in both pyrotag datasets, with six archaeal pyrotags in three OTUs in Arzakan and nine pyrotags in six OTUs in Jermuk. The *Methanosarcinales* were represented in both pyrotag datasets, with *Methanomethylovorans* detected in Jermuk; *Methanosaeta* and a sequence that could not be classified below the order level were detected in Arzakan (Table S1). In addition, the yet-uncultivated group MCG was detected in both springs and the yet-uncultivated group DHVC1 was detected in Jermuk (Table S1).

Nearly-complete 16S rRNA genes of Archaea

To gain more insight into the archaea in these springs, pyrotag analysis was augmented with analysis of near-full length archaeal small subunit rRNA gene sequences. The clone library analysis uncovered a low richness and diversity of archaea in both springs, although the archaeal communities were substantially more even than the aggregate communities (Table 1). Although some archaea phylotypes were novel, a relatively high percentage of the total archaeal sequences were confidently assigned to known genera (68 % at Arzakan and 45 % at Jermuk; Table 3). All sequences obtained in these archaeal 16S rRNA gene libraries contained the consensus binding site for primer 515F and all but one (Arz_04H_P1, mismatch at 6th position for the 3′ end) contained the consensus binding site for primer 1391R, the oligonucleotides utilized for generating the pyrotags described above. The low abundance in pyrotag datasets of archaeal sequences identical or similar to those obtained in the clone libraries is therefore apparently not due to bias in PCR amplification because of primer mismatches, but may reflect a low overall abundance of Archaea in these hot spring samples.

Analysis of the near full-length sequences revealed considerable overlap between the two springs and broad agreement with the few archaeal pyrotags that were recovered. Both springs contained close relatives of methanogenic archaea, AOA, and yet-uncultivated phylogenetic groups (Table 3; Fig. 3). Close relatives of *Methanospirillum hungatei*, in the order *Methanomicrobiales*, were inferred to be abundant in both springs. In addition, two phylotypes in Arzakan (Arz_02H_P1, Arz_01A_P12) were related to the genus *Methanoregula*, also in the *Methanomicrobiales*. Members of both genera use H_2/CO_2 and/or formate as methanogenic substrates; however, their presence in geothermal systems was somewhat surprising since they are not reported to grow above 37 °C (Liu and Whitman 2008).

The other order of methanogens present in both springs was *Methanosarcinales*, represented by *Methanosaeta* and *Methanomethylovorans*. *Methanosaeta* was abundant in Jermuk (Jer_11H_P2) and includes obligate acetoclastic species known to grow up to 60 °C (Liu and Whitman 2008). *Methanomethylovorans* species use a variety of substrates, including methanol, dimethylsulfide (DMS), methanethiol (MT), and methylamines, and are proposed to be important DMS and MT consumers in nature (Jiang et al. 2005; Lomans et al. 1999). *Methanomethylovorans thermophila* is capable of growth up to 58 °C (Jiang et al. 2005).

A third order-level group in the Euryarchaeota was detected in both springs, but was particularly abundant in Jermuk. The most abundant OTU in this clade (Jer_02H_P1) was very distant from the closest cultivated relative. However, it was found to be part of a larger clade that included other Armenian hot spring phylotypes and "*Methanomassiliicoccus luminyensis*", which was recently isolated from human feces. "*M. luminyensis*" requires both H_2 and methanol for methanogenic growth, a rare trait among known methanogens (Dridi et al. 2012).

Both springs also contained a low inferred abundance of close relatives of the AOA "*Candidatus* Nitrososphaera gargensis". "*Ca.* Nitrososphaera gargensis" and related Thaumarchaeota are known to inhabit soil (Tourna et al. 2011) and moderate temperature geothermal habitats (Hatzenpichler et al. 2008). They likely play a role in the oxidation of ammonia supplied either by the spring source water or deamination of proteins during decay of mat biomass, and/or the oxidation of ammonia groups following the removal of ammonia groups from simple organic molecules such as urea (Alonso-Sáez et al. 2012). The resulting nitrite may be oxidized in situ by the nitrite-oxidizing bacteria (NOB) *Nitrospira calida* and *Nitrospira moscoviensis,* since these thermophilic NOB have recently been identified in enrichments from Jermuk (Edwards et al. 2013).

Both springs also hosted archaeal phylotypes that belong to lineages with no cultivated relatives, as

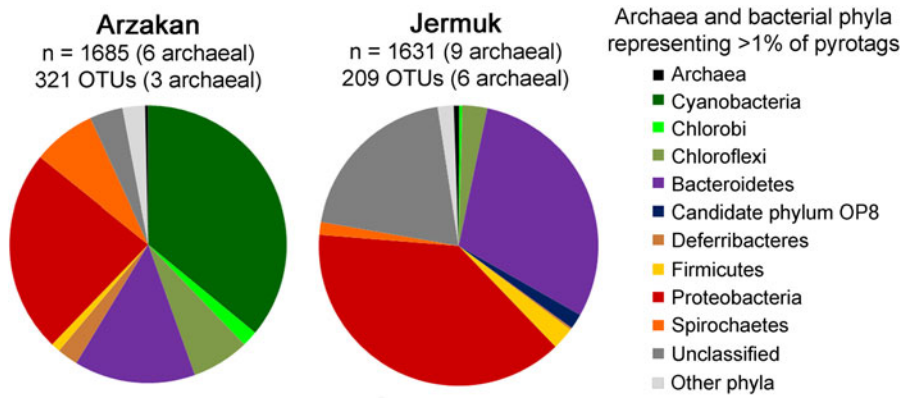

Fig. 2 Relative abundance of phylogenetic groups in "universal" pyrotag datasets. The number (*n*) of quality-filtered pyrotags is indicated. OTUs were defined at the 97 % identity level and phylum-level taxonomic assignments were given based comparison to Silva database using the Wang (Bayesian) method with a 50 % confidence cut-off; those below the cut-off were binned as "Unclassified"

Table 2 Dominant OTUs in pyrotag datasets (>2 % of total)

% of total	Closest cultured relative	% id	Phylum
Arzakan			
17.5	*Spirulina subsalsa*	86	Cyanobacteria
7.95	*Stanieria cyanosphaera*	100	Cyanobacteria
5.10	*Spirochaeta zuelzerae*	87	Spirochaetes
4.69	*Leptolyngbya* sp. CCMEE6132	100	Cyanobacteria
2.67	*Leptolyngbya* sp. CCMEE6132	98	Cyanobacteria
2.31	*Rivularia* and *Calothrix* spp.	87	Cyanobacteria
2.43	*Deferribacter desulfuricans*	91	Deferribacteres
2.79	*Parabacteroidetes distasonis*	84	Bacteroidetes
Jermuk			
17.7	*Aminomonas paucivorans*	90	Synergistetes[a]
17.2	*Croceimarina litoralis*	98	Bacteroidetes
12.1	*Thiobacillus thioparus*	99	Proteobacteria
6.19	*Sulfuricurvum kujiense*	98	Proteobacteria
4.54	*Sideroxydans lithotrophicus*	99	Proteobacteria
3.99	*Prolixibacter bellariivorans*	92	Bacteroidetes

[a] This OTU representative was classified as "Synergistetes" with only 39 % confidence by mothur, and thus is grouped as "unclassified" in Fig. 2

suggested by the pyrotag analysis. Several phylotypes from both springs belonged to the MCG. The MCG is a diverse and ubiquitous lineage that is extremely abundant in low-energy, deep marine sediments (Kubo et al. 2012). Although a role for MCG archaea in methanotrophy has been suggested based on isotopic data (Biddle et al. 2006), more recent studies suggest a role in heterotrophy, possibly the fermentation of refractory carbon (Kubo et al. 2012). A second yet-uncultivated lineage detected in Arzakan was DHVC1, which has been found in diverse environments such as freshwater sediments (Kato et al. 2012), hydrothermal vent sediments (Takai and Horikoshi 1999), and hypersaline mats (Robertson et al. 2009).

Methanogenic enrichments

Methanogenic enrichments were set up in order to definitively test some of the physiological functions that were predicted. Enrichments in defined media produced active and stable methanogenic cultures on acetate and H_2/CO_2 in both springs. Both metabolisms were active in enrichments from Jermuk at 45 and 55 °C, but only at 45 °C in Arzakan (Table 4). Defined methylotrophic enrichments were only successful with samples from Jermuk; however, parallel methylotrophic enrichments amended with yeast extract were positive from Arzakan (data not shown), suggesting H_2-dependent methylotrophy, based on consumption

Table 3 Archaeal OTUs from Arzakan and Jermuk represented by nearly complete 16S rRNA gene sequences

Representative sequence	# in OTU[a]	Cultured microorganism whose 16S rRNA gene has the highest BLASTn hit to the OTU representative sequence			Phylogenetic affiliation of OTU[d]
		Organism name and strain[b]	Accession #	Id.[c]	
Arzakan					
Arz_11B_P2	25	*Methanospirillum hungatei* JF-1	CP000254.1	99	*Methanospirillum*
Arz_12B_P2	8	*Thermofilum pendens* Hw3	NR_029214.1	83	MCG*
Arz_09H_P1	3	*Thermofilum pendens* Hw3	NR_029214.1	82	MCG*
Arz_11H_P1	3	*Methanospirillum hungatei* JF-1	CP000254.1	99	*Methanospirillum*
Arz_11A_P2	1	*Methanomethylovorans hollandica* ZB	AY260433.1	99	*Methanomethylovorans*
Arz_01A_P2	1	*Methanoregula formicicum* SMSP	CP003167.1	96	*Methanomicrobiales*
Arz_01H_P1	1	*Methanomassiliicoccus luminyensis* B10	HQ896499.1	87	Unaffiliated
Arz_02H_P1	1	*Methanoregula formicicum* SMSP	CP003167.1	98	*Methanoregula*
Arz_04H_P1	1	*Thermofilum pendens* Hw3	NR_029214.1	82	MCG*
Arz_07B_P2	1	"*Ca*. Nitrososphaera gargensis" Ga9.2	CP002408.1	94	*Thaumarchaeota*
Arz_10H_P1	1	*Thermofilum pendens* Hw3	NR_029214.1	80	DHVC1*
Arz_12G_P1	1	*Methanothermobacter thermautotrophicus* ΔH	NR_074260.1	78	DHVC1*
Jermuk					
Jer_12D_P1	25	*Aciduliprofundum boonei* T469	NR_074217.1	80	Unaffiliated
Jer _10A_P1	12	*Methanospirillum hungatei* JF-1	CP000254.1	99	*Methanospirillum*
Jer _11H_P2	12	*Methanosaeta thermophila* PT	CP000477.1	99	*Methanosaeta*
Jer _06F_P1	2	*Methanomassiliicoccus luminyensis* B10	HQ896499.1	88	Unaffiliated
Jer _11C_P1	2	*Thermofilum pendens* Hw3	NR_029214.1	83	MCG*
Jer _04D_P1	1	"*Ca*. Nitrososphaera gargensis" Ga9.2	CP002408.1	94	*Thaumarchaeota*
Jer _07A_P1	1	*Methanomethylovorans hollandica* ZB	AY260433.1	98	*Methanomethylovorans*
Jer _09A_P1	1	*Methanomassiliicoccus luminyensis* B10	HQ896499.1	88	Unaffiliated
Jer _02C_P1	1	*Thermofilum pendens* Hw3	NR_029214.1	83	MCG*

[a] Number of sequences in the 16S rRNA gene library represented by the OTU, defined at 97 % identity level

[b] The closest relative based on BLASTn to the Nucleotide collection (nr/nt) using Megablast default parameters with Max target sequences set to 250. In cases where sequence identity to cultivated organisms was low, no cultivated relatives were identified; therefore, the search was repeated to the reference RNA sequences (refseq_rna)

[c] Percent identity to the OTU representative sequence. 100 % of the query sequence was used except where identity to closest relatives was <90 %

[d] Affiliation at the finest taxonomic level (class, order, family, or genus) based on the default RDP classification in Greengenes. Affiliations inferred instead by phylogenetic analysis (Fig. 3) are also shown (OTUs indicated with an asterisk)

Table 4 Methane production from secondary methanogenic enrichments in mineral medium

Methanogenic substrate	Methanol			Acetate			$H_2 + CO_2$		
Cultivation temperature (°C)	45	55	65	45	55	65	45	55	65
Arzakan	−	−	−	+++	−	−	+++	−	−
Jermuk	+++	++	−	+++	+	−	+++	++	−

+++, headspace methane concentration >5,000 ppm; ++, headspace methane concentration <5,000 ppm but >1,000 ppm; +, headspace methane detected but <1,000 ppm; −, no methane detected (detection limit 2 ppm)

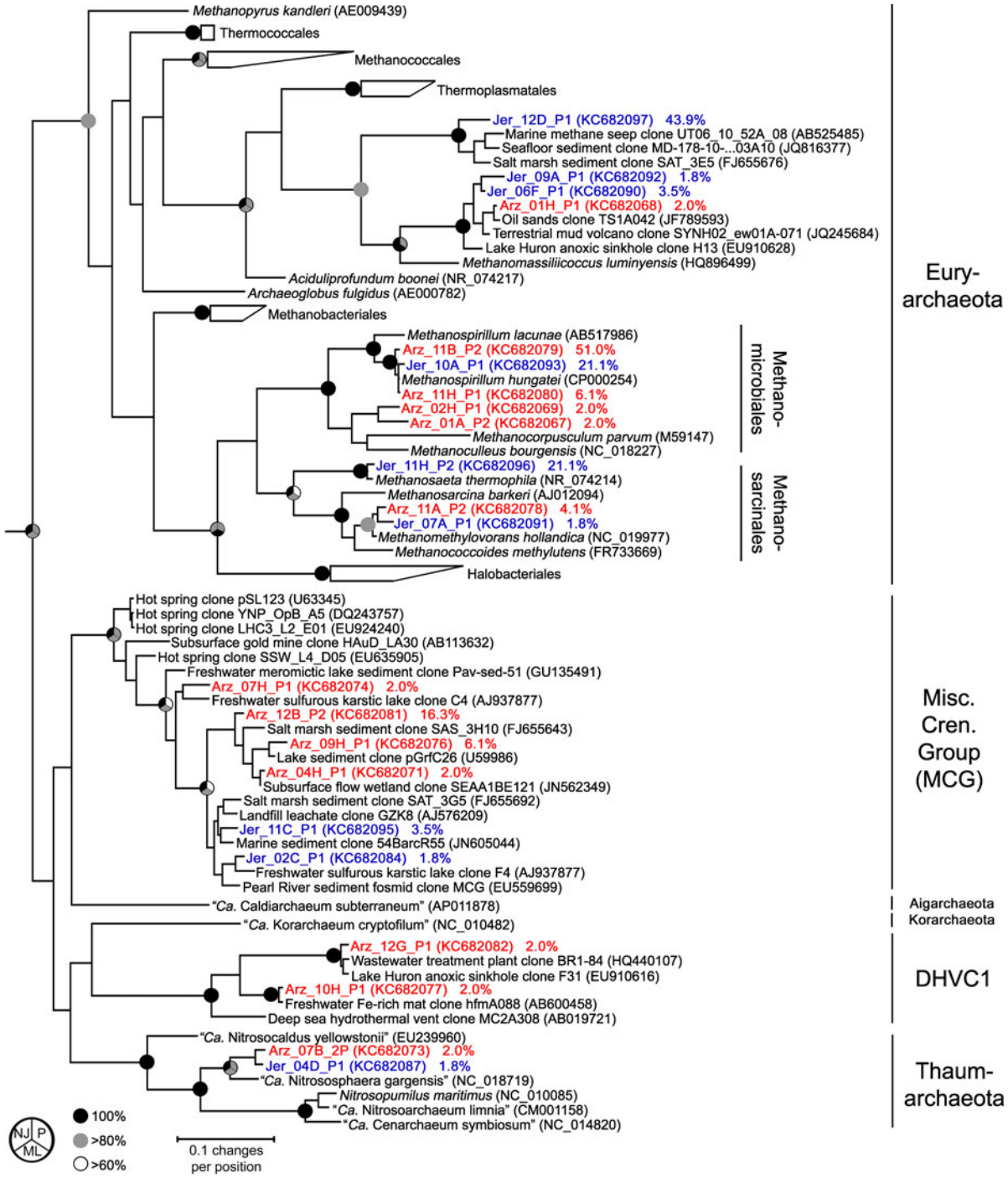

Fig. 3 Maximum-likelihood phylogeny depicting relationships between near-complete archaeal 16S rRNA genes recovered from Arzakan (*red*) and Jermuk (*blue*) and closely related sequences, including well-studied microbial isolates. Percent values for each OTU represent the percent abundance of the OTU in the clone library. Bootstrap support is indicated at major nodes for maximum-likelihood (ML; 100 replicates), parsimony (P; 1,000 replicates), and distance (neighbor-joining, NJ; 1,000 replicates) methods. Taxonomic designations for major phylogenetic groups are shown at the *right*

of H_2 from yeast extract fermentation. If so, this would be similar to the physiology recently described for "*M. luminyensis*", which is unusual among known methanogens (Dridi et al. 2012). The enrichment results are consistent with the roles predicted by the cultivation-independent census for the diverse assemblage of methanogenic Euryarchaeota and suggest a role for methanogens in processing low-energy fermentation products produced by bacteria.

Conclusions

This study is one of few published studies focusing on Archaea in moderate-temperature geothermal systems (Huang et al. 2007) and fewer on the microbiology of diverse and abundant geothermal systems in the Minor Caucasus (Edwards et al. 2013; Panosyan 2010). Both springs were dominated by Bacteria, the photoautotrophic Cyanobacteria in Arzakan and the chemolithotrophic Proteobacteria and heterotrophic Synergistetes and Bacteroidetes in Jermuk. The cause for the apparent dominance of potential chemolithotrophs, rather than phototrophs, in Jermuk is unclear. The archaeal communities described here are broadly similar to those inhabiting moderate-temperature geothermal systems in the U.S. Great Basin (Huang et al. 2007). Both are inhabited by methanogenic Euryarchaeota and close relatives of the AOA "*Ca.* Nitrososphaera gargensis", suggesting these two phylogenetic groups and their physiological roles are broadly conserved in geographically distant, moderate-temperature, circumneutral geothermal systems. These two systems also share a diversity of phylotypes in the MCG (called "Great Basin Crenarchaeota Group 1"; Huang et al. 2007), which play a yet-undefined role in these ecosystems. Both archaeal and bacterial communities described here include a high diversity and inferred abundance of yet-uncultivated microorganisms and justify continued synergy of cultivation-dependent and -independent studies to better understand the microbiology of moderate-temperature geothermal systems.

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