



Microbial Diversity of Terrestrial Geothermal Springs in Lesser Caucasus

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Abstract

The geology of the Lesser Caucasus is complex, owing to accretion of terrains through plate-tectonic processes and to ongoing tectonic activity and volcanism. Numerous geothermal springs of different geotectonic origins and with different physicochemical properties are found on the territory of the Lesser Caucasus. Despite intensive microbiological studies on terrestrial geothermal springs in various regions of the globe, very little is known about microbial diversity of similar ecosystems in the Lesser Caucasus. Recently the phylogenetic diversity of the prokaryotic community thriving in some geothermal springs located on the

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territory of Armenia, Georgia, and Nagorno-Karabakh has been explored following both cultivation-based and culture-independent approaches. Despite previous efforts, a comprehensive census of the microbial communities in the Lesser Caucasus hot springs is still lacking. This chapter contains a review of the results of microbial diversity analyses of 11 geothermal springs of the Lesser Caucasus with special emphasis to its distribution, ecological significance, and biotechnological potential.

Keywords

Lesser Caucasus · Geothermal springs · Microbial diversity · Thermophiles · Culture-dependent and culture-independent techniques

4.1 Introduction

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. These habitats have attracted broad interest since they are analogs for primitive Earth (Stan-Lotter and Fendrihan 2012). Geothermal springs offer a new source of fascinating microorganisms with unique properties well adapted to these extreme environments (Hreggvidsson et al. 2012; Deepika and Satyanarayana 2013). The adaptation to these harsh habitats makes thermophiles and their thermostable proteins suitable for various industrial and biotechnological applications (Raddadi et al. 2015; DeCastro et al. 2016).

The scientific interest in the microbial diversity of these exotic niches has increased during the last decades. With time, the tools used for microbial exploration have improved. Initially, studies were incepted with culture-based approaches. In recent time, culture-independent techniques (16S rRNA gene-based clone library analysis, denaturing gradient gel electrophoresis (DGGE), pyrosequencing, metagenomics, and metatranscriptomics) are mostly being used (Bhaya et al. 2007; Liu et al. 2011; López-López et al. 2013; DeCastro et al. 2016). This has shifted the cultivation-based narrow view into a more detailed and holistic insight of hot spring microbial habitats in terms of diversity, adaptation, functions, and ecological significance. Using a combination of several approaches of traditional microbiology with state-of-the-art molecular biology techniques has substantially increased our understanding of the structural and functional diversity of the microbial communities. Such approaches has been extensively used to study microbiota of the geothermal springs located in Iceland (Krebs et al. 2014), Azores (Sahm et al. 2013), the United States (Meyer-Dombard et al. 2005; Bowen De León et al. 2013), Bulgaria (Stefanova et al. 2015), Russia (Kublanov et al. 2009), China (Hedlund et al., 2012; Hou et al. 2013), India (Singh and Subudhi 2016; Saxena et al. 2017; Poddar and Das 2017), Malaysia (Chan et al. 2015), Argentina (Urbieta et al. 2015), Turkey (Cihan et al. 2011), Italy (Maugeri et al. 2009), Thailand (Portillo et al. 2009), New Zeland (Hetzler et al. 2007), Tunisia (Sayeh et al. 2010), Marocco (Aanniz et al.

2015) Romania (Coman et al. 2013), Spain (López-López et al. 2015) and other parts of world.

Thermal springs located in the Lesser Caucasus still represent a challenge for exploring biodiversity and searching of undescribed biotechnological resource. The geology of the region where Armenia, Georgia, and Nagorno-Karabakh are situated is complex, owing to accretion of terrains through plate-tectonic processes and to ongoing tectonic activity and volcanism (Henneberger et al. 2000; Badalyan 2000). Numerous geothermal springs with different geochemical properties are found on the territory of Lesser Caucasus. Despite a wide distribution of hot springs throughout Lesser Caucasus with hints of intrinsic scientific interest, limited attention has been paid toward microbiological analysis of these hot springs. With the best of information available, it was noted that data of microbial communities of several hot springs distributed on the territory of Armenia and Nagorno-Karabakh were published to date (Panosyan 2010; Hedlund et al. 2013; Panosyan and Birkeland 2014; Panosyan 2017; Panosyan et al. 2017). Despite these previous efforts, a comprehensive census of the microbial communities in Lesser Caucasus hot springs is still lacking.

The primary objective of this chapter is to review the findings of microbiological studies of several geothermal springs in the Lesser Caucasus and to summarize investigations on relationships between thermophilic microbial communities and geochemical conditions of their habitats. The results of this study expand the current understanding of the microbiology of hot springs in Lesser Caucasus and provide a basis for comparison with other geothermal systems around the world.

4.2 Geographical Distribution and Physiochemical Profiling of Geothermal Springs

The Caucasus Mountains include the **Greater Caucasus** in the north and **Lesser Caucasus** in the south (Stokes 2011). The **Lesser Caucasus** Mountains are formed predominantly of the **Paleogene** rocks with a smaller portion of the Jurassic and Cretaceous rocks. The formation of the Caucasus began from the **Late Triassic** to the **Late Jurassic** during the **Cimmerian orogeny** at the active margin of the **Tethys Ocean** while the uplift of the Greater Caucasus is dated to the **Miocene** during the **Alpine orogeny**. The Caucasus Mountains formed largely as the result of a **tectonic plate collision** between the **Arabian plate** moving northwards with respect to the **Eurasian plate**. This collision caused the uplift and the **Cenozoic** volcanic activity in the Lesser Caucasus Mountains. This region is regularly subjected to strong **earthquakes** from this activity (Reilinger et al. 1997). While the Greater Caucasus Mountains have a mainly folded sedimentary structure, the Lesser Caucasus Mountains are largely of **volcanic** origin (Philip et al. 1989). The geology of the region is complex, owing to accretion of exotic terranes through plate-tectonic processes and to ongoing tectonic activity and volcanism which have taken place more or less continuously since Lower Pliocene or Upper Miocene time.

The distribution of natural geothermal springs, including terrestrial hot springs (with water temperature higher than 21.1 °C), in various regions of our planet are primarily associated with tectonically active zones in areas where the Earth’s crust is relatively thin. On the territory of the Lesser Caucasus, where traces of recently active volcanic processes are still noticeable, many geothermal springs with different geotectonic origins and physicochemical properties are found (Mkrtchyan 1969, Kapanadze et al. 2010).

Although no high-temperature geothermal resources have been identified in Armenia, numerous low-temperature resource areas (cooler than 100 °C) are present. Geothermal springs distributed on the territory of Armenia have been catalogued and described, and hundreds of shallow wells have been drilled to investigate mineral water sources throughout the country (Mkrtchyan 1969).

Three main heat flow zones (northeastern, central, and southwestern) have been distinguished on the basis of heat flow and temperature gradients (Fig. 4.1). The central zone (Zone II), which coincides closely with the belt of Quaternary

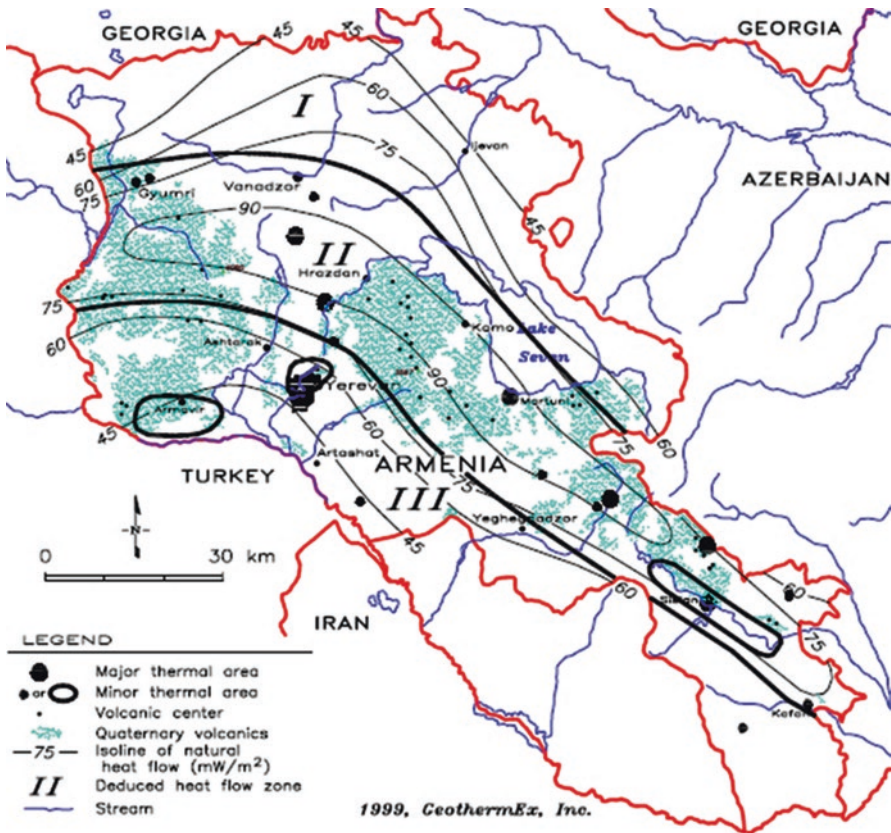


Fig. 4.1 Contour of heat flow with deduced heat flow zones in Armenia. (From Henneberger et al. 2000)

volcanoes, has highest heat flow (75 to more than 90 mW/m²) and elevated temperature gradients (generally greater than 50 °C/km). The Zone I is considered to have no significant potential for geothermal resources. In Zone III there are scattered occurrences of thermal water, despite the overall low heat flow in this region (Karakhanian et al. 1997; Henneberger et al. 2000).

Nagorno-Karabakh is located in the southeastern part of the Lesser Caucasus. It is typically mountainous, embracing the eastern part of the Karabakh Plateau with the Artsakh valley, forming the great part of the Kura-Araks lowland. The Artsakh plateau like all Armenian plateaus is characterized by seismic activity. Volcanic rocks that appeared in ancient times are gaining ground: limestone and other sedimentary rocks from the Jurassic and Cretaceous period. Numerous geothermal springs at high elevations with different physicochemical properties are found also on the territory of Nagorno-Karabakh.

Georgia is located in the central and western parts of the Trans-Caucasus and lies between the Euro-Asiatic and Afro-Arabian plates. Apart from the Precambrian and Paleozoic formations that cover a smaller area, Mesozoic and Cenozoic rock assemblages mainly make up the geological structure of Georgia (Moore and Fairbridge 1998). Three major tectonic units can be distinguished according to the geologic development of Georgia: (1) the Greater Caucasus fold system, which represents a marginal sea in the geological past, (2) the Trans-Caucasus intermountain area which marks the northern part of the Trans-Caucasus island arc, and (3) the Lesser Caucasus fold system, the southern part of the ancient Trans-Caucasus island arc. The amount of thermal flow for the main parts of Georgia can be listed as follows:

1. The south flank of Caucasus Mountains, 100 mWm²
2. Plate of Georgia:
 - (a) For the west zone 40 mWm²
 - (b) For the east zone 30mWm²
3. Adjara-Trialeti folded system:
 - (a) Central part 90 mWm²
 - (b) East zone 50 mWm²
4. Artvin-Bolnisi platform 60 mWm (Achmadova 1991)

The maximum heat flow is observed for the central zone of folded part of Georgia and the minimum for the plate, while the Adjara-Trialeti folded system is characterized by the middle range (Bunterbart et al. 2009).

Physical conditions, especially temperature, are regarded as a key factor for correlating microbial abundance and diversity of a spring (Everroad et al. 2012). Hot springs in the Lesser Caucasus could be grouped into three categories based on intrinsic temperature: warm springs (20–37 °C), moderately hot or mesothermal springs (37–50 °C), and hot springs (>50 °C). Using a cutoff temperature of 20 °C to distinguish thermal from nonthermal waters, several thermal areas are known to exist in Armenia (Mkrtchyan 1969). Hot springs at Uyts have the lowest temperature (25.8 °C). The highest temperature has been recorded for hot springs at Jermuk (>53 °C) and Karvachar (70 °C) (Fig. 4.2). The studies of some higher-temperature



Fig. 4.2 Map of the locations of microbiologically explored terrestrial geothermal springs in the Lesser Caucasus. Closeup photographs of some geothermal springs. (1) Samtredia (2) Tbilisi sulfur spring (3) Akhurik (4) Hankavan (5) Bjni (6) Arzakan (7) Jermuk (8) Tatev (9) Uyts (10) Karvachar (11) Zuar. The source of the map is <http://www.geocurrents.info/place/russia-ukraine-and-caucasus/where-is-the-caucasus>

geothermal springs (for instance, Jermuk spring, located in the Karabakh Upland along Armenia's eastern border) using various geophysical surveys indicated that temperature at deeper levels (from 600 to 1000 m) can reach up to 99 °C (Karakhanian et al. 1997; Henneberger et al. 2000).

Geothermal springs found on the territory of Nagorno-Karabakh are also mainly classified as springs with moderate temperature. Two of Nagorno-Karabakh geothermal springs located in Karvachar (≥ 70 °C) and Zuar (42 °C) are characterized with higher water temperature (Fig. 4.2).

Up to 250 natural thermal springs and artificial wells are known in Georgia with water temperature ranging between 30–108 °C (Fig. 4.3) (Kapanadze et al. 2010). The lowest water temperature geothermal springs (30–35 °C) are distributed all over the territory of Georgia but are mainly found in Borjomi, Tsikhisjvari, Tskaltubo, and Saberio areas, while the highest water temperatures (78–108 °C) have been recorded for the waters from the artificial wells and boreholes in West Georgia, such as the Zugdidi-Tsaishi, Kvaloni, and Kindgi regions (Tsertsvadze et al. 1998).

All studied Armenian and Nagorno-Karabakhian hot springs are neutral, moderately alkaline, or alkaline in nature. Most of the spring samples have neutral pH (7–7.5), but hot springs at Tatev, Ajhurik, and Uyts have pH lower than 7. The hot

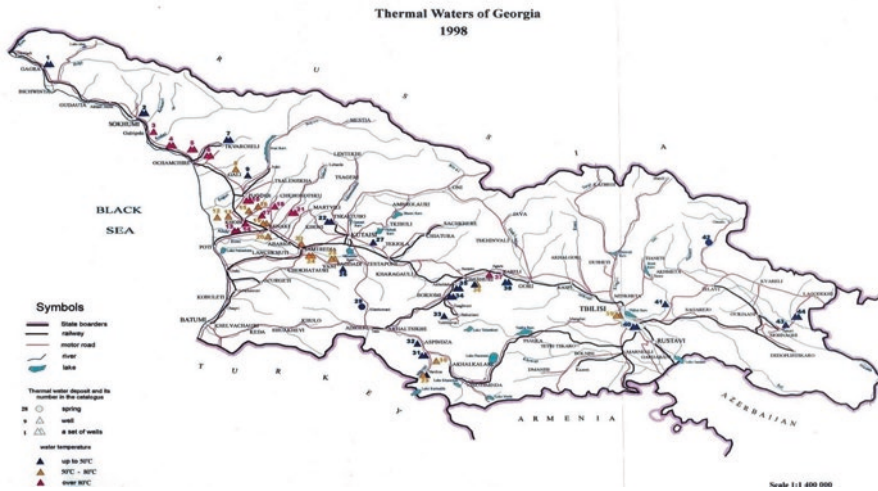


Fig. 4.3 Distribution of thermal waters in Georgia (Kapanadze et al. 2010)

springs in Georgia range from alkaline to acidic, but most of them are close to neutral or weak alkaline. The highly acidic hot spring in Georgia with pH 2.2 is located in Vani region, village Tsikhesulori, while the alkaline springs are found in Tbilisi area with pH 9.7 (Tsertsvadze et al. 1998).

Compared to physical analysis, limited attention has been paid to chemical profiling of hot spring water or sediment samples. Hot spring water usually has high concentrations of various elements owing to mineralization of dissolved solid elements from the adjacent areas. The composition of hot water is mainly determined by chemical interactions with reservoir rocks and rock-forming minerals along the ascent path, which may cause the spring water to be acidic or alkaline. All of the Armenian and Nagorno-Karabakhian thermal waters studied have mixed-cation mixed-anion compositions. Total dissolved solids contents tend to be less than about 0.5 mg/l but are occasionally higher. As is typically the case, the hotter and more saline samples tend to have higher ratios of $(Na + K)/(Ca + Mg)$ and relatively high ratios of chloride to bicarbonate (Cl/HCO_3) or sulfate to bicarbonate (SO_4/HCO_3). The cooler waters tend to be higher in $Ca + Mg$ and bicarbonate (Mkrtchyan 1969; Henneberger et al. 2000). For a few springs' major and minor elements, anions were analyzed by ionic coupled plasma optical emission spectrometry (ICP-OES; Thermo Iris), by mass spectrometry (ICP-MS; Thermo Element 2), and by ion chromatography (IC; Metrohm). Analyses of major and minor elements in the water sampled from the Arzakan geothermal spring 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; and 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; and Zn, 6.73 (Panosyan and Birkeland 2014). The Georgian hot springs are characterized by diverse chemical composition, with mineralization ranging

from 0.2 mg/L (Borjomi region) to 11.3 mg/L (Aspindza region). Similar to Armenian and Nagorno-Karabakhian region, the Georgian thermal waters also have mixed-cation and mixed-anion ratios mainly composed of hydrocarbonate, chloride, sulfate, sodium, potassium, magnesium, and calcium ions (Tsertsvadze et al. 1998). All studied springs are rich in heavy metals. Some of the springs contain gasses such as hydrogen sulfide, methane, nitrogen, and carbon dioxide (Mkrtchyan 1969; Tsertsvadze et al. 1998; Melikadze et al. 2010).

Most of the studies were focused on the hot springs at higher altitude and with high temperature. A majority of the hot springs found in the Lesser Caucasus are anthropogenically influenced and often used by tourists and local people for bath. Some of the geothermal springs are used for balneology (Mkrtchyan 1969; Melikadze et al. 2010).

The geographical locations, physicochemical profiling, and brief characteristic of main geothermal springs distributed on the territory of the Lesser Caucasus are summarized in Table 4.1.

4.3 Microbiological Analysis

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 (Amann et al. 1995; DeLong and Pace 2001; Amann and Ludwig 2000; Zhou 2003; Bhaya et al. 2007; Liu et al. 2011; López-López et al. 2013; DeCastro et al. 2016). Using a combination of several approaches of traditional microbiology with state-of-the-art molecular biology techniques has substantially increased our understanding of the structural and functional diversity of microbial communities. Both culture-based and not culture-independent approaches have been used for addressing microbial diversity associated with geothermal springs. 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 biotechnological potential (Antranikian and Egorova 2007). Here we have summarized data of the phylogenetic diversity of the prokaryotic communities thriving in some of the geothermal springs in the Lesser Caucasus based on molecular- and culture-based methods (Tables 4.2 and 4.3).

4.3.1 Cultivation-Independent Studies

Up to date, two Armenian, two Georgian, and two thermal springs from Nagorno-Karabakh region have been analyzed using cultivation-independent approaches. Studies based on sequence analysis of 16S rRNA gene clone libraries from the mixed water and sediment sampled from the Arzakan (Armenia) geothermal spring have been done recently (Panosyan and Birkeland 2014). It was the first

Table 4.1 Geographical location, physiochemical profiling, and brief characteristic of main geothermal springs distributed on the territory of the Lesser Caucasus

Thermal mineral spring	Spring GPS location	Altitude, m, above sea level	pH	Conductivity, $\mu\text{S}/\text{cm}$	Temperature of water in the outlet, T, °C	Description
<i>Armenia</i>						
Akhurik	40°44'34.04"N	1490	6.5	2490	30	Near the village of Akhurik. This geothermal spring is a result of geological drillings. Geothermal water coming from a pipe. A shallow pool with a small continuous outflow from a 2-m man-made cement-fountain landscape. In composition, it belongs to the hydrocarbonate-sulfate sodium-magnesium type of spring. Slightly degassing. Sands at bottom. Multiple thick biomats (1–5 cm) of various colors (dark brown, red, dark green, and orange) are formed on the fountain, while a dark brown and green filamentous mat is present on the bank of the collecting pool. Tourist spot, believed to have medicinal values
	43°46'53.95"E					
Arzakan	40°27'36.10"N	1490	7.2	4378.3	44	Near the village of Arzakan. This geothermal spring is a result of geological drillings. Small pool source with a high flow rate. The hot spring belongs to the hydrocarbonate sodium class of mineral springs and possesses a high concentration of dissolved minerals (of which >20% is HCO_3^- and > 20% is Na^+). Slightly degassing. Silicate sands at bottom. Biofilms with yellow, light brown, and light green colors are formed in the spring. Tourist spot, believed to have medicinal values
	44°36'17.76"E					
Bjni	40°45'94.44"N	1610	6.2–7.0	4138.3	30–37	Near the village of Bjni. This geothermal spring is a result of geological drillings. Geothermal water coming from a pipe. Small pool source with a low flow rate. In composition, it belongs to the chloride-hydrocarbonate sodium springs. Sands at bottom. Biofilms with yellow, light brown, and light green colors are formed in the spring
	44°64'86.11"E					

(continued)

Table 4.1 (continued)

Thermal mineral spring	Spring GPS location	Altitude, m, above sea level	pH	Conductivity, $\mu\text{S}/\text{cm}$	Temperature of water in the outlet, T, $^{\circ}\text{C}$	Description
Hankavan	40°63'26.50"N	1900	7.0–7.2	6722.9	42–44	Near the village of Hankavan located on the bank of Marmarik river. This geothermal spring is a result of geological drillings. Geothermal water coming from a pipe. Small pool source with a high flow rate. In composition, it belongs to the hydrocarbonate-chloride sodium springs. Vigorously degassing. Silicate sands at bottom. Biofilms with yellow, orange, light brown, and light green colors are formed in the spring tourist spot, believed to have medicinal values
	44°48'46.00"E					
Jermuk	39°96'63.90"N	2080	7.5	4340	>53	Near the town of Jermuk. Small pool with a high flow rate. In composition, it belongs to the carbon hydro-sulfate-sodium water sources. Sands at bottom. Biofilms with orange and light green are formed in the spring. Tourist spot, has medicinal value. Medicinal properties are similar to the springs in Karlovy Vary, Czech Republic
	45°68'52.80"E					
Tatev	39°23'76.00" N	960	6.0	1920	27.5	Hot spring located in Syunik region, near Satana's bridge (Satani Kamur) on the bank of Vorotan River. In composition, it belongs to the carbon-bicarbonate calcium water sources. Many bubbling sources and no visible outflow. Roughly round-shaped pool with diameter ~3 m, and depth ~0.5 m. Clays and sands at the bottom. Biofilms with light green color are formed in the spring. Source is left in its natural form; no trace of human intervention is found. However, there are traces of ancient baths, which testify to the settlements dating BC
	46°15'48.00" E					
Uyte(Uz)	39°31'00" N	1600	6.23	2700	25.8	Near the village of Uz. This geothermal spring is a result of geological drillings. Big pool (5–6 m diameter, 20 cm depth) source with a high flow rate. In composition, it belongs to the hydrocarbonate-chloride-sulfate-sodium sources. Sands at bottom. Biofilms with dark green color are formed in the spring. Tourist spot, believed to have medicinal values
	46°03'09" E					

<i>Nagorno-Karabakh</i>						
Karvachar	40°17'41.00"N	1584	7.3	4600	70	The spring is located on the bank of Tartar River, not far from Karvachar City (about 20 km). The hottest spring in Nagorno-Karabakh. Man-made round-shaped big pool (5 m diameter, 1 m depth) source with a high flow rate. Clear water and fine clays at the bottom. In composition, it belongs to the hydrocarbonate-sulfate sodium sources. Biofilms with yellow, orange, light brown, and light green colors are formed in the spring. Tourist spot, believed to have medicinal values
	46°27'50.00" E					
Zuar	40°02'47.60"N 46°14'09.30"E	1520	7.0	4300	42	The spring is located on the bank of Turon River. Round-shaped small pool (2 m diameter, 0.7 m depth) source with a high flow rate. Clays and sand at the bottom. In composition, it belongs to the hydrocarbonate-sulfate-sodium sources. Biofilms with yellow, orange, light brown, and light green colors are formed in the spring. Tourist spot, believed to have medicinal values
<i>Georgia</i>						
Samtredia borehole #1	42°10'12.04"N	24	7.15	5380	58	The spring is located in the town Samtredia, West Georgia. The spring resulted from geological drilling in 1969 to depth of 3045 m. In composition it belongs to chloride-hydrocarbonate-sulfate-calcium-sodium-potassium-magnesium sources. A yellow greenish biofilm is formed at the spring pool bottom. The water is used in greenhouses.
	42°19'44.44"E					
Tbilisi sulphur spring	41°41'18.87"N 44°48'52.30"E	405	7.8	737	37.7	The spring is located in Tbilisi and used for baths and balneology. In composition, it belongs to the sulfate-chloride-sodium type and contains hydrogen sulfide. The water has yellowish color

Table 4.2 Culture-independent studies of some geothermal spring microbiome in the Lesser Caucasus

Geothermal spring	Approach	Population proportion	Accession number	References
<i>Armenia</i>				
Arzakan	Shotgun pyrosequencing of V4 region on 454 GS FLX platform	Dominant bacterial phyla were cyanobacteria, in addition to Proteobacteria, Bacteroidetes, Chloroflexi, and Spirochaeta Dominant archaeal pyrotags which were affiliated with Euryarchaeota (<i>Methanosarcinales</i> and <i>Methanosaeata</i>) and Crenarchaeota (the yet-uncultivated group MCG)	SRR747863	Hedlund et al. (2013)
	Bacterial 16S rRNA gene library	Detected bacterial groups were Bacteroidetes (48%), Cyanobacteria (35%), Betaproteobacteria (22%), Gammaproteobacteria (13%), Epsilonproteobacteria (9%), Firmicutes (9%), and Alphaproteobacteria (8%)	JQ929026–JQ929037	Panosyan and Birkeland (2014)
	Archaeal 16S rRNA gene library	Archaeal population was presented by Euryarchaeota (methanogenic Archaea belonging to <i>Methanospirillum</i> , <i>Methanomethylivorans</i> , and <i>Methanoregula</i>), AOA Thaumarchaeota <i>Ca. Nitrososphaera gargensis</i> , and yet-uncultivated Crenarchaeota (MCG and DHVCI groups)	KC682067–KC682083	Hedlund et al. (2013)
	DGGE	Dominant bacterial populations were related to Proteobacteria (affiliated with the Beta-, Epsilon-, and Gammaproteobacteria), Bacteroidetes, and Cyanobacteria	JX456536–JX456538	Panosyan and Birkeland (2014) and Panosyan (2017)

Jermuk	Shotgun pyrosequencing of V4 region on 454 GS FLX platform Illumina HiSeq2500 paired-end sequencing	Dominant bacterial pyrotags were affiliated with Proteobacteria and Bacteroidetes, and Synergistetes-dominant archaeal pyrotags were affiliated with Euryarchaeota (<i>Methanosarcinales</i> , <i>Methanosaeata</i>) and the yet-uncultivated Crenarchaeota groups MCG and DHVC1 Dominant sequence reads were affiliated with Proteobacteria, Firmicutes, Bacteroidetes, candidate division WS6, and candidate phylum Ignavibacteria. Archaeal community (~1%) was dominated by Euryarchaeota, followed by Crenarchaeota, unclassified groups, and a minor fraction of Thaumarchaeota	SRR747864	Hedlund et al. (2013) Poghosyan (2015)
	Archaeal 16S rRNA gene library construction	Dominant archaeal populations were related to Euryarchaeota (methanogenic Archaea belonging to <i>Methanospirillum</i> , <i>Methanomethylovorans</i> , and <i>Methanosaeata</i>), AOA Thaumarchaeota <i>Ca. Nitrososphaera gargensis</i> , and yet-uncultivated Crenarchaeota (MCG group)	KC682084-KC682097	Hedlund et al. (2013)
	DGGE	Detected dominant groups were Epsilonproteobacteria, Bacteroidetes, Spirochaetes, Ignavibacteriae, and Firmicutes		Panosyan (2017)
<i>Nagorno-Karabakh</i>				
Karvachar	Bacterial 16S rRNA gene library construction	Dominant bacterial phyla were Proteobacteria (48.6%), Cyanobacteria (29.7%), Bacteroidetes (5.4%), Chloroflexi (5.4%), Verrucomicrobia (2.7%), and Planctomycetes (2.7%)	-	Saghatelyan and Panosyan (2015)
	DGGE	Detected bacterial groups were Bacteroidetes and Firmicutes	-	Panosyan (2017)
	Whole-metagenome shotgun sequencing, using Illumina HiSeq 4000 platform	Dominant sequence reads were affiliated with Actinobacteria, Alpha-, Beta-, Delta-, Epsilon-, and Gammaproteobacteria, Bacteroidetes/Chlorobi, Firmicutes, Chlamydiae, Cyanobacteria/Melainabacteria, Fusobacteria, and Synergistia	-	Unpublished data
Zuar	Bacterial 16S rRNA gene library construction	Dominant bacterial groups were Proteobacteria (42.3%), Firmicutes (19.2%), Bacteroidetes (15.4%), Cyanobacteria (3.8%), Tenericutes (3.8%), and yet-unclassified phylotypes (15.4%)	-	Saghatelyan et al. (2014)

(continued)

Table 4.2 (continued)

Geothermal spring	Approach	Population proportion	Accession number	References
<i>Georgia</i> Samtredia	Whole-metagenome shotgun sequencing, using Illumina HiSeq 2500 platform	Dominant bacterial sequence reads were affiliated with Firmicutes (33%), Gammaproteobacteria (32%), Actinobacteria (15.5%), Betaproteobacteria (9.1%), Alphaproteobacteria (2.9%), Chlamydia (1.6%), Bacteroidetes (1.5%). Archaeal sequence reads were affiliated with Crenarchaeota (1.4%) and Euryarchaeota (0.2%)	–	Unpublished data
Tbilisi sulfur spring	Whole-genome shotgun sequencing using Illumina MiSeq platform	Bacterial sequence reads were affiliated with Firmicutes (20.6%), Gammaproteobacteria (46.4%), Actinobacteria (6.4%), Betaproteobacteria (16.4%), Alphaproteobacteria (5.7%), Chlamydia (1.7%), Bacteroidetes (1.5%), Deinococcus-Thermus (0.1%), delta/epsilon subdivisions (0.9%), Acidithiobacillia (0.2%), Cyanobacteria/Melainabacteria group (0.2%), and Synergistia (0.1%)	–	Unpublished data
	DGGE	Archaeal sequence reads were affiliated with Euryarchaeota (0.2%) Detected dominant bacterial group was Betaproteobacteria (<i>Sulfurisoma</i> , <i>Thiobacillus</i> , <i>Oxalicibacterium faecigallinarum</i>) Detected archaeal group was Euryarchaeota (<i>Methanoseta harundinacea</i>)	–	Unpublished data

– data not available

Table 4.3 Summary of thermophilic bacteria isolated from geothermal springs of the Lesser Caucasus

Geothermal spring	Bacterial and Archaeal genera	Comments	References
Armenia			
Akhurik	<i>Bacillus</i> (<i>B. licheniformis</i> , <i>B. pumilus</i> , <i>B. murimartini</i> , <i>Bacillus</i> sp.), <i>Geobacillus</i> (<i>G. pallidus</i>), <i>Brevibacillus</i> (<i>B. borstelensis</i>), <i>Thermoactinomyces</i> (<i>Thermoactinomyces</i> sp.)	Used for studies of extracellular amylase, lipase, and protease. Lipase-producing <i>B. licheniformis</i> strain Akhourik 107 (accession number KY203975) consist genes encoding thermostable esterase GDLSL (family II)	Panosyan (2010), Panosyan (2017), Shahinyan et al. (2017) and Shahinyan et al. (2015)
	<i>Rhodobacter</i> (<i>R. sulfidophilus</i>), <i>Thiospirillum</i> (<i>T. jenense</i>)	Used for studies of aspartase, aminoacylase, glucose isomerase, and inulinase activities, as well as sources of protein, carbohydrates, and vitamins	Paronyan (2002a)
	<i>Methylocaldum</i> - <i>Methylococcus</i> - <i>Methylolaparacoccus</i> - <i>Methylogaea</i> <i>Methylocaldum</i> (<i>Methylocaldum</i> sp.)	<i>Methylocaldum</i> sp. strain AK-K6 (accession number KP272135) had a temperature range for growth of 8–35 °C (optimal 25–28 °C) and a pH range of 5.0–7.5 (optimal 6.4–7.0). 16S rRNA gene sequences showed that it was a new gammaproteobacterial methanotroph, which forms a separate clade in the family <i>Methylococcaceae</i>	Islam et al. (2015)
Arzakan	<i>Bacillus</i> (<i>B. licheniformis</i> , <i>B. simplex</i>), <i>Anoxybacillus</i> (<i>A. rупiensis</i>), <i>Geobacillus</i> (<i>G. toebii</i> , <i>G. thermodenitrificans</i> , <i>G. stearothermophilus</i> , <i>G. caldioxilyolyticus</i>), <i>Paenibacillus</i> (<i>Paenibacillus</i> sp.), <i>Sporosarcina</i> (<i>Sporosarcina</i> sp.), <i>Arcobacter</i> (<i>Arcobacter</i> sp.), <i>Methylocaldum</i> (<i>Methylocaldum</i> sp.)	Used for studies of extracellular amylase, lipase, and protease. The strain <i>A. rупiensis</i> Arzakan 2 produces thermostable amylases at optimal growth temperature 65 °C and pH 7. Two isolates, <i>G. thermodenitrificans</i> Arza-6 (accession number JQ929020) and <i>G. toebii</i> Arza-8 (accession number JQ929022), produce EPSs with high specific production yield (0.271 g g ⁻¹ dry cells and 0.222 g g ⁻¹ dry cells, respectively) at 65 °C and pH 7.0. Purified EPSs displayed a high molecular weight: 5 × 10 ⁵ Da for <i>G. thermodenitrificans</i> Arza-6 and 6 × 10 ⁵ Da for <i>G. toebii</i> Arza-8. Chemical composition and structure of the biopolymers, determined by GC-MS, HPAAE-PAD, and NMR showed that both EPSs are heteropolymers with mannose as major monomer unit	Hovhannisyanyan et al. (2016), Panosyan and Birkeland (2014), Panosyan (2010), Panosyan (2017) and Panosyan et al. (2014)

(continued)

Table 4.3 (continued)

Geothermal spring	Bacterial and Archaeal genera	Comments	References
	<i>Rhodobacter</i> (<i>R. sphaeroides</i>), <i>Rhodopseudomonas</i> (<i>R. palustris</i>), <i>Thiocapsa</i> (<i>T. roseopersicina</i>)	Used for studies of aspartase, aminoacylase, glucose isomerase, and inulinase activities	Paronyan (2002a)
	Acetoclastic and hydrogenotrophic methanogenic enrichment, <i>Methanoculleus</i> (<i>Methanoculleus</i> sp.)	Enrichment in defined media produced active and stable methanogenic cultures on acetate and H ₂ /CO ₂ at 45 °C	Hedlund et al. (2013)
	Enrichment of nitrite-oxidizing bacteria (NOB)	Successful NOB enrichment incubated at 44 °C was obtained	Edwards et al. (2013)
Bjni	<i>Bacillus</i> (<i>B. licheniformis</i> , <i>B. aestuarii</i>), <i>Ureibacillus</i> (<i>U. thermosphaericus</i>), <i>Anoxybacillus</i> (<i>Anoxybacillus</i> sp.), <i>Geobacillus</i> (<i>G. toebii</i>)	Used for studies of extracellular amylase, lipase, and protease	Panosyan (2017)
	<i>Rhodobacter</i> (<i>R. sphaeroides</i>), <i>Rhodopseudomonas</i> (<i>R. palustris</i>), <i>Thiocapsa</i> (<i>T. roseopersicina</i>)	Used for studies of aspartase, aminoacylase, glucose isomerase, and inulinase activities, as well as sources of protein, carbohydrates, and vitamins	Paronyan (2002a)
Hankavan	<i>Bacillus</i> (<i>B. licheniformis</i> , <i>B. cirulans</i>), <i>Brevibacillus</i> (<i>B. thermoruber</i>), <i>Geobacillus</i> (<i>G. stearothermophilus</i>), <i>Anoxybacillus</i> (<i>Anoxybacillus</i> sp.)	Used for studies of extracellular amylase, lipase, and protease. The thermotolerant and metalotolerant bacilli <i>B. cirulans</i> (3A) is able to grow at different concentrations (from 10 to 300 mg/l) of Cd ²⁺ , Cu ²⁺ , Zn ²⁺ , and Ni ²⁺	Margaryan et al. (2010) and Panosyan (2017)
Jermuk	<i>Bacillus</i> (<i>B. licheniformis</i> , <i>B. amyloliquefaciens</i>), <i>Anoxybacillus</i> (<i>A. gonensis</i> , <i>A. kestanbolensis</i> , <i>Anoxybacillus</i> sp.), <i>Geobacillus</i> (<i>G. stearothermophilus</i> , <i>G. caldxylosilyticus</i>)	Used for studies of extracellular amylase, lipase, and protease. <i>Anoxybacillus</i> sp. is a candidate of new species	Hovhannisyan et al. (2016) and Panosyan (2017); Poghosyan (2015)

	<p><i>Desulfomicrobium</i> (<i>D. thermophilum</i>) <i>Desulfovibrio</i> (<i>D. psychrotolerans</i>) <i>Treponema</i> (<i>Treponema</i> sp.)</p>	<p>One of the isolates is claimed to be a novel <i>Spirochaetes</i> species, <i>Treponema thermophilum</i> sp. nov., and two Deltaproteobacterial SRB strains possibly also representing novel species. The <i>T. thermophilum</i> genome was sequenced to near completion and the 3.2 Mb draft sequence supports the description of this isolate as a separate species</p>	Poghosyan (2015)
	<p><i>Rhodobacter</i> (<i>R. Capsulatus</i>, <i>R. shpaeroides</i>) <i>Rhodopseudomonas</i> (<i>R. palustris</i>) <i>Thiospirillum</i> (<i>T. jenense</i>)</p>	<p>Used for studies of aspartase, aminoacylase, glucose isomerase, and inulinase activities, as well as sources of protein, carbohydrates, and vitamins. The strain <i>R. palustris</i> D-6 produces aspartase (with activity 33.05% per 100 mg dry biomass, at 37 °C, pH 6.0–9.0) The strain <i>R. capsulatus</i> D-4 produces L (+) lactic acid by 90% conversing sugars. The strain <i>R. shpaeroides</i> D-10 is a good producer of spheroiden and β-carotin</p>	Paronyan (2002a), Paronyan (2002b) and Paronyan (2007)
	Methylothermic, acetoclastic, and hydrogenotrophic methanogenic enrichments	Enrichments in defined media produced active and stable methanogenic cultures on methanol, acetate, and H ₂ CO ₂ , at 45 and 55 °C	Hedlund et al. (2013)
	Enrichment of NOB, <i>Nitrospira</i> (<i>N. calida</i> and <i>N. moscoviensis</i>)	The enrichment had a wide temperature range (25–60 °C, with a temperature optimum of 45–50 °C) of nitrite oxidation yielding nitrite oxidation rates of 7.53 ± 1.20 to 23.0 ± 2.73 fmoles cell ⁻¹ h ⁻¹ . The highest rates of NOB activity occurred with initial NO ⁻² concentrations of 0.5–0.75 mM; however, lower initial nitrite concentrations resulted in shorter lag times	Edwards et al. (2013)
Tatev	<p><i>Bacillus</i> (<i>B. licheniformis</i>), <i>Geobacillus</i> (<i>G. toebii</i>, <i>Geobacillus</i> sp.), <i>Anoxybacillus</i> (<i>Anoxybacillus</i> sp.), <i>Thermoactinomyces</i> (<i>T. vulgaris</i>)</p>	Used for studies of extracellular amylase, lipases, and protease. The strains <i>Geobacillus</i> sp. Tatev N5 and Tatev N6 showed high lipase activity (70.3 U/ml) at 65 °C after 5 hours of incubation. Lipase-producing <i>Geobacillus</i> sp. Tatev 4 (KY203974) contains genes encoding thermostable lipases (family I). Lipase contains Zn ²⁺ and Ca ²⁺ as ligands	Vardanyan et al. (2015), Shahinyan et al. (2015), Panosyan (2017) and Shahinyan et al. (2017)

(continued)

Table 4.3 (continued)

Geothermal spring	Bacterial and Archaeal genera	Comments	References
Uyts	<i>Bacillus</i> (<i>B. licheniformis</i>), <i>Ureibacillus</i> (<i>U. terrenus</i> , <i>U. thermosphaericus</i>), <i>Anoxybacillus</i> (<i>Anoxybacillus</i> sp.), <i>Geobacillus</i> (<i>G. toebii</i>)	Used for studies of extracellular amylase, lipases or protease	Panosyan (2017)
Nagorno-Karabakh			
Karvachar	<i>Bacillus</i> (<i>B. Licheniformis</i>), <i>Anoxybacillus</i> (<i>Anoxybacillus</i> sp., <i>A. flavithermus</i> , <i>A. rупiensis</i>), <i>Geobacillus</i> (<i>G. toebii</i>), <i>Aeribacillus</i> (<i>A. pallidus</i>)	Used for studies of extracellular amylase, lipase, and protease. Lipase-producing <i>A. flavithermus</i> strain Karvachar QB2 contains genes encoding thermostable esterase GDSL (family II). Draft genome of amylase producer <i>Anoxybacillus</i> sp. strain K103 (accession number MQAD00000000) was sequenced using a HiSeq 400 Illumina genome sequencer and contains alpha-amylase and alpha-glucosidase genes. Sequence analysis supports the description of this isolate as a separate species	Shahinyan et al. (2015), Hovhannisyan et al. (2016), Hovhannisyan et al. (2017), Shahinyan et al. (2017) and Panosyan (2017)
	<i>Thermus</i> (<i>T. scotoductus</i> , <i>T. ruber</i> , <i>T. flavus</i>)	Draft genome of <i>T. scotoductus</i> K1 (accession number LJJR00000000) sequenced and assembled with PacBio RS technology and Celera Assembler. Used to study of DNA polymerase. <i>T. ruber</i> and <i>T. flavus</i> identified based only phenotypic characteristics	Achmadova (1991), Saghatelyan et al. (2015) and Saghatelyan et al. (2016)
Zuar	Enrichment of NOB <i>Anoxybacillus</i> (<i>A. rупiensis</i>), <i>Geobacillus</i> (<i>G. toebii</i>)	Successful NOB enrichment incubated at 56 °C was obtained Used for studies of extracellular amylase, lipase, and protease	Edwards et al. (2013) Hovhannisyan et al. (2016)

microbiological investigation on any hot spring in the Lesser Caucasus. The study indicated a predominance of Alphaproteobacteria (8%), Betaproteobacteria (22%), Gammaproteobacteria (13%), Epsilonproteobacteria (9%), Firmicutes (9%), Bacteroidetes (48%), and Cyanobacteria (35%). In addition, DGGE was employed to reveal the microbial profile of sediments of this hot spring. The authors reported an abundance of bacterial populations related to Proteobacteria (affiliated with the Beta-, Epsilon-, and Gammaproteobacteria), Bacteroidetes, and Cyanobacteria based on the DGGE profile, which was in good agreement with the clone library results. The sequence of dominating DGGE bands showed affiliation to *Rhodofera* sp., a phototrophic, purple non-sulfur betaproteobacterium and to *Sulfurimonas* sp., a hydrogen-oxidizing chemolithoautotrophic bacterium isolated from a rearing tank with dissolved hydrogen (Panosyan and Birkeland 2014; Panosyan et al. 2017).

Samples from the Arzakan spring were screened also with advanced metagenomic approaches. Amplification of small-subunit rRNA genes using “universal” primers followed by pyrosequencing (pyrotags) on 454 GS FLX platform also revealed highly diverse microbial communities in Arzakan mat samples (Hedlund et al. 2013). 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. It was shown that in Arzakan spring, relatively few (16%) of the total pyrotags could be assigned to known genera, underscoring the novelty of these ecosystem and the need for continued efforts to cultivate and describe microorganisms in geothermal systems.

The phylogenetic analysis of Bacteria identified the dominant phylotypes as members of Proteobacteria. The phylogeny for Proteobacteria revealed considerable diversity. While it is not possible to predict their metabolism from environmental sequences alone, the closest phylogenetic affiliations were to aerobic and anaerobic heterotrophs and methanotrophs (within the Proteobacteria lineage). It was established that the primary production of the Arzakan geothermal system supports by a complex microbial community composed of chemolithotrophs (hydrogen- and sulfide-oxidizing Epsilonproteobacteria and methanotrophic Gammaproteobacteria) and phototrophs (Cyanobacteria and purple non-sulfur anoxygenic phototrophic Betaproteobacteria). The most abundant Cyanobacteria OTUs were confidently assigned to the genera *Spirulina*, *Stanieria*, *Leptolyngbya*, and *Rivularia/Caldithrix*.

To study bacterial diversity of the hot spring in Jermuk (Armenia), 454 GS FLX pyrosequencing of V4–V8 variable regions of the small-subunit rRNA was applied. As reported, the most abundant phyla represented in the pyrotag dataset from Jermuk were the Proteobacteria, Bacteroidetes, and Synergistetes (Hedlund et al. 2013). 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*, *Sideroxydans*, and *Hydrogenophaga*, suggesting the importance of chemolithotrophy in primary productivity (Kampfer 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 OTUs 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.

DGGE analysis of the partial bacterial 16S rRNA gene PCR amplicons also was used to profile bacterial populations inhabiting the sediment and water fractions in the Jermuk geothermal spring. The sequence analysis of DGGE bands showed affiliation with Epsilonproteobacteria, Bacteroidetes, Spirochaetes, Ignavibacteriae, and Firmicutes. The sequences obtained from bands were related to anaerobic or facultatively anaerobic organotrophic or H₂-utilizing and thiosulfate-/sulfur-reducing bacteria. Heterotrophic microorganisms detected in the DGGE profile clustered among fermentative microorganisms, which are actively involved in C-cycle (Panosyan 2017).

Culture-independent technique with an emphasis on members of the Archaea was used to determine the composition and structure of microbial communities inhabiting microbial mats in the source pools of two geothermal springs, Arzakan and Jermuk. Based on an analysis of near full-length small-subunit rRNA genes amplified using Archaea-specific primers, it was shown that these 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 (Fig. 4.4) (Hedlund et al. 2013). 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, and *Methanosaeta* and a sequence that could not be classified below the order level were detected in Arzakan. Close relatives of *Methanospirillum hungatei*, in the order *Methanomicrobiales*, were inferred to be abundant in both springs. In addition, two phylotypes in Arzakan were related to the genus *Methanoregula*, also in the *Methanomicrobiales*. Members of both genera use H₂/CO₂ and/or format as methanogenic substrates; however, their presence in the geothermal systems was somewhat surprising since they are not reported to grow above 37 °C. The other order of methanogens present in both springs was *Methanosarcinales*, represented by *Methanosaeta* and *Methanomethylovorans*. *Methanosaeta* was abundant in Jermuk and includes obligate acetoclastic species known to grow up to 60 °C (Liu and Whitman 2008).

Recently, Illumina HiSeq2500 paired-end sequencing of metagenomic DNA also was used to analyze water/sediment samples of the Jermuk hot spring. Taxonomic analyses of the metagenomic rRNA sequences revealed a prevalence of *Proteobacteria*, *Firmicutes* and *Bacteroidetes*. However, many of the largest contigs represented uncharacterized or poorly characterized groups such as candidate division WS6 and candidate phylum Ignavibacteria. The archaeal community, constituting a minor fraction (~1%) of the community, was dominated by Euryarchaeota, followed by

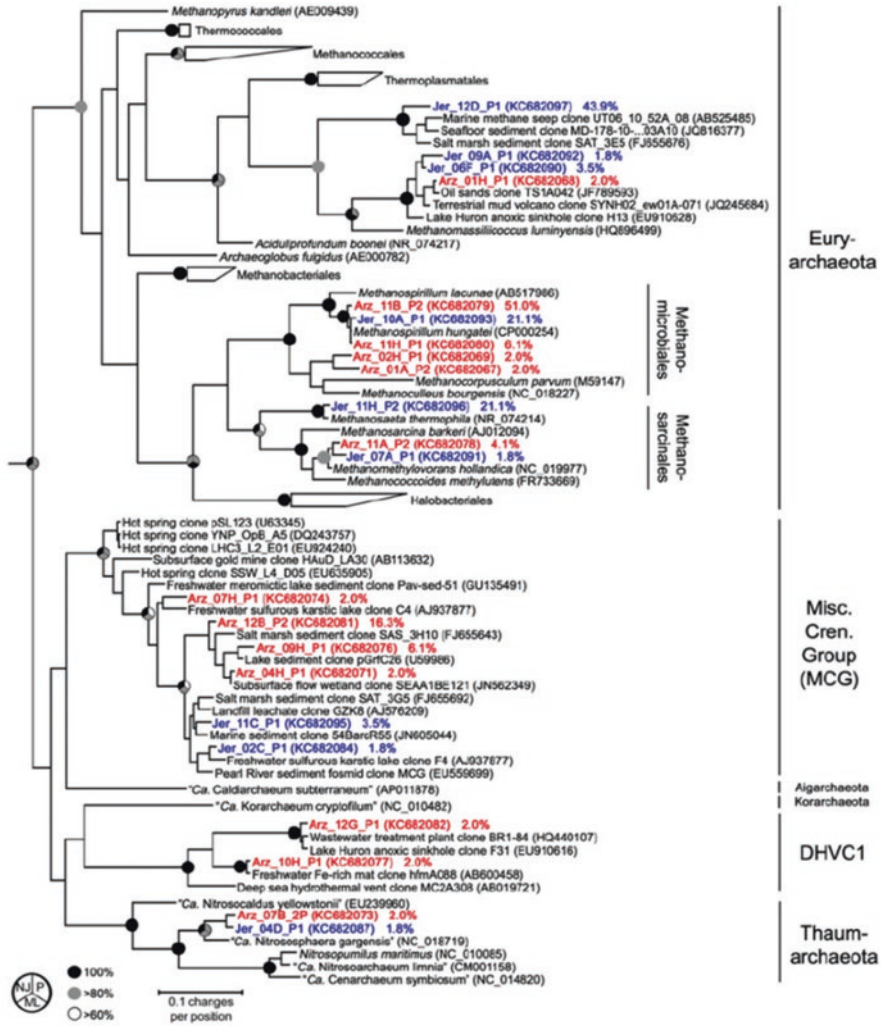


Fig. 4.4 Maximum-likelihood phylogeny depicting relationships between near-complete archaeal 16S rRNA genes recovered from Arzakan (red) and Jermuk (blue) and closely related sequences, including well-studied microbial isolates. Percent values for each OTU represent the percent abundance of the OTU in the clone library. Bootstrap support is indicated at major nodes for maximum-likelihood (ML; 100 replicates), parsimony (P; 1000 replicates), and distance (neighbor-joining, NJ; 1000 replicates) methods. Taxonomic designations for major phylogenetic groups are shown at the right (Hedlund et al. 2013)

Crenarchaeota, unclassified groups, and a minor fraction of Thaumarchaeota. The functional composition based on metagenomics sequence information indicated a dominance of heterotrophic types of metabolism (Poghosyan 2015).

For investigation of the bacterial composition of sediment and water samples from the Zuar geothermal spring (Nagorno-Karabakh), only a bacterial clone library

based on 16S rRNA genes was constructed. It was shown that clones obtained from the Zuar geothermal spring originated from phyla Proteobacteria (42.3%), Firmicutes (19.2%), Bacteroidetes (15.4%), Cyanobacteria (3.8%), Tenericutes (3.8%), and yet-unclassified phylotypes (15.4% for Zuar) (Saghatelyan et al. 2014).

According to the recent report of sequence analysis of clones obtained from bacterial 16S rRNA gene libraries, the presence of Proteobacteria (48.6%), Cyanobacteria (29.7%), Bacteroidetes (5.4%), Chloroflexi (5.4%), Verrucomicrobia (2.7%), and Planctomycetes (2.7%) in sediment and water samples in Karvachar (Nagorno-Karabakh) hot spring (Fig. 4.5) was indicated (Saghatelyan and Panosyan 2015). The dominating bacterial group was the phylum Proteobacteria. A few phylotypes belonging to the phylum Bacteroidetes were obtained. One of the dominating groups was Cyanobacteria, representatives of which dominate especially on top layer of microbial mats and are the most important primary producers in hot spring ecosystems (Roeselers et al. 2007).

Representatives of phylum Firmicutes were not detected in the clone library, while DGGE profiling of the same samples indicated presence of Firmicutes (genus *Geobacillus*) as a one of the major components in bacterial community of Karvachar geothermal spring (Panosyan 2017). This has been confirmed later by metagenome analysis of the Karvachar hot spring samples.

Based on recent data (unpublished data) obtained from the whole-genome shotgun sequencing of sediment samples of Karvachar, using an Illumina HiSeq 2500 platform, 580 bacterial sequences were aligned to reference genes (NCBI RefSeq), belonging to the following bacterial taxonomical groups: Actinobacteria; Alpha-, Beta-, Delta-, Epsilon-, and Gammaproteobacteria; Bacteroidetes/Chlorobi; Firmicutes; Chlamydiae; Cyanobacteria/Melainobacteria; *Fusobacteria*; and Synergistia. Among these groups, Proteobacteria (Alpha-, Beta-, and Gammaproteobacteria) and Firmicutes were the major components in the total bacterial sequence reads (Fig. 4.5). The sequences affiliated with Gammaproteobacteria were predominant (48.96% of Proteobacteria, 235 out of 480), and most of them were closely (98–100%) related to cultured Gammaproteobacteria. Representative of the groups of *Porphyrobacter*, *Paracoccus*, and *Oceanibaculum* was predominant Alphaproteobacteria found in study samples. The majority of sequences derived from spring were closely related (95–99% identity) to *Porphyrobacter cryptus*, a slightly thermophilic, aerobic, bacteriochlorophyll a-containing species isolated from a hot spring at Alcafache in Central Portugal (Rainey et al. 2003).

Betaproteobacterial-related sequences were the third major group of obtained bacterial sequences (20.6% of Proteobacteria, 99 out of 480). The majority of the obtained sequences showed 92–100% similarity to *Caldimonas taiwanensis*, an aerobic amylase-producing heterotrophic bacterium isolated from a hot spring located in Taiwan (Chen et al. 2005) and 94–99% of similarity to representatives of genus *Tepidimonas*, particularly to the species *T. taiwanensis*, *T. thermophilus*, and *T. fonticaldi*, isolated from hot springs in Taiwan and India (Chen et al. 2013; Poddar et al. 2014). Forty sequences (6.9%, 40 out of 580) were affiliated with Firmicutes. Around 10.3% (60 out of 580) of the total bacterial clone sequences were affiliated with some minor groups, such as Actinobacteria, Bacteroidetes/Chlorobi, Chlamydia,

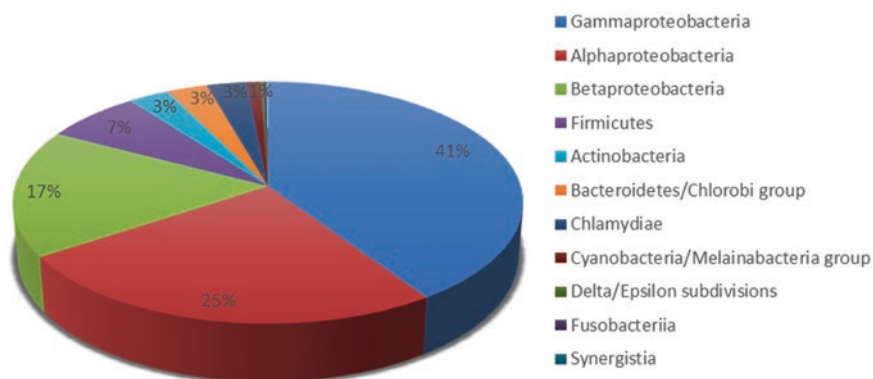


Fig. 4.5 Phylum level grouping of bacterial sequence read obtained from Karvachar geothermal spring

Cyanobacteria/Melainabacteria, Fusobacteria, and Synergistia. Most of these sequences were closely (98–99%) related to clones retrieved from water environments and different habitats (Anil Kumar et al. 2010; Yoon et al. 2009). Phototrophic bacteria belonging to genera *Neosynechococcus*, *Pseudanabaena*, and *Fischerella* represented the three most abundant and metabolically active primary producers of the analyzed community. Most Cyanobacteria detected were related to others previously reported in thermophilic environments (Portillo et al. 2009). Representatives of genus *Rhodobacter* (purple non-sulfur anoxygenic phototrophs) and other phototrophic microbes were found to share these environments with the cyanobacteria.

The sequence reads from the Samtredia geothermal spring (Georgia) water sample, obtained from the whole-genome shotgun sequencing on Illumina HiSeq 2500 platform, showed high similarity (>90%) to 938 bacterial and 15 archaeal reference sequences (Fig. 4.6). The majority of bacterial sequence reads were affiliated with the Firmicutes (33%) and Gammaproteobacteria (32%), followed by Actinobacteria (15.5%), Betaproteobacteria (9.1%), Alphaproteobacteria (2.9%), Chlamydia (1.6%), and Bacteroidetes (1.5%). Other groups of Prokaryotes (Aquificae, Deinococcus-Thermus, Deltaproteobacteria, Epsilonproteobacteria, Acidithiobacillia, Planctomycetes, Cyanobacteria/Melainabacteria group) comprised a minority, less than 1% of the communities. Archaeal sequence reads were affiliated with Crenarchaeota (1.4%) and Euryarchaeota (0.2%) (unpublished data).

The most dominant phylum, *Firmicutes*, was represented by genera *Streptococcus*, *Enterococcus*, *Clostridioides*, *Bacillus*, and *Listeria*. The majority of these bacteria can be recovered from a wide range of habitats. Firmicutes representatives considered as inhabitants of thermal waters include genera such as *Geobacillus*, *Thermoanaerobacter*, *Desulfotomaculum*, and *Desulfovirgula* have been revealed in the Samtredia hot spring. Similarly to other above described thermal waters, Proteobacteria were largely represented in the sequence reads. Four hundred and twenty two sequences (45%, 422 out of 938) were affiliated with Proteobacteria,

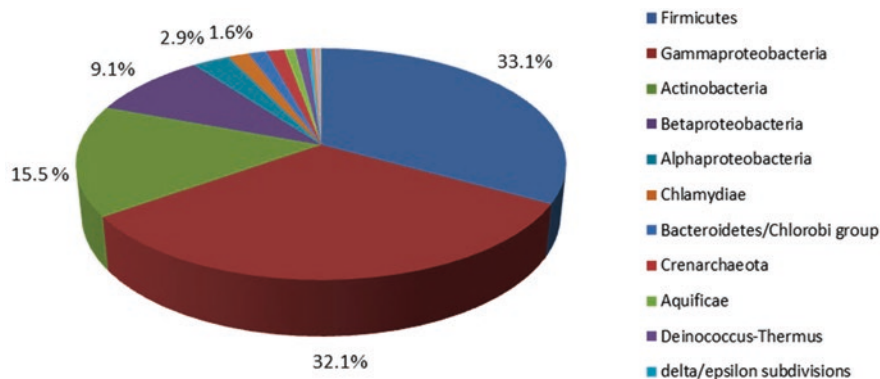


Fig. 4.6 Phylum level grouping of sequences obtained from Samtredia geothermal spring water sample

belonging to following subgroups: Alpha-, Beta-, Gamma-, and Deltaproteobacteria. The sequences affiliated with Gammaproteobacteria were predominant (72.5% of Proteobacteria, 306 out of 422). The dominant groups were *Escherichia*, *Acinetobacter*, *Pseudomonas*, *Salmonella*, and *Legionella*. Surprisingly most gammaproteobacterial sequences were *Escherichia*-related sequences. These microbes are not autochthons for hot springs and could be considered as contaminants.

Betaproteobacterial-related sequences were the second major group of obtained proteobacterial sequences (20.6% of Proteobacteria, 87 out of 422). The genera of *Caldimonas* and *Tepidiphilus*, representing the hot spring microbiota, were one of the minor groups of Betaproteobacteria found in the study samples. Alphaproteobacterial-related sequences comprised 6.6% of Proteobacteria and were represented mainly by nonindigenous bacteria. Actinobacteria accounted for a significant portion of bacteria, composing 15.8% (148 out of 938) of total bacterial populations dominated by *Mycobacteria*, while the Deinococcus-Thermus group was mainly represented by thermophilic bacteria belonging to the genus *Thermus*. Aquificales accounted for 0.8% of the reads, affiliated to facultatively anaerobic, hydrogen- or sulfur-/thiosulfate-oxidizing, thermophilic bacteria belonging to genus *Sulfurihydrogenibium*. Less than 5% of the total bacterial sequences were aligned with some other minor groups, such as Acidithiobacillia, Bacteroidetes/Chlorobi, Chlamydia, Cyanobacteria/Melainabacteria, and Planctomycetes. Most of these sequences were closely related to clones retrieved from water and soil environments.

The microbial diversity of the Tbilisi sulfur spring (Georgia) was analyzed using whole-genome shotgun sequencing using Illumina MiSeq platform (unpublished data). The sequences obtained from metagenomic DNA showed high similarity (>90%) to 1090 RefSeq database reference sequences, revealing 240 species. The thermal water was dominated by Gammaproteobacteria (46.4% of total reads) followed by Firmicutes (20.6%), Betaproteobacteria (16.4%), Actinobacteria (6.1%), Alphaproteobacteria (5.7%), Chlamydiae (1.7%), and Bacteroidetes (1.5%).

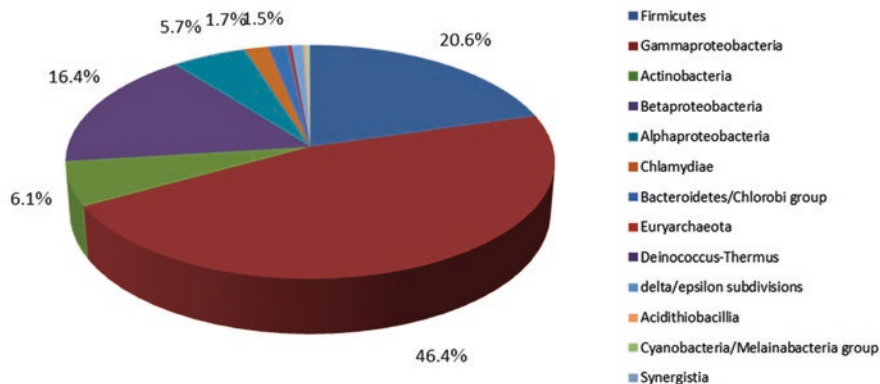


Fig. 4.7 Phylum level grouping of sequences obtained from Tbilisi sulfur spring water sample

Deinococcus-Thermus, delta/epsilon subdivisions, Acidithiobacillus Cyanobacteria/Melainabacteria group, and Synergistia comprised a minority of the prokaryotic populations accounting for less than 1% of total reads for each group (Fig. 4.7). Archaeal sequence reads were also in minority, belonging to the Euryarchaeota and comprising 0.2% of total reads.

Gammaproteobacteria were represented by 35 bacterial genera, dominated by *Escherichia* (30%), *Pseudomonas* (24%), *Xanthomonas* (11%), *Legionella* (5%), *Salmonella* (3.5%), and *Acinetobacter* (2.8%). Some of these bacteria are found in diverse habitats and may also cause diseases in humans. Interestingly, *Pseudomonas*, *Legionella*, and *Acinetobacter* were reported in a variety of geothermal springs (Lin et al. 2007; Petursdottir et al. 2009; Saxena et al. 2017). The sulfur spring also harbored *Silanimonas lenta* (3.5%), belonging to moderately thermophilic alkaliphilic bacteria isolated from a hot spring in Korea (Lee et al. 2005), purple sulfur bacteria *Ectothiorhodospira*, and thermophilic bacterium *Thermomonas hydrothermalis* isolated from a hot spring in Central Portugal (Alves et al. 2003).

The second most abundant group of bacteria was Firmicutes, including representatives belonging to the *Bacillus* and related genera such *Geobacillus* and *Tepidibacillus*.

Betaproteobacteria were dominated by *Neisseria* (29%), presumably allochthonous bacteria. The other two most prevalent betabacteria inhabiting the studied spring were amylase-producing *Caldimonas taiwanensis* (22%) and alkaline-protease-producing *Tepidimonas taiwanensis* (14%), thermophilic bacteria reported in geothermal springs in Taiwan (Chen et al. 2005; Chen et al. 2006) and, as described above, have been found in the Karvachar hot spring as well.

Actinobacteria were represented by 12 genera dominated by *Streptomyces* and *Mycobacteria* that may inhabit thermal spring environments. *Streptomyces* spp. are known to produce various enzymes and biological active compounds, including antimicrobials, and can be readily isolated from geothermal environments (Al-Dhabi

et al. 2016). Mycobacteria, with potential to cause diseases in humans, have been also found and isolated from the sulfur hot springs (Lee et al. 2015).

Alphaproteobacteria of the sulfur spring comprised 18 genera dominated by *Mesorhizobium* (23% of total Alphaproteobacteria reads) and *Thalassobius* (16%) species. Though these bacteria were not described in the hot springs, they have been found in diverse environments such as marine waters and soils (Arahal et al. 2005; Yuan et al. 2016), indicating possibility of their presence in thermal waters as well.

The Bacteroidetes/Chlorobi group was represented by 12 genera with 25% and 12% of sequence reads aligned to *Bacteroides* and *Pedobacter* reference genes, respectively. *Bacteroides* spp. have not been described in geothermal waters and can be considered as a contaminant, while the presence of *Pedobacter* has been reported in an alkaline hot spring in Thermopolis (Buckingham et al. 2013).

The delta/epsilon subdivision comprised a minority of the sulfur spring microbial population represented only with five genera, including the sulfur-reducing microaerophilic bacterium *Sulfurospirillum* that could be considered as natural inhabitant of this geothermal spring. The Deinococcus-Thermus group was also in minority, represented by *Meiothermus taiwanensis*, aerobic, thermophilic, non-sporulating, filamentous bacteria reported in a hot spring in Taiwan (Chen et al. 2002).

The sulfur spring was inhabited by two methanogenic Euryarchaeota species, *Methanolacinia paynteri* and *Methanosarcina mazei*. The optimum growth conditions for *Methanolacinia paynteri* are pH 6.6–7.2, temperature 40 °C, and the sulfide may serve as the sulfur source (Zellner et al. 1989), thus presence of this archaeon in the sulfur spring is not surprising. *Methanosarcina* spp. can survive in a variety of habitats, including extreme environments and may use different metabolic pathways to produce methane (Assis das Graças et al. 2013).

In addition to whole metagenomic DNA sequencing, the microbial diversity of the sulfur spring was also analyzed using a PCR/DGGE approach. The majority of DGGE bands were affiliated with Betaproteobacteria involved in sulfur cycle, such as species belonging to the genera *Sulfurisoma*, *Thiobacillus*, and oxalotrophic bacterium *Oxalicibacterium faecigallinarum*.

The study has also revealed the presence of the methanogen *Methanosaeta harundinacea*, belonging to Euryarchaeota, confirming that the methanogenic Euryarchaeota dominate archaeal populations of the sulfur spring.

4.3.2 Cultivation-Dependent Studies

Cultivable approaches have been used for analysis of microbial diversity associated with hot springs. Several studies have been performed on the description of novel genera, species and strains, characterization of different bio-resources, and whole-genome analysis of a few isolates from geothermal springs in the Lesser Caucasus. Many thermostable enzymes, including lipase, protease, amylase, DNA polymerase, aspartase, aminoacylase, glucose isomerase and inulinase, producers of EPS, protein and vitamins, enrichments of nitrite-oxidizing bacteria (NOB), and

methylophilic, acetoclastic, and hydrogenotrophic methanogens with potential biotechnological applications have been reported by several authors (Table 4.3).

Overall, all isolates of bacteria and Archaea from the Lesser Caucasus belong to more than 40 distinct species of 21 different genera, namely, *Bacillus*, *Geobacillus*, *Anoxybacillus*, *Paenibacillus*, *Brevibacillus*, *Aeribacillus*, *Ureibacillus*, *Thermoactinomyces*, *Sporosarcina*, *Thermus*, *Rhodobacter*, *Thiospirillum*, *Thiocapsa*, *Rhodopseudomonas*, *Methylocaldum*, *Desulfomicrobium*, *Desulfovibrio*, *Treponema*, *Arcobacter*, *Nitrospira*, and *Methanoculleus*. The members of phylum Firmicutes were most dominant among the identified bacteria isolated from all thermal springs. Culture-dependent studies indicate that *Bacillus* and related genera were ubiquitous and predominant in harsh environments of high temperatures. Representatives of the genera *Geobacillus* and *Anoxybacillus* are the most highly distributed obligate thermophiles in the Lesser Caucasus hot springs. All isolates from the hot springs that belonged to the genus *Bacillus* were thermotolerant microorganisms among which *B. licheniformis* appeared as the dominating species. All studied springs demonstrated significantly lower content of species belonging to genera *Brevibacillus*, *Ureibacillus*, *Paenibacillus*, *Thermoactinomyces*, and *Sporosarcina*.

Bacteria belonging to the genera *Bacillus* and *Thermus* were mostly reported as aerobic, heterotrophic thermophiles and found in thermal systems with neutral to alkaline pH (Spanevello and Patel 2004). Although *Thermus* spp. may be predominant heterotrophs in many hot springs (Hjorleifsdottir et al. 2001), they were isolated only from the Karvachar hot spring.

Several strains representing potentially novel species were reported from the Akhurik, Jermuk, and Karvachar geothermal springs. Two novel strains belonging to genera *Anoxybacillus* and *Treponema* were reported from the hot spring at Jermuk. A novel species belonging to genus *Anoxybacillus* and a new strain belonging to *Thermus scotoeductus* were reported from the Karvachar spring (Saghatelyan et al. 2015; Hovhannisyan et al. 2017). 16S rRNA gene sequences of a methanotrophic isolate from Akhurik geothermal spring showed that it was a new gammaproteobacterial methanotroph, forming a separate clade in the *Methylococcaceae* family. It fell into a cluster with thermotolerant and mesophilic methanotrophs, comprising the genera *Methylocaldum*-*Methylococcus*-*Methyloparacoccus*-*Methylogaea*. The genes *pmoA*, *mxoF*, *cbbL*, and *nifH* were detected, but no *mmoX* gene was found. The strain probably represents a novel methanotrophic genus (Islam et al. 2015).

Whole-genome analysis of the hot spring isolates was a major thrust area of investigation. Whole-genome shotgun sequencing of novel species isolated from hot springs at Jermuk (*Treponema thermophilum* sp. nov) and Karvachar (*Anoxybacillus* sp. strain K103) was performed (Poghosyan 2015; Hovhannisyan et al. 2017). Similarly, the whole-genome sequence of *Thermus scotoeductus* K1 was reported following its isolation from the Karvachar spring (Saghatelyan et al. 2015).

Attention was also paid to the bioprospecting of geothermal spring's microbes with an intention of using these resources for commercial applications. In total, 135 thermophilic and thermotolerant bacilli strains were isolated under aerobic conditions at 55–65 °C and identified based on 16S rRNA gene sequence analysis

as representatives of genera *Bacillus*, *Geobacillus*, *Anoxybacillus*, *Paenibacillus*, *Brevibacillus*, *Aeribacillus*, *Ureibacillus*, *Thermoactinomyces*, and *Sporosarcina*. These thermophilic bacilli were tested for hydrolytic enzyme production capacities, and biotechnologically valuable enzyme producers were selected (Panosyan 2017). The majority of the studies focused on hydrolytic enzymes like lipase (Vardanyan et al. 2015; Shahinyan et al. 2017), amylase (Hovhannisyan et al. 2016), and protease (Panosyan 2017).

Some phototrophic bacteria isolated from Armenian hot springs were good producers of enzymes such as aspartase, aminoacylase, glucose isomerase, and inulinase, as well as sources of protein, carbohydrates, and vitamins (Paronyan 2003). Two isolates belonging to the genus *Geobacillus* are able to produce heteropolymeric EPSs with high molecular weight (Panosyan et al. 2014).

Prospective microbes from hot springs offer a major advantage of preserving those strains for future studies and exploring them in due course for potential biotechnological applications in medical, industrial, and agricultural processes.

4.4 Correlation Between Geophysiology and Microbiology of the Hot Springs in the Lesser Caucasus

Understanding the microbial community structure in hot springs with different ecologies is important to elucidate community functions and their importance for the maintenance of hot spring ecosystems.

In general, microbial diversity was inversely correlated with temperature, and temperature has been shown to be a key factor in controlling the microbial diversity in hot springs (Wang et al. 2013). Thermophilic or hyperthermophilic Bacteria are commonly present in high-temperature hot springs (>75 °C) (Hou et al. 2013). When temperature is suitable for photosynthesis (<75 °C), moderately thermophilic and mesophilic phototrophic Bacteria are important members in terrestrial thermal springs, such as Cyanobacteria, Chloroflexi, and phototrophic representatives of Proteobacteria (Cox et al. 2011). In addition to Bacteria, members of the archaeal phyla Crenarchaeota, Euryarchaeota, and Thaumarchaeota are also commonly detected in geothermal systems (Ochsenreiter et al. 2003; Zhang et al. 2008).

The comparison of microbial species abundance and diversity in the Lesser Caucasus hot springs with those available internationally displays similar patterns. It was shown earlier that there is a negative correlation between spring temperature and diversity of microbes (Wang et al. 2013; Poddar and Das 2017).

Prokaryotic diversity was found to be low at high-temperature springs in contrast to low-temperature springs. Temperature has also been shown to drive phylum diversity in hot springs. Most of the studied hot springs in the Lesser Caucasus have a temperature below 50 °C and harbor bacterial species pertaining to phyla Firmicutes, Proteobacteria, Bacteroidetes, and Cyanobacteria, although with varying abundance between springs. Springs with higher temperatures also contained thermophiles belonging to Actinobacteria, Deinococcus-Thermus, and Aquificae. Representatives of the phylum Firmicutes were most versatile in the

investigated hot springs and could populate hot springs with a wide range of temperatures. These observations are in accordance with many global studies indicating that thermophilic bacteria belonging to phyla Aquificae, Deinococcus-Thermus, and Firmicutes were abundant in the hot springs with high temperatures, whereas mesophilic bacterial members of Cyanobacteria, Chloroflexi, and Proteobacteria mostly occupy mesothermal hot springs (Wang et al. 2013). 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 (Castenholtz 1973). It was shown earlier that moderate-temperature geothermal systems cool enough to permit phototrophy at the source with neutral or alkaline pH are often colonized by visible microbial growth that forms laminated mats or streamers dominated by phototrophic bacteria (Klatt et al. 2011). Relatively low-temperature (>75 °C) and neutral pH in all studied springs can support growth of phototrophic bacteria due to obvious light effect in the outlet of the spring.

A comparison of the optimum growth temperature of the closest cultivated relatives of the microorganisms detected in the clone libraries, DGGE profiles, or pyro-tags suggested that most of the 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 metagenomic DNA sequences also affiliated with taxa that are not described in the literature as being associated with geothermal environments. This can be explained by the presence of contamination from surrounding soils. Although most of the retrieved sequences were most similar to environmental sequences representing uncultured bacteria from various habitats, some of them were phylogenetically associated with environmental clones obtained from similar habitats.

Archaea appeared to be a minority in the prokaryotic community. High-temperature environments were previously generally believed to be the realm of Archaea (Li et al. 2015; Urbieta et al. 2015; Chan et al. 2017). However, recent studies applying molecular methods have revealed that bacteria rather are the predominant prokaryotic communities in such environments (Badhai et al. 2015; López-López et al. 2015). The factors that allow bacteria to dominate in high-temperature habitats are not well understood.

All reported Lesser Caucasus springs have circumneutral pH and, therefore, harbor a microbial community different from acidic hot springs environments (Purcell et al. 2007; Poddar and Das 2017). Acidic springs have been reported to contain chemolithotrophic acidophiles belonging to genera *Acidithiobacillus*, *Sulfobacillus*, *Hydrogenobaculum*, *Acidobacteria*, *Acidimicrobium*, etc. that participate in Fe and sulfur oxidation in those environments (Burgess et al. 2012; Urbieta et al. 2015; Skirmisdottir et al. 2000). Acidophiles were hardly detected in Lesser Caucasian hot springs. Bacterial species isolated from the studied hot springs exhibited optimal growth at neutral pH and could not grow at low pH conditions. It was shown by many investigators that Firmicutes and Proteobacteria are the phyla consistently present in circumneutral hot springs. Results obtained from Lesser Caucasus geothermal springs also are in line with observations of microbial assemblages distributed in hot springs with $\text{pH} \geq 7$ globally (Nakagawa and Fukui 2002; Wang et al.

2013). In general, Archaea are not dominant in circumneutral hot springs, which is in agreement with several recent reports with similar pH ranges (Wang et al. 2013; Merkel et al. 2017).

Environmental conditions and the nutritional status in a natural habitat may drive the development of a particular microbial group or population. The set of abiotic factors allow natural selection of a few species that can dominate and multiply in the ecologically relevant niche. Limited carbon and nitrogen sources and high temperature of the springs located in the Lesser Caucasus allowed also the development of a unique population dominated by a large number of bacilli including *Geobacillus* and *Anoxybacillus* spp.

Besides temperature and pH, the limiting factor for microbial diversity and biomass could be a combination of abiotic factors including dissolved gasses (H_2 , CO_2 , H_2S , CH_4) and high mineralization. The geothermal systems of the Lesser Caucasus are known to contain high concentrations of minerals, and thus, the mineralization 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 (Whitaker et al. 2003; Takacs-Vesbach et al. 2008).

4.5 Conclusion

Investigations of the geothermal springs' microbiome are important for understanding the microbe-mediated biogeochemical cycles and ecosystem functioning as well as exploring the biotechnological potency of thermophilic isolates. This is the first comprehensive census of the microbial communities thriving in 11 geothermal springs of the Lesser Caucasus. Firmicutes, Proteobacteria, Bacteroidetes, and Cyanobacteria were the signature phyla in all 11 hot springs that along with the presence of site-specific taxa contributed to the uniqueness of each spring. Archaea appeared to be a minority in the prokaryotic community composing less than 1% of all microbial population. Overall, microbial diversity and richness were negatively affected by increasing temperature. Other influential factors shaping the microbiota of the studied Lesser Caucasus circumneutral geothermal springs appear to be pH and mineralization. Biogeography and geological history should not be ignored in microbial ecology studies, as all abiotic factors collectively contribute to the dynamics of the microbial populations. Many new thermophilic microbes mainly belonging to the *Bacillus* and related genera have been isolated, identified, and evaluated taking into account their biotechnological potency.

The present work, therefore, extends the previous sphere of information regarding the thermophilic bacterial diversity of thermal springs in the Lesser Caucasus.

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