Biofilm forming bacteria and archaea in thermal karst springs of Gellért Hill discharge area (Hungary)

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Abbreviations: BTKS, Buda Thermal Karst System; RT, Rudas-Török spring cave; DH, Diana-Hygieia thermal spring; RN, Rác Spa Nagy spring; GO, Gellért-Ősforrás; ARDRA, Amplified Ribosomal DNA Restriction Analysis; BLAST, Basic Local Alignment and Search Tool; SEM, Scanning Electron Microscopy

ABSTRACT

The Buda Thermal Karst System (BTKS) is an extensive active hypogenic cave system located beneath the residential area of the Hungarian capital. At the river Danube, several thermal springs discharge forming spring caves. To reveal and compare the morphological structure and prokaryotic diversity of reddish-brown biofilms developed on the carbonate rock surfaces of the springs, scanning electron microscopy (SEM) and molecular cloning were applied. Microbial networks formed by filamentous bacteria and other cells with mineral crystals embedded in extracellular polymeric substances were observed in the SEM images. Biofilms were dominated by prokaryotes belonging to phyla Proteobacteria, Chloroflexi and Nitrospirae (Bacteria) and Thaumarchaeota (Archaea) but their abundance showed differences according to the type of the host rock, geographic distance and different water exchange. In addition, representatives of phyla Acidobacteria, Actinobacteria, Caldithrix, Cyanobacteria, Firmicutes Gemmatimonadetes and several candidate divisions of Bacteria as well as Crenarchaeota and Euryarchaeota were detected in sample-dependent higher abundance. The results indicate that thermophilic, anaerobic sulfur-, sulfate-, nitrate- and iron(III)-reducing chemoorganotrophic as well as sulfur-, ammonia- and nitrite-oxidizing chemolithotrophic prokaryotes can interact in the studied biofilms adapted to the unique and extreme circumstances (e.g. aphotic and nearly anoxic conditions, oligotrophy and radionuclide accumulation) in the thermal karst springs.

1 INTRODUCTION

The study of biofilm forming microorganisms living in karst caves characterized by constant temperatures, complete darkness and relatively stable geochemical conditions has been in the focus of research interest in the last decades [1-4]. The Buda Thermal Karst System (BTKS) is situated in the NE part of the Transdanubian Central Range, and its discharge area is located in Budapest, the capital of Hungary. Based on the location of spring groups, the origin of their water, their temperature and dissolved mineral concentrations, BTKS can be divided into three discharge areas [5,6]. In the BTKS a special geomicrobiological environment has been explored where microbial biofilms developed on the carbonate rock surfaces of the spring caves. These biofilms contain inorganic materials and can accumulate different trace elements [6-8]. The presence of mainly iron accumulating biogeochemical layers was recognized in the BTKS, even though bacterial cell morphological structures of biofilms are characteristically different [9]. Storage capacity of biogeochemical layers was measured recently by calculating the enrichment factors [7]. Biofilms developing in the discharge areas of BTKS are presumed to contribute to hypogenic karstification processes, as well [10]. Preliminary microbiological examinations on the biofilms and thermal waters from different parts of the BTKS revealed the existence of extremophilic prokaryotic composition adapted to the special environmental conditions [9,11,12]. Biofilm bacterial communities at all studied sites proved to be somewhat more diverse than that of the surrounding thermal waters [11,12]. The reddish-brown biofilms were dominated by facultative anaerobic, hydrogen or sulfur/thiosulfate-oxidizing (chemolithoautotrophic) and thermophilic *Sulfurihydrogenibium* (Aquificae) in the well of Széchenyi Thermal Bath [11] while multilayer filamentous structure forming representatives of the phylum Chloroflexi inhabited the Molnár János hypogene cave [12]. However, regarding the biofilm community composition in the Gellért Hill of BTKS, our knowledge is still rather incomplete. In the biofilm communities studied to date, dominance of phylotypes affiliated with

Deltaproteobacteria and Nitrospira was detected [9,13]. The discovered complex microbial community structures involving phylotypes closely related to both meso- and thermophilic species indicate the importance of special and interconnected hydrogen, sulfur and nitrogen metabolizing prokaryotic networks in this part of the BTKS.

This study focused on the Southern discharge area (Gellért Hill) of the BTKS. Based on preliminary hydrogeological results, the Rose Hill area are discharged by lukewarm and thermal water, while only thermal water appears in the springs of the Southern area [6,8,10]. Thermal water contains not only karst water but also so called basinal fluid component differing for the two systems; the Rose Hill can be characterized by dominantly NaCl-type water, while SO_4^{2-}-rich water is characteristic in the Gellért Hill discharge zone [8,10,14]. Sulfur appears in the thermal water of the Rose Hill but more enhanced in the form of H_2S [8]. Consequently, our hypothesis was that both the morphological structure and genetic diversity of biofilm communities formed on the carbonate rock surfaces of the springs located in the Gellért Hill discharge zone differ from that of Rose Hill of BTKS. Therefore, the aim of this research was to explore and compare the bacterial and archaeal composition and morphological structures of biofilms developed on the carbonate rock surfaces in springs for the Gellért Hill discharge zone, Budapest.

2 MATERIALS AND METHODS

2.1 Description of the sampling sites

Some thermal springs of the Gellért Hill area used for therapeutic purposes were mentioned in documents originated from the 13th century. Nevertheless, the first prosperity of the so-called Turkish spas located, at the right side of river Danube was in the 16th century. These famous baths, today called Gellért, Rudas and Rác Spas were established at the discharge area of deep groundwater flow systems, and were supplied in the past by water of the spring group of Gellért Hill. The location and overview map with sampling points of the BTKS are

presented elsewhere [7]. Since the late 1970s, the springs have been drained in the artificial tunnel of the Gellért Hill, and four operating wells were drilled which provided water for the Spas of Gellért and the Rudas. The Rác Spa has not been operating since 2002. However, the original springs of the area are remained, they were captured, and their water is collected and diverted to the Danube. Four of them were involved into the study: the so called Gellért-Ősforrás, the Rudas-Török and the Diana-Hygieia springs belonging to the Rudas Spa and the Nagy spring of Rác Spa. The water of most springs flows from the Triassic-dolomite, while the Nagy spring emerges from the enlarged fracture of the Upper Eocen Buda Marl Formation [5].

For microbiological research of this study, biofilm samples developed on the carbonate rock surfaces of thermal springs were collected as described by Borsodi et al. [9] from the Rudas-Török spring cave (RT), the Diana-Hygieia thermal spring (DH), the Rác Spa Nagy spring (RN) and the Gellért-Ősforrás (GO).

2.2 Determination of physical and chemical parameters of the water

The temperature, pH, electric conductivity and dissolved oxygen concentration of the thermal water were measured using a Multi 350i Portable Multi Meter (WTW GmbH, Weilheim, Germany). For the determination of salinity, samples were evaporated and dried at 105 °C to constant weight, and the resulting residue was used to calculate sample salinity. All other parameters were determined according to standard methods [15]. Alkalinity (ASTM2320-B), hardness (ASTM 2340-C), and the concentration of chloride (4500-Cl^--B) were measured by titrimetric methods. Ammonium (ASTM 4500-NH_3-D), iron (3500-Fe-D), nitrite ion (ASTM 4500-NO_2^--B), nitrate ion (ASTM4500-NO_3^--B), and sulfate ion (ASTM 4500-SO_4^{2-}-E) were measured photometrically. Orto phosphate was determined by ascorbic acid method (ASTM 4500-P-E). The concentration of total organic carbon (TOC), total inorganic carbon (TIC), and total bounded nitrogen (TN) was determined by a Multi N/C 2100S analyzer (Analytik Jena, Germany). During TOC measurements, the TIC content of the previously

122 acidified samples (pH 2 was set by 1 M sulfuric acid) was purged with oxygen to enhance the
123 measurement of the relatively low organic carbon content in sample.

124 **2.3 Scanning electron microscopy**

125 For scanning electron microscopy (SEM), biofilm samples were filtered onto 0.2 μm
126 polycarbonate filter (Millipore), and fixed in glutaraldehyde (5% in 0.1 M phosphate buffer)
127 for 3-4 h at room temperature. The fixed samples were rinsed twice with phosphate buffer
128 solution (pH 7), shock frozen in liquid nitrogen and freeze-dried (until 2 x 10-2 mbar, at -60 °C
129 for 6-8 h). After lyophilization, the dried samples were mounted on metal stubs, and sputter-
130 coated with gold. The samples were examined using an EVO MA 10 Zeiss scanning electron
131 microscope at an accelerating voltage of 10 kV.

132 **2.4 Bacterial DNA extraction and PCR amplification**

133 The community DNA from the biofilm samples was isolated using Ultra Clean Soil Kit
134 (MO Bio Inc., CA, USA) according to the manufacturer's instructions, detected in 1% agarose
135 gel stained with ECO Safe Nucleic Acid Staining Solution (Avegene, Taiwan) and visualized
136 by UV excitation. The 16S rRNA gene was amplified by PCR using Bacteria-specific 27 f (5'-
137 AGAGTTTGATCMTGGCTCAG-3') and 1492 r (5'-TACGGYTACCTTGTTACGACTT-3')
138 primers [16], and Archaea-specific A109 f (5'-ACKGCTCAGTAACACGT-3') and A958 r
139 (5'-YCCGGCGTTGAMTCCAATT-3') primers [17]. The following temperature protocol was
140 used for bacterial PCR: initial denaturation at 98°C for 3 min, followed by 32 cycles of
141 denaturation at 94°C for 30 s, annealing at 52°C for 30 s and elongation at 72°C for 90 s, and a
142 final extension at 72°C for 30 min. For the archaeal PCR, a touch-down temperature protocol
143 was used: initial denaturation at 98°C for 3 min, 20 cycles of denaturation at 94°C for 30 s,
144 annealing at 60°C for 30 s (in each cycle, the annealing temperature was decreased by 0.5°C)
145 and elongation at 72°C for 90 s followed by 15 cycles of denaturation 94°C for 30 s, annealing
146 at 50°C for 30 s and elongation at 72°C for 90 s and a final extension at 72°C for 30 min. The

147 PCR reaction mixture contained 200 mM of each deoxynucleoside triphosphate, 1 U of LC *Taq*
148 DNA Polymerase (recombinant) (Fermentas, Lithuania), 1 x *Taq* buffer with $(NH_4)_2SO_4$
149 (Fermentas, Lithuania), 2 mM $MgCl_2$, 0.65 mM of each primer, and about 20 ng of genomic
150 DNA template in a total volume of 50 µL.

151 **2.5 Construction of 16S rRNA gene based clone libraries**

152 The PCR products were purified using the EZ-10 Spin Column PCR Purification Kit
153 (Bio Basic, Canada), ligated into a TA-cloning vector (pGEM-T Vector System, Promega, WI,
154 USA), and transformed into competent *E. coli* JM109 cells. The transformed cells were spread
155 on LB agar plates containing 100 µg ml^{-1} ampicillin, 80 µg ml^{-1} X-Gal and 0.5 mM IPTG and
156 incubated overnight at 37°C. Recombinant plasmids were extracted from the *E. coli* cells by
157 incubating the cultures at 98°C for 5 min, and pelleting the cell fragments by centrifugation
158 with 4500 rcf for 5 min. Insert sequences were amplified with standard primers M13 f (5'-
159 GTAAAACGACGGCCAGT-3') and M13 r (5'-CAGGAAACAGCTATG-3') primers [18]
160 followed by a nested PCR with 27 f and 1492 r as well as A109 f and A958 r primers. The
161 thermal profiles of PCRs were the same as described previously.

162 PCR amplicons were grouped based on their Amplified Ribosomal DNA Restriction
163 Analysis (ARDRA) patterns produced with enzymes *Msp*I and *Bsu*RI (Fermentas, Lithuania)
164 as described by Massol-Deya et al. [19] Digestion products were separated in 2% agarose gel,
165 stained with ECO Safe Nucleic Acid Staining Solution (Avegene, Taiwan) and visualized by
166 UV excitation using a Micromax CCD camera.

167 **2.6 Sequencing and identification of molecular clones**

168 The partial 16S rRNA sequencing of the selected ARDRA representatives was
169 performed with the 27 f (Bacteria specific) and A109 f (Archaea specific) primers using the
170 automated Sanger-method by LGC Ltd (Berlin, Germany). The quality of chromatograms was
171 checked with the help of the Chromas software, and low-quality ends were trimmed

(Technelysium Pty Ltd., Australia). Taxonomic relationships of the sequences were determined using the EzBioCloud database [20] and Basic Local Alignment and Search Tool (BLAST) program [21]. Maximum Likelihood phylogenetic trees based on the V1-V4 region of the 16S rRNA gene were constructed by MEGA 7.0 software [22] after ClustalW alignment, using Kimura 2-parameter model and Bootstrap method which was set to 500 replications. Sequences giving the highest similarity to ours after the alignment by EzTaxon-e and type strains of the genera were chosen as references.

The 16S rRNA gene sequences (in average 800-900 bp long) were deposited into the GenBank under accession numbers LN680106-LN680152 and HG974481-HG974492 for the Diana-Hygieia Bacteria (DHB) clones, LN680153-LN680225 for the Gellért-Ősforrás Bacteria (GOB) clones, LN680226-LN680256 for the Rác-Nagy Bacteria (RNB) clones, LK936198-LK936243 for the Rudas-Török Bacteria (RTB), LN864926-LN864934 for the Diana-Hygieia Archaea (DHA) clones, LN864935-LN864948 for the Gellért-Ősforrás Archaea (GOA) clones, LN864949-LN864962 for the Rác-Nagy Archaea (RNA) clones, LN864963-LN864971 for the Rudas-Török Archaea (RTA) clones.

To reveal the correlation between bacterial diversity of biofilms and abiotic characteristics of the water, environmental factors were fitted as vectors using "envfit" function of vegan (package vegan) onto the Bray-Curtis similarity index based NMDS (Non-Metric Multidimensional Scaling) ordination of relative abundance of bacterium phyla and Proteobacteria classes. The significance of fittings was tested by random permutations in R programming environment [23, 24].

3 RESULTS

3.1 Physical and chemical characterization of the water samples

The measured physical and chemical parameters of the thermal waters are shown in Table 1. The water temperature ranged between 29.1 °C and 38.7 °C in the studied four springs.

From the on-site measurement results, the pH was circum-neutral (the mean ± SD value was 6.8±0.1), and the average electric conductivity was 1794±99 μS cm^{-1}. The thermal waters were nearly anoxic due to the low dissolved oxygen levels (an average of 2.3±1.7 mg l^{-1}). All water samples were dominated by sulfate (354±15 mg l^{-1} on average) and chloride anions (129±13 mg l^{-1} on average), and were relatively low in nitrogen forms and orthophosphate ions (the average TN and PO_4^{3-}values were 0.5±0.3 mg l^{-1} and 0.4±0.7 mg l^{-1}, respectively). The total organic carbon content of the well waters was also low (2.6±2.6 mg l^{-1} on average). Among the sampling sites no significant differences were found in the alkalinity, salinity and hardness (the average values were 8.1±0.5 mval l^{-1}, 1232±24 mg l^{-}1 and 32.6±1.6 nK°, respectively). The water physical and chemical profile of the studied Gellért Hill discharge zone is considerably differed from the Rose Hill area of BTKS. It can be traced back mainly to the dissimilarity in the water temperature, sulfate concentration, salinity and electric conductivity values [11,12].

The microchemical characterization of biogeochemical precipitates collected from the two sampling sites (Gellért Ősforrás and Rác Spa Nagy spring) has been published by Dobosy et al. [7]. From the other two sites (Diana-Hygieia thermal spring and Rudas-Török spring cave) the amount of the available material was not enough for such analysis.

3.2 Scanning electron microscopic observations

Scanning electron microscopy (SEM) was used to examine the morphological structure of mucilaginous, reddish-brown colored biofilms from different sampling sites. The low magnification scanning electron microscope images showed that network architecture structure formed by filamentous bacteria and other cells with mineral crystals embedded in extracellular polymeric substances (EPS) (e.g. Fig. 1A, C, E and G). The high-resolution SEM images reflected the morphological variability of the biofilm forming bacterial cell assemblages. The Gellért-Ősforrás (Fig. 1C, D), Diana-Hygieia (Fig. 1G, H) and Rudas-Török (Fig. 1A, B) spring

samples contained numerous filamentous structures, and their morphology structure was similar to that produced by the known iron-oxidizing bacteria (FeOB). Sheath-forming morphotypes similar to *Leptothrix* were common in the studied samples. *Leptothrix* species (Betaproteobacteria), members of Fe/Mn-oxidizing bacteria, are capable of oxidizing Fe(II) and producing extracellular, microtubular, Fe-encrusted sheaths. *Leptothrix*-like fragmented filamentous structures often can be seen as hollow constructions (Fig. 1A and C). Unusually large reticulated, prokaryotic filaments (Fig. 1C and H) were detected in the Gellért-Ősforrás sample, and these structures were also abundant in the Diana-Hygieia thermal spring sample. Spiral-shaped bacteria, typical of *Nitrospira* were also observed in the microscopic images (Fig. 1D) from the Gellért-Ősforrás sample. The higher magnification micrographs of filamentous microbial biofilms (Fig. 1C, G and H) clearly showed that characteristic, reticulated filaments (approximately 0.6 μm in diameter) can be found among the filamentous forms. Anda et al. [13] previously detected these reticulated formations from the Diana-Hygieia thermal spring sample. Based on the results of microscopic and analytical techniques used for the chemical and morphological characterization of these reticulated filaments, they can be regarded as biogenic [13,25]. In the Rác-Nagy thermal spring sample, microbial biofilm was made up of thin (0.2-0.3 μm in diameter) filamentous structures (Fig. 1E-F).

3.3 Molecular clones of Bacteria

From the biofilms developed in the thermal karst springs four bacterial clone libraries (GOB, DHB, RTB and RNB) were constructed. Following the ARDRA grouping, 208 representatives (GOB: 73; DHB: 58; RTB:47; RNB: 30) were sequenced and identified (Supplementary Figures 1-4) from the altogether 510 molecular clones (GOB: 124; DHB: 131; RTB: 123; RNB: 132). In the studied biofilm samples, members of 14 different phyla (Chloroflexi, Nitrospirae, Cyanobacteria, Chlorobi, Proteobacteria, Firmicutes, Actinobacteria, Acidobacteria, Bacteroidetes, Armatimonadetes, Spirochaetes, Caldithrix, Gemmatimonadetes

and Elusimicrobia), 7 candidate phyla (Gracilibacteria, Parcubacteria, Acetothermia, Omnitrophica, Aminicenantes, Saccharibacteria and Latescibacteria), formerly candidate divisions (GN02, OD1, OP1, OP3, OP8, TM7 and WS3) and 2 candidate divisions (GN04 and WS1) were detected (Fig. 2). At phylum level the highest diversity was revealed from the Gellért-Ősforrás (GO) sample (with 14 phylogenetic divisions) whereas the genetic diversity was the lowest (with 10 phylogenetic divisions) in the Rác Spa Nagy spring (RN) sample. Sequences belonging to phyla Proteobacteria (29%), Chloroflexi (28%) and Nitrospirae (16%) dominated the clone libraries but their distribution differed in each sample. Among the molecular clones affiliated with the phylum Proteobacteria, members of classes Beta- (11%) and Deltaproteobacteria (11%) were the most represented. The occurrence of sequences related to phyla Acidobacteria (5%) and Gemmatimonadetes (2%) was also typical, apart from the Rác Spa Nagy spring (RN) sample. However, members of Cyanobacteria (1%) and Chlorobi (<1%) were present only in the Gellért-Ősforrás (GO) sample and in low abundance. The relative proportion of clone sequences related to Firmicutes and Actinobacteria was also low (1% and 2%, respectively). Concerning the abundance of candidate divisions, their proportion was less than 1%, except for OP3 characteristic to Diana-Hygieia thermal spring (DH) and Rudas-Török spring cave (RT) samples.

3.4 Molecular clones of Archaea

In the four archaeal clone libraries (GOA, DHA, RTA and RNA) constructed from the biofilms of the thermal karst springs, the altogether 374 molecular clones (GOA: 94; DHA: 95; RTA: 93; RNA: 92) resulted in 46 ARDRA groups (GOA: 14; DHA: 9; RTA: 9; RNA: 14) (Supplementary Figure 5). The overall distribution of sequences at phylum level ranged from 82% for Thaumarchaeota, 16% for Euryarchaeota and 2% for Crenarchaeota (Fig. 3). The diversity of archaeal clone libraries was dominated by phylum Thaumarchaeota, except for the Rác Spa Nagy spring (RN) sample where sequences belonging to Euryarchaeota were the most

abundant. Phylotypes affiliated with phylum Crenarchaeota were also present only in the Rác Spa Nagy spring (RN) sample.

4 DISCUSSION

Biofilms developed on the carbonate rock surfaces in the studied thermal springs of Gellért Hill discharge areas showed high morphological and taxonomic diversity based on the electron microscopic and molecular cloning results. A high portion of the molecular clones exhibited the highest sequence matches to environmental clones from similar habitats (e.g. karst spring waters, microbial biofilm from cave systems, iron rich microbial mats, hot springs). Nevertheless, hardly any molecular clones could be identified at species or genus levels (Supplementary Figures 1-5) because the 16S rRNA gene sequence matches were far below the accepted cut-off values [26]. All these suggest that several uncultivated prokaryotes are present in the biofilms developed on the carbonate rock surfaces in the thermal springs (Supplementary Figures 1-5) similarly those found in other cave environments [27-29].

Prevalence of the higher taxonomic ranks of prokaryotes as phyla Proteobacteria, Chloroflexi and Nitrospirae (Bacteria) and Thaumarchaeota (Archaea) was common in all four biofilm samples (Fig. 2 and 3). Although all studied thermal springs belong to the Gellért Hill of the BTKS based on their hydrogeological properties, both the distribution of molecular clones and the morphological structure of biofilms showed differences according to the sampling sites (Figs. 1-3). The observed differences in the composition and organization of biofilms primarily can reflect the type of host rock and different water exchange, i.e. volume discharge of the springs. The distribution of dominant bacterial and archaeal taxa and the arrangements of prokaryotic cells in the biofilms were the most similar in the adjacent Rudas-Török spring cave (RT) and Diana-Hygieia thermal spring (DH) (Fig. 2 and 3). However, there was an anticorrelation with the geographical distance, as the abundance of phyla Proteobacteria decreased while Chloroflexi increased from Gellért-Ősforrás (GO) spring towards Rác Spa

297 Nagy spring (RN) spring. The largest deviation was found in the Rác Spa Nagy spring (RN),
298 the water of which comes from the Buda Marl Formation. The lowest bacterial and the highest
299 archaeal diversity together with the thinnest and the least structured biofilm were observed in
300 the Rác Spa Nagy spring (RN) sample. In contrast, the Gellért-Ősforrás (GO) was characterized
301 by the highest bacterial diversity and the morphologically most complex biofilm formation.
302 Therefore, it can be assumed that at the sampling sites not only the different physical and
303 chemical characteristics and the flow rate of the thermal waters but the parent rock from which
304 the thermal waters discharge, can also influence the appearance and composition of biofilms.
305 According to the results of NMDS analysis of bacterial phyla and Proteobacteria classes
306 (Fig. 4), similar separation of the sampling sites was observed as obtained by the UPGMA
307 dendrogram (Fig. 2.) However, fitting environmental characteristics onto the NMDS plot did
308 not reveal any significant ($p<0.05$) parameter that could correlate well with the separation
309 pattern of the samples.

310 Similarly, to other aphotic karst cave environments in the world [2,4,30,31], the BTKS
311 biofilms were populated mainly by chemoorganotrophic and chemolithotrophic prokaryotes
312 belonging to phyla Proteobacteria and Chloroflexi as well as Nitrospirae. However, in the
313 BTKS samples where circum-neutral pH values were measured in the thermal water
314 surrounding the biofilms, phylotypes closely related to *Thiobacillus* species
315 (Betaproteobacteria) represented the chemolithotrophic sulfur-oxidizing bacteria unlike the
316 extremely acidic hypogenic caves (e.g. Lechuguilla Cave and Carlsbad Cavern, New Mexico;
317 Frasassi cave system, Italy) where the sulfuric acid speleogenesis is driven by higher diversity
318 of sulfur-oxidizing bacteria belonging to Beta-, Gamma- and Epsilonproteobacteria [1,2,4,32].
319 Nevertheless, in accordance with the low oxygenation and the high sulfate concentrations of
320 the thermal waters in the Southern discharge area of BTKS, high diversity of phylotypes
321 affiliated with the anaerobic sulfur-, sulfate-, nitrate- and iron(III)-reducing taxa (e.g.

Deferribacter, *Desulfobacter*, *Desulfuromonas*, *Deferrisoma*) of Deltaproteobacteria was found in the biofilm samples. The portion of chemoorganotrophic Alpha- and Gammaproteobacteria was less than 5% in the BTKS samples except for the Gellért-Ősforrás (GO) biofilm where high number of phylotypes closely related to Rhodospirillales (Alphaproteobacteria) capable of anaerobic fermentative metabolism in the dark was uncovered.

Members of the phylum Chloroflexi are frequently retrieved from thermal waters and cave environments [25,33,34], and dominated the bacterial community of Molnár János cave [12], as well. In this hypogenic cave the host rock is the Upper Eocen Buda Marl Formation similarly to that found in the Rác Spa Nagy spring (RN) of Gellert Hill discharge area. Molecular clones affiliated with the Anaerolineae were detected in all four biofilm samples of the Southern part of BTKS. The typical multicellular filaments of Anaerolineae were also observed by electron microscopy. The thermophilic, strictly anaerobic chemoorganotrophic lifestyle of this classis [35] is well suited to the conditions provided by the BTKS. It can be assumed that phylotypes of Anaerolineae are permanent members of the Hungarian thermal karst systems, as besides the present research, representatives were uncovered from the thermal water of Harkány, Villány Mountains [36] and biofilms formed in the Városliget-II well of Széchenyi Thermal Bath [11] and Molnár János cave [12].

In the new millennium, a growing number of studies report the presence of phylotypes belonging to phylum Nitrospirae in subsurface karst environments [3,29,30] including the Molnár János cave belonging to the Rose Hill area of BTKS [12]. In the present study, molecular clones closely related to all three known metabolic types of Nitrospirae (the autotrophic nitrite-oxidizing Nitrospirales, the anaerobic methane-oxidizing Methylomirabilis and the anaerobic, thermophilic, sulfate-reducing Thermodesulfovibrio) were detected, the highest proportions in the adjacent Diana-Hygieia thermal spring (DH) and Rudas-Török spring

cave (RT) biofilm samples. It is interesting to note that the strain of *Candidatus* Nitrospira inopinata species isolated from a biofilm of a geothermal spring (Aushiger, North Caucasus, Russia) can perform the complete nitrification (ammonia oxidation to nitrate) [37,38]. Based on the high similarity of the habitats and the common occurrence of Nitrospirae with ammonia-oxidizing bacteria and archaea, it can be assumed that comammox organisms may also be present in biofilms of BTKS. This highlights the importance of the high variety of microbial metabolic processes taking part in the carbon-, nitrogen- and sulfur cycles in such a low autochthonous organic carbon containing, nitrogen limited but sulfate rich environment (Table 1).

Due to the lack of light, no phototrophic prokaryotes were detected three out of the four biofilms in the studied wells. Presence of Cyanobacteria and Chlorobi was observed only in the Gellért-Ősforrás (GO) sampling site where sometimes artificial lighting happens due to the operational interventions. It is interesting to note that members of the detected Ignavibacteriae (Chlorobi) appear to be capable of dissimilatory iron reduction [39].

The general occurrence of *Caldithrix* related phylotypes in almost all studied biofilm samples can be traced back to the special hydrogeological characteristics (e.g. the high temperature water from the deep regional flow system) in the Southern part of BTKS [6,8]. According to our knowledge, representatives of the thermophilic anaerobic chemoorganotrophic bacteria of this phylogenetic lineage were retrieved mainly from different geothermally heated and/or active volcanic environments [40,41] but not from hypogene, thermal water affected karst ecosystems to date.

A relatively high diversity of phylotypes related to Acidobacteria was present in the biofilms of those Gellért Hill springs discharged from the Triassic-dolomite (Gellért-Ősforrás, Rudas-Török spring cave and Diana-Hygieia thermal spring samples). According to other cultivation independent geomicrobiological studies, Acidobacteria constitutes a decisive

proportion of the karst microbial communities [2,3,31,42] but their eco-physiological role is largely unknown due to the very small number of cultivated strains.

Bacterial molecular clones of Gellért Hill discharge area represented a large variety of candidate phyla (Gracilibacteria, GN02; Parcubacteria, OD1; Acetothermia, OP1; Omnitrophica, OP3; Aminicenantes, OP8; Saccharibacteria, TM7; Latescibacteria, WS3) and divisions (GN04 and WS1), even though the abundance of these novel lineages was low in the studied biofilms (except for the Diana-Hygieia thermal spring sample where the ratio of OP3 was greater than 5%). Similar high microbial phylotype richness of the deeply branching OP3 lineage was described only from shallow pools of a Swiss karst cave system [42], so far.

The diversity of Archaea in karst cave environments is still largely unexplored, compared to Bacteria [25,41,43,44]. In the present study, phylotypes related to the deep-branching phylum of Thaumarchaeota dominated the biofilms in three out of the four samples. The results enhance the potential importance of aerobic ammonia-oxidation (AOA) in the biofilms of Triassic-dolomite springs (Gellért-Ősforrás, Rudas-Török spring cave and Diana-Hygieia thermal spring samples) in the Southern part of the BTKS. These molecular clones showed the highest sequence matches to *Nitrososphaera viennensis* [45] and "*Candidatus* Nitrososphaera gargensis" [46] similarly to Molnár János cave [12]. In the Rác Spa Nagy spring biofilm sample originated from the Buda Marl Formation, more than 50% of the molecular clones was members of the phylum Euryarchaeota but they could not be identified more precisely because of the low sequence matches to known taxa. The other part of molecular clones of Rác Spa Nagy spring was closely related to environmental clones of phylum Thaumarchaeota and Crenaechaeota revealed also from phreatic limestone sinkholes in cenote La Palita, Mexico [47].

According to the results, both the morphological structure and the composition of biofilms developed on the carbonate rock surfaces of thermal springs, especially for the Gellért

Hill discharge area are greatly influenced by the groundwater flow systems, the discharging thermal water with basinal fluids and the type of the host rock and the flow rate, i.e. water exchange. In addition, molecular clones of this study showed the highest sequence matching to uncultured clones from karst cave and thermal spring environments, reflecting the special hydrogeological characteristics of the Southern discharge area of BTKS. Based on the known metabolic properties of closely related species, it is presumable that thermophilic, anaerobic sulfur-, sulfate-, nitrate- and iron(III)-reducing chemoorganotrophic as well as sulfur-, ammonia- and nitrite-oxidizing chemolithotrophic prokaryotes form complex metabolic networks in the studied biofilms adapting to the unique and extreme environmental circumstances.

ACKNOWLEDGEMENTS

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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547

548 **Figure legends**

549 **Figure 1**. Comparison of SEM images of mucilaginous, reddish-brown colored biofilms from
550 Rudas-Török spring cave (A, B), Gellért-Ősforrás (C, D), Rác Spa Nagy spring (E, F) and
551 Diana-Hygieia thermal spring (G, H). Morphotypes similar to *Leptothrix*, reticulated filaments
552 and spiral-shaped bacteria are indicated by orange, red and yellow arrows, respectively.

553 **Figure 2**. Distribution of phylotypes among bacterial phyla, candidate phyla and divisions
554 based on the 16S rRNA gene sequences of clone libraries constructed from the biofilms formed
555 on the karst rock surfaces in the wells of Budapest thermal spas (Sample abbreviations: Gellért-
556 Ősforrás, GOB; Diana-Hygieia thermal spring, DHB; Rudas-Török spring cave, RTB; Rác Spa
557 Nagy spring, RNB)

558 **Figure 3**. Distribution of phylotypes among archaeal phyla based on the 16S rRNA gene
559 sequences of clone libraries constructed from the biofilms formed on the karst rock surfaces in
560 the wells of Budapest thermal spas (Sample abbreviations: Gellért-Ősforrás, GOA; Diana-
561 Hygieia thermal spring, DHA; Rudas-Török spring cave, RTA; Rác Spa Nagy spring, RNA)

562 **Figure 4** Two dimensional non-metric multidimensional scaling (NMDS) plot of bacterium
563 phyla and Proteobacteria classes obtained from the biofilms of the thermal karst springs. The
564 environmental factors were fitted as vectors onto the PCA ordination. (Stress<0.1)

565 **Supplementary Figure 1a** Maximum likelihood phylogenetic tree based on the 16S rRNA
566 gene sequence data of Alpha- and Betaproteobacteria molecular clones from the biofilms
567 developed in thermal karst springs of Gellért Hill discharge area (Hungary). (Representative
568 molecular clones sequenced in this study appear in bold. The number of members of the
569 ARDRA groups is indicated after the representative molecular clones. U. means uncultured
570 molecular clones.)

571 **Supplementary Figure 1b** Maximum likelihood phylogenetic tree based on the 16S rRNA
572 gene sequence data of Gamma- and Deltaproteobacteria molecular clones from the biofilms

developed in thermal karst springs of Gellért Hill discharge area (Hungary). (Representative molecular clones sequenced in this study appear in bold. The number of members of the ARDRA groups is indicated after the representative molecular clones. U. means uncultured molecular clones.)

Supplementary Figure 2 Maximum likelihood phylogenetic tree based on the 16S rRNA gene sequence data of Chloroflexi, Chlorobi and Cyanobacteria molecular clones from the biofilms developed in thermal karst springs of Gellért Hill discharge area (Hungary). (Representative molecular clones sequenced in this study appear in bold. The number of members of the ARDRA groups is indicated after the representative molecular clones. U. means uncultured molecular clones.)

Supplementary Figure 3 Maximum likelihood phylogenetic tree based on the 16S rRNA gene sequence data of Nitrospira and Acidobacteria molecular clones from the biofilms developed in thermal karst springs of Gellért Hill discharge area (Hungary). (Representative molecular clones sequenced in this study appear in bold. The number of members of the ARDRA groups is indicated after the representative molecular clones. U. means uncultured molecular clones.)

Supplementary Figure 4 Maximum likelihood phylogenetic tree based on the 16S rRNA gene sequence data of other bacterial molecular clones from the biofilms developed in thermal karst springs of Gellért Hill discharge area (Hungary). (Representative molecular clones sequenced in this study appear in bold. The number of members of the ARDRA groups is indicated after the representative molecular clones. U. means uncultured molecular clones.)

Supplementary Figure 5 Maximum likelihood phylogenetic tree based on the 16S rRNA gene sequence data of Archaea molecular clones from the biofilms developed in thermal karst springs of Gellért Hill discharge area (Hungary). (Representative molecular clones sequenced in this study appear in bold. The number of members of the ARDRA groups is indicated after the representative molecular clones. U. means uncultured molecular clones.)

Figure 1.

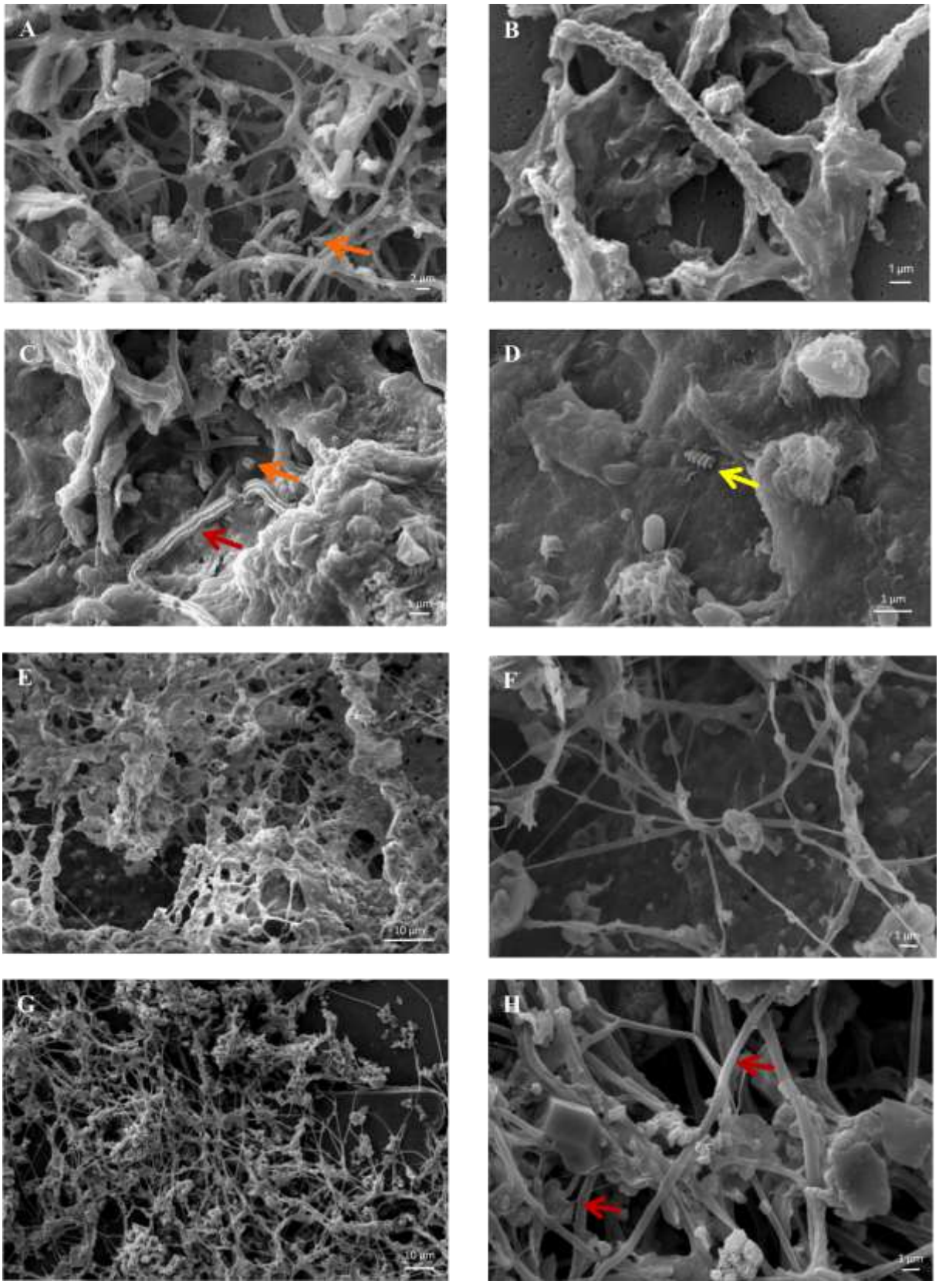


Figure 2.

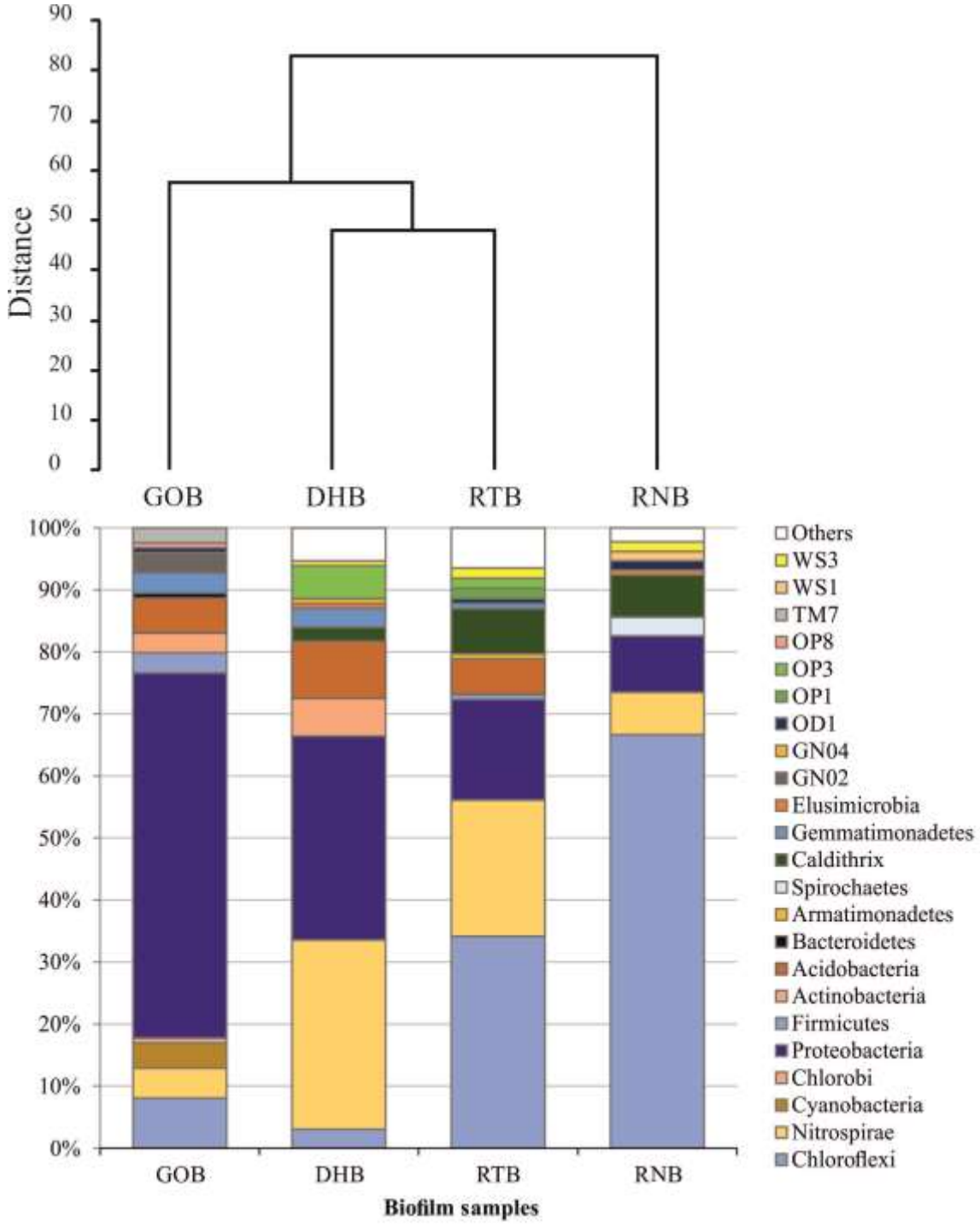
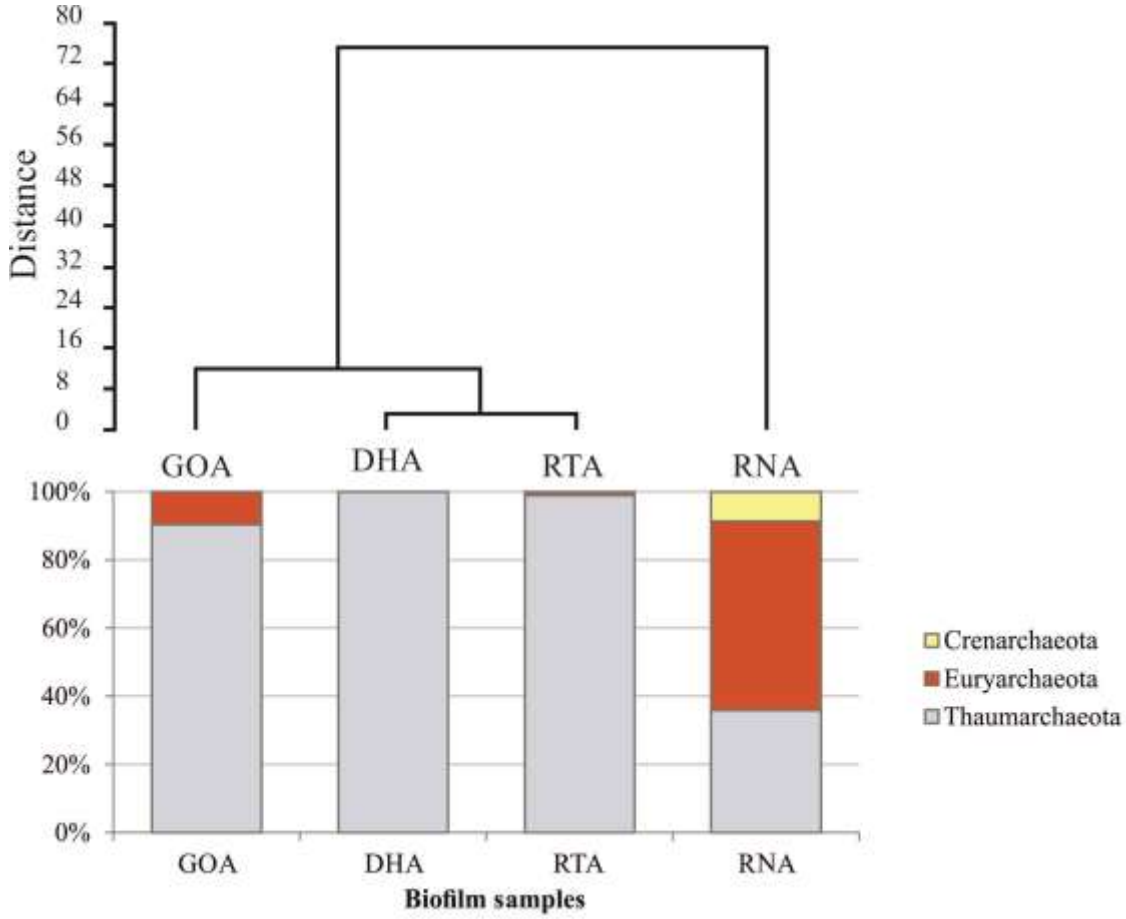
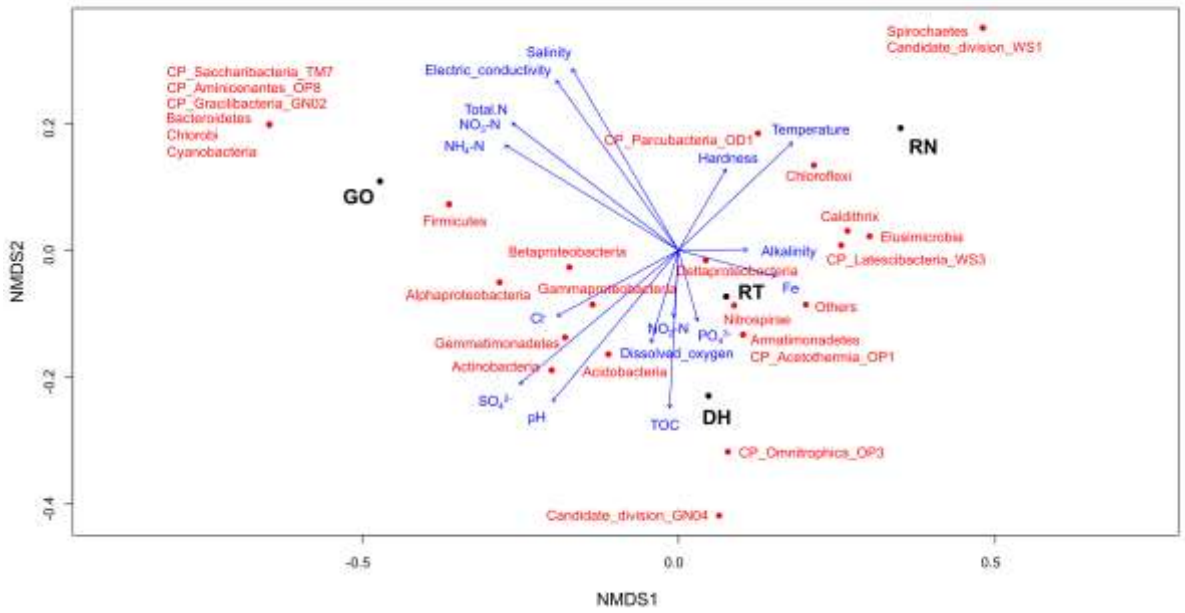

Distance
90
80
70
60
50
40
30
20
10
0
GOB
DHB
RTB
RNB
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Others
WS3
WS1
TM7
OP8
OP3
OP1
OD1
GN04
GN02
Elusimicrobia
Gemmatimonadetes
Caldithrix
Spirochaetes
Armatimonadetes
Bacteroidetes
Acidobacteria
Actinobacteria
Firmicutes
Proteobacteria
Chlorobi
Cyanobacteria
Nitrospirae
Chloroflexi
GOB
DHB
RTB
RNB
Biofilm samples

Figure 3.


Distance
80
72
64
56
48
40
32
24
16
8
0
GOA
DHA
RTA
RNA
100%
80%
60%
40%
20%
0%
GOA
DHA
RTA
RNA
Biofilm samples
Crenarchaeota
Euryarchaeota
Thaumarchaeota

607 **Figure 4.**



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609

610 **Table 1.** Physical and chemical characteristics of the water samples taken from the wells of
611 Budapest thermal spas (Sample abbreviations: Gellért-Ősforrás, GO; Diana-Hygieia thermal
612 spring, DH; Rudas-Török spring cave, RT; Rác Spa Nagy spring, RN)

	GO	DH	RT	RN
Temperature (°C)	29.6	29.1	38.7	37.6
pH	6.8	7.0	6.8	6.7
Alkalinity (mval l^{-1})	7.8	7.7	8.9	8.1
Salinity (mg/L)	1266	1212	1218	1232
Electric conductivity (μS/cm) 20°C	1908	1708	1715	1845
Hardness (nK°)	31.9	30.8	34.5	33
Dissolved oxygen (mg/L)	2.6	4.3	0.3	1.8
Total N (mg/L)	0.9	0.4	0.4	0.4
NH_4^+-N (mg/L)	0.14	<0.01	0.06	<0.01
NO_2^--N (mg/L)	0.017	0.011	0.076	<0.001
NO_3^--N (mg/L)	0.5	<0.2	<0.2	<0.2
Cl^- (mg/L)	137	122	142	114
SO_4^{2-} (mg/L)	369	362	350	336
PO_4^{3-} (mg/L)	0.01	0.09	1.42	<0.01
Fe (mg/L)	0.08	0.18	0.04	0.17
TOC (mg/L)	1.8	6.4	0.8	1.1

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614

615 **Supplementary Figure 1a.**

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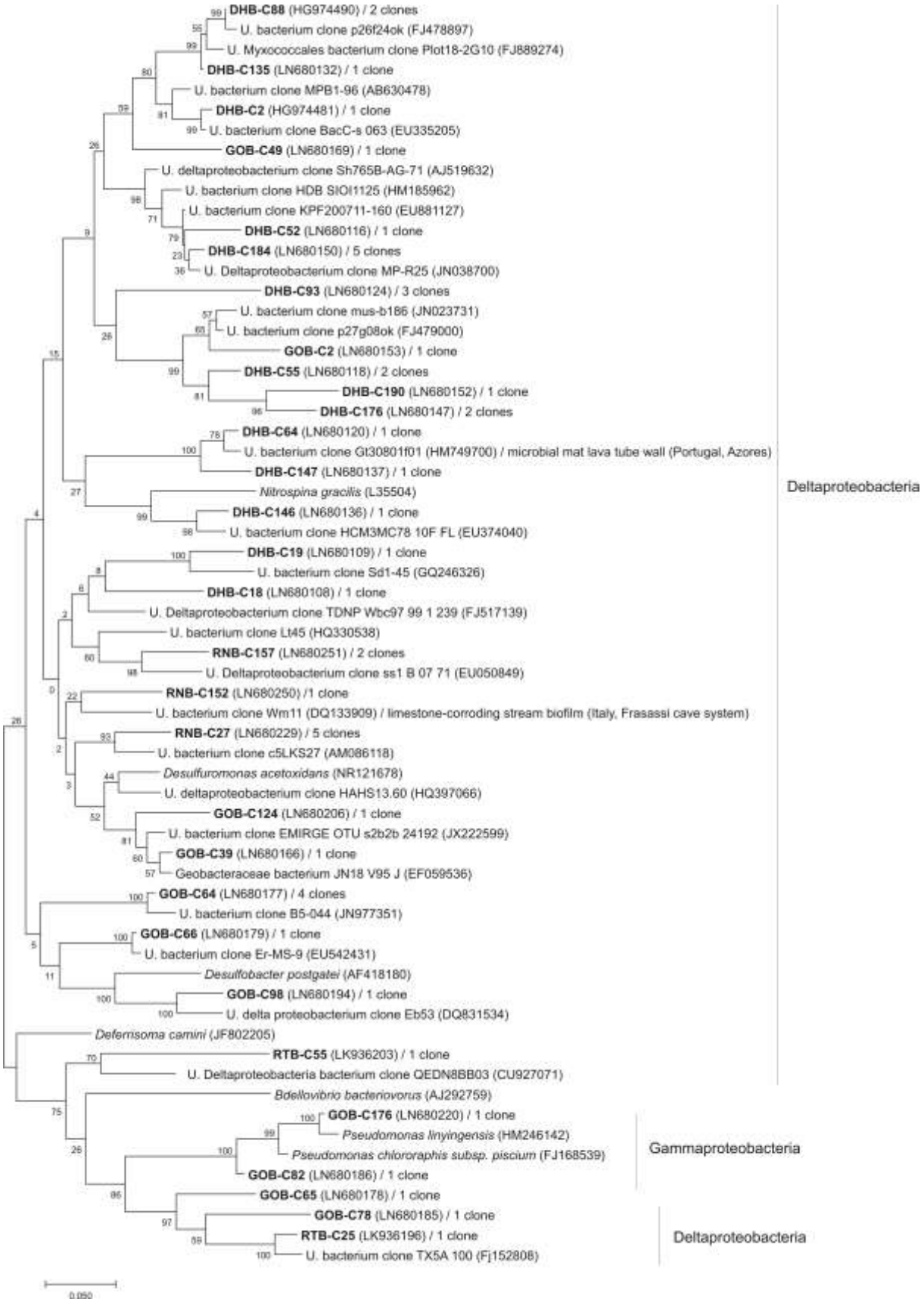
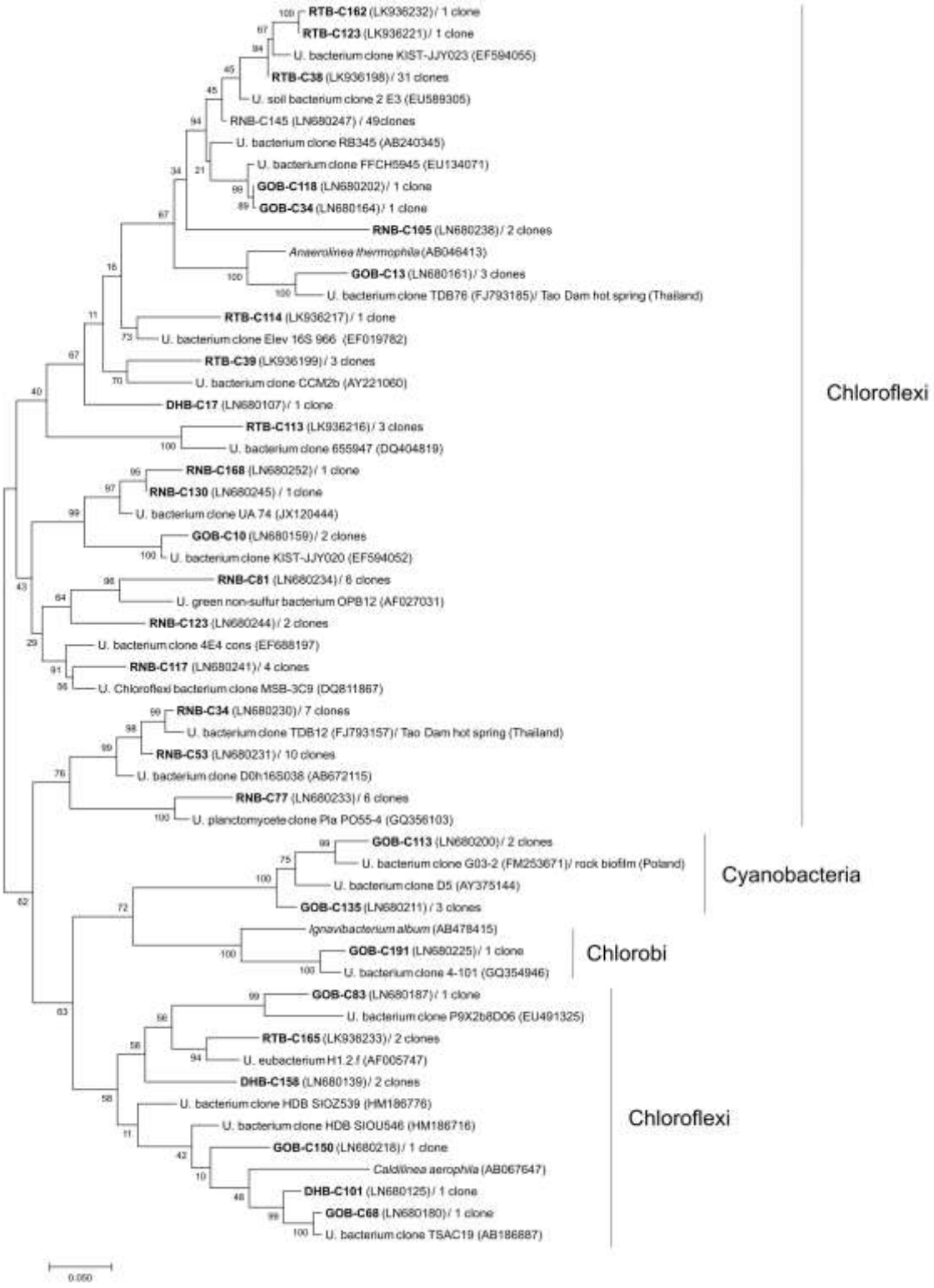
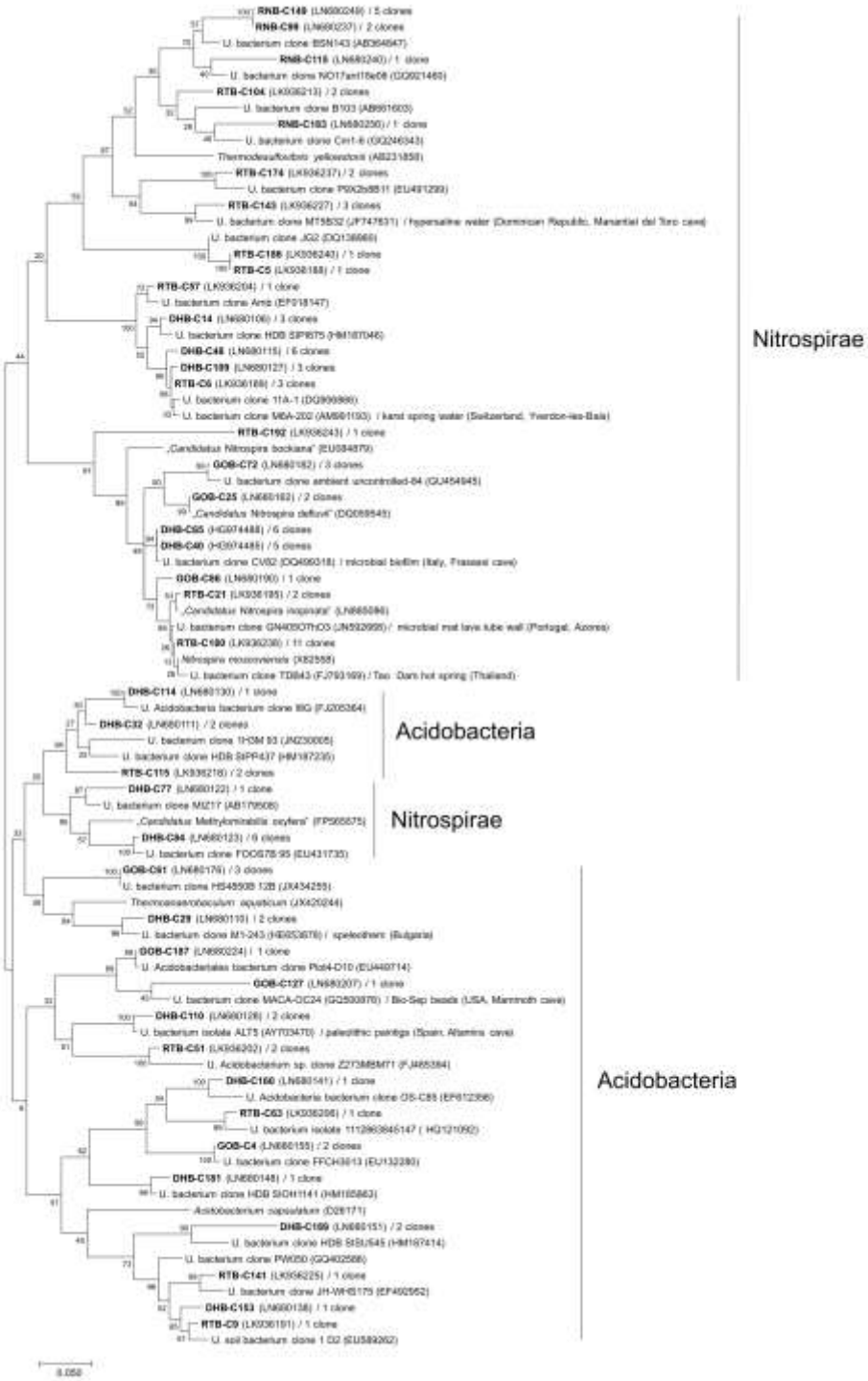
Supplementary Figure 1b.



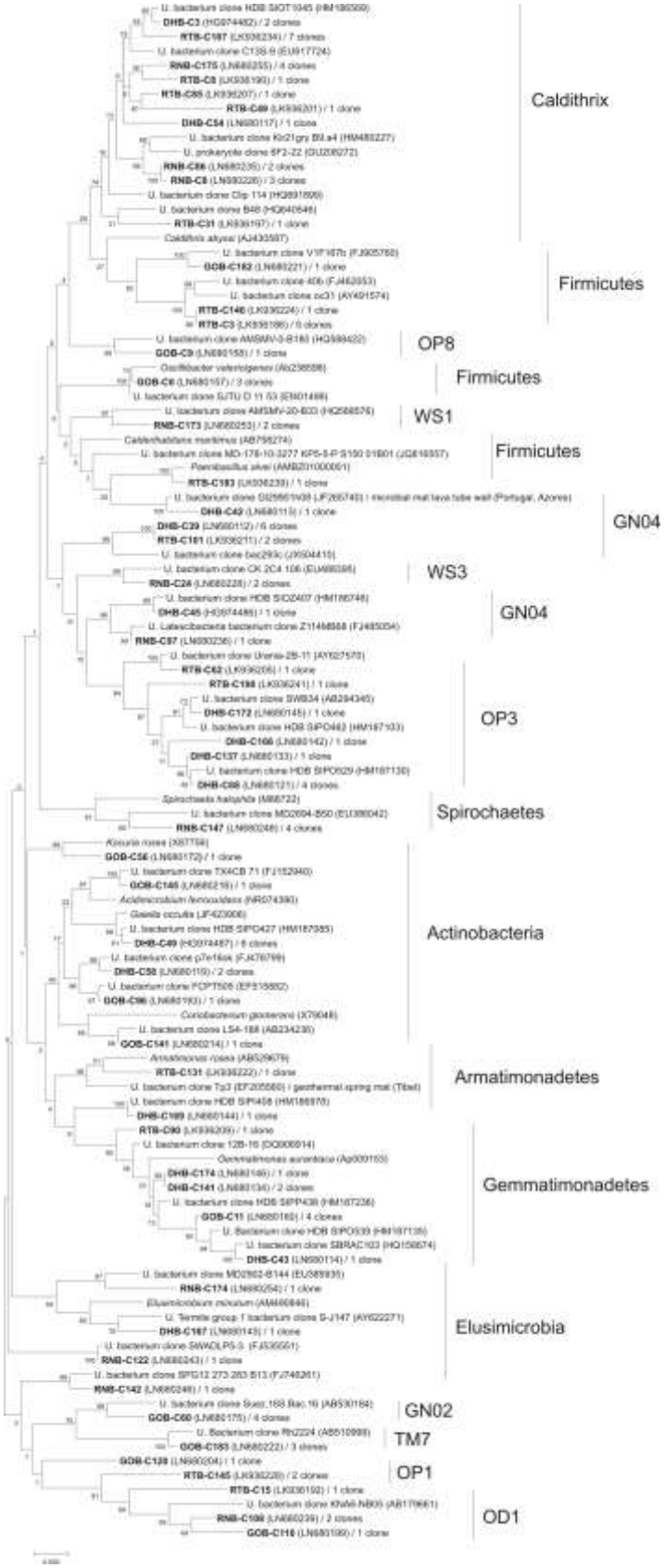
Supplementary Figure 2.



Supplementary Figure 3.



Supplementary Figure 4.



Supplementary Figure 5.