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Bacterial diversity in a microbial mat colonizing a man-made geothermal spring from Romania

Cecilia M. Chiriac[1,2], Lucian Barbu-Tudoran[3], Andreea Baricz[1,2], Edina Szekeres[1,2], Tiberiu Szoke-Nagy[1,2], Nicolae Dragoș[1,2] and Cristian Coman[1,2, ✉]

SUMMARY. Some of the oldest evidence of life on Earth comes from microbialites, or biologically induced carbonate deposits. Modern lithified microbial mats are considered analogues to some of the earliest Archaean ecosystems. This study investigated the bacterial diversity in a microbial mat developed on the surface of a hot spring carbonate deposit from Romania. A clone library was constructed and more than 200 partial 16S rRNA gene sequences were obtained. Phylogenetic analysis showed the existence of nine major groups. Gammaproteobacteria, Cyanobacteria and Betaproteobacteria were dominant, comprising 75% of the clone library. Verrucomicrobia, some Cyanobacteria (*Phormidium*, *Oscillatoria* and *Leptolyngbya*), Chloroflexi, Firmicutes and Deltaproteobacteria taxa observed in the investigated mat are common inhabitants of this type of environments. *Arthrospira platensis* and *Desertifilum tharense* (Cyanobacteria) were described for the first time in association with a geothermal habitat. Also, the representatives of Gammaproteobacteria, Betaproteobacteria, Bacteroidetes and Chrysiogenetes identified in the mat have not been described in geothermal habitats, but are known to prevail in saline, neutral to alkaline environments.

Keywords: bacterial diversity, community structure, cyanobacterial mat, hot springs.

[1] *Taxonomy and Ecology, Algology, National Institute of Research and Development for Biological Sciences, Institute of Biological Research, Cluj-Napoca, Romania*

[2] *Molecular Biology and Biotechnology Department, Faculty of Biology and Geology, Babeș-Bolyai University, Cluj-Napoca, Romania*

[3] *Electron Microscopy Center, Faculty of Biology and Geology, Babeș -Bolyai University, Cluj-Napoca, Romania*

✉ ***Corresponding author: Cristian Coman**, Institute of Biological Research, 48 Republicii Street, 400015 Cluj-Napoca, Romania, Email: cristian.coman@icbcluj.ro*

Introduction

Modern microbial mats, especially those dominated by cyanobacteria, are often considered analogues to some of the earliest communities on Earth (Wacey, 2009). They have been present on our planet for at least 3 billion years and are examples of self-sustainability (Noffke *et al.*, 2008, Allwood *et al.*, 2009). They had a substantial impact on the evolution of life forms as we see them today, especially due to oxygenic photosynthesis of cyanobacteria (Kasting and Howard, 2006). One of the features of microbial mats is their laminary structure in which certain groups of microorganisms are distributed in different layers. In rare cases, mineral precipitation (mainly calcite) can be observed, the process leading, in time, to the formation of stratified rocks (microbialites), thus trapping the microorganisms between layers. These microbialites are considered modern analogues of ancient stromatolites and are of great importance for studies regarding the evolution of life on Earth.

In natural aquatic settings, microorganisms form benthic biofilms that may develop into thick microbial mats (Pagaling *et al.*, 2012). Initially, the biofilm is composed of cells appertaining to few microbial groups and their extracellular polymeric substances (EPS). Over time, the biofilm becomes highly diverse with more and more microorganisms migrating into the consortium. Mature microbial mats include photosynthetic microorganisms (e.g., cyanobacteria, diatoms) and a wealth of chemoorganotrophic and chemolithotrophic bacteria (Konhauser, 2007).

Cyanobacterial communities of coccoidal and/or filamentous groups constitute major microbial mat builders (Chacon, 2010). Hot spring cyanobacterial mats are excellent model systems for biodiversity studies and are intensively surveyed worldwide (Dupraz and Visscher, 2005; Couradeau *et al.*, 2011).

As contemporary microbial mats are believed to hold the key to the past and to provide insight into the role of microbes in mineral precipitation, this paper focuses on the description of the microstructure and the bacterial diversity of a non-mineralised microbial mat that colonizes the surface of a hot spring carbonate deposit near the village of Ciocaia (Bihor County, Romania). A culture-independent approach was undertaken, as it was proven successful in other similar studies (Huang *et al.*, 2011; Pagaling *et al.*, 2012). Currently, there are very few studies on the microbial diversity in the thermophilic mats from the Western Plain of Romania where sedimentary structures were observed (Coman *et al.*, 2011; 2012). Therefore, this study further increases our knowledge of microbial diversity in this geothermal region.

Materials and methods

Sampling

Microbial mat samples were obtained from Ciocaia village (Bihor County, Romania) (470 19’ 97” N; 220 03’ 09” E). The samples were collected from the

blue-green layer directly in contact with the surface of the geothermal water flow from the drilling situated in the vicinity of the village and immediately frozen in liquid nitrogen. One sample was used for SEM (Scanning Electron Microscopy) and another for DNA extraction and clone library construction.

Optical and electronic microscopy

Microbialites lamellar structure was investigated by optical microscopy performed using a Nikon TE-2000 apparatus with a Nikon D90 digital camera. For electronic microscopy the samples were fractured in liquid nitrogen, fixed on copper holders, covered with a 10 nm gold layer and observed with a Jeol JSM 5510LV electron microscope.

DNA extraction

DNA was purified from fresh samples using the ZR Soil Microbe DNA Kit (ZymoResearch, Orange, CA, USA) according to the manufacturer's instructions. Briefly, samples were added to lysis tubes and the microbes were rapidly lysed. After centrifugation, the supernatant was transferred to a spin column and the DNA was washed twice for contaminants removal. The DNA was eluted in 35µL DNase/RNase-Free Water.

16S rRNA gene clone library construction, sequencing and phylogenetic analysis

Bacterial 16S rRNA gene fragments were amplified using universal primers 27F-1492R (Lane, 1991). The PCR mix consisted of: 1X DreamTaq Buffer (Fermentas, Vilnius, Lithuania), 1µM dNTP mix (Fermentas, Vilnius, Lithuania), 0.25 µM of each primer (synthesized at Eurogentec - Liège, Belgium), 1.5 units of DreamTaq polymerase (Fermentas, Vilnius, Lithuania), and 50 ng of DNA in a final volume of 50 µl. The PCR program included 1 cycle of initial denaturation at 94°C for 3 min, followed by 30 cycles with a denaturing step of 45 sec at 94°C, an annealing step of 1 min at 53°C, and an elongation step of 2 min at 72°C. The final elongation was performed for 10 min at 72°C.

The PCR products were purified using the GeneJET Gel Extraction Kit (Fermentas, Vilnius, Lithuania) and a clone library (C3b) was constructed using the InsTAclone PCR cloning kit (Fermentas, Vilnius, Lithuania). The clones were partially sequenced at Macrogen (The Netherlands) using the M13F-pUC primer. The resulting 16S rRNA gene sequences, containing the V1-V4 variable regions, were tested for chimeras using Bellerophon (Huber *et al.*, 2004) and compared to sequences stored in the GenBank nucleotide database using the blastn algorithm (Altschul *et al.*, 1990, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Operational Taxonomic Units (OTUs) were designated based on the highest scores after Basic Local Alignment Search Tool (BLAST) interrogation, with a threshold for species delimitation (where possible) of 97% or higher identity between the 16S rRNA gene sequences.

A multiple alignment was performed using ClustalX algorithm in MEGA5.1 (Tamura *et al.*, 2011). JModelTest software (Guindon and Gascuel, 2003; Posada et al. 2003) was used to select an appropriate model of sequence evolution for phylogenetic inference. Generalized Time Reversible with gamma distribution (GTR+G) was found to be the best fit model that can be applied to our 16S rRNA gene sequences. A Maximum Likelihood tree was constructed with MEGA version 5. The bootstrap analysis included 500 replicates. The 16S rRNA gene sequence from *Methanosaeta thermophila* was used as outgroup.

Accession numbers of nucleotide sequences

Partial 16S rRNA gene sequences obtained from bacterial clones used for phylogenetic analyses as described above, have been deposited in GenBank under accession numbers JX575076- JX575101.

Results

Study site and material

The Ciocaia drilling site dates from 1970 and the well is part of the Lower Pontian thermal aquifer from Săcuieni. Generally, the thermal aquifer from Săcuieni is located at depths between 1250 and 1700 m. The surface water temperature varies between 50 and 85°C (Antics and Roșca, 2003). In situ, the measured temperature was between 55°C-60°C, and the pH was ~7.5.

Because the chemical characterization of the thermal water from Ciocaia was performed by Țenu *et al.* (1981) and, in time, its composition was proven relatively constant with only minor fluctuations (Romanian Waters Administration, personal communication), repeating the chemical analysis would be redundant. The chemical composition of the geothermal water is as follows (in $mg \cdot L^{-1}$): Cl^- - 812; HCO_3^- - 7,283; SO_4^{2-} - 27.9; NH_4^+ - 7.9; Na^+ - 3,525; K^+ - 30; Ca^{2+} - 790; Mg^{2+} - 2.6; Fe^{2+} - 0.2; total mineralization: 12,106; TDS - 1,600. A particularity of the geothermal water from Ciocaia is the increased HCO_3^- concentration, which is 3 to 4 times higher than in other similar springs from this area (Coman *et al.*, 2011; 2012).

The mat presented the typical three-layered structure, observation based on the ratio between filamentous and coccoid bacteria (Fig. 1): i) an upper layer of net-like arranged cyanobacterial filaments; ii) a middle layer of filaments and coccoid cells of possibly aerobic bacteria; iii) a third layer, with very few filaments and an increased number of round-shaped, probably anaerobic taxa. The mat structure resembles that of microbial mats described by Ward *et al.*, (1998) and Pagaling *et al.*, (2012).

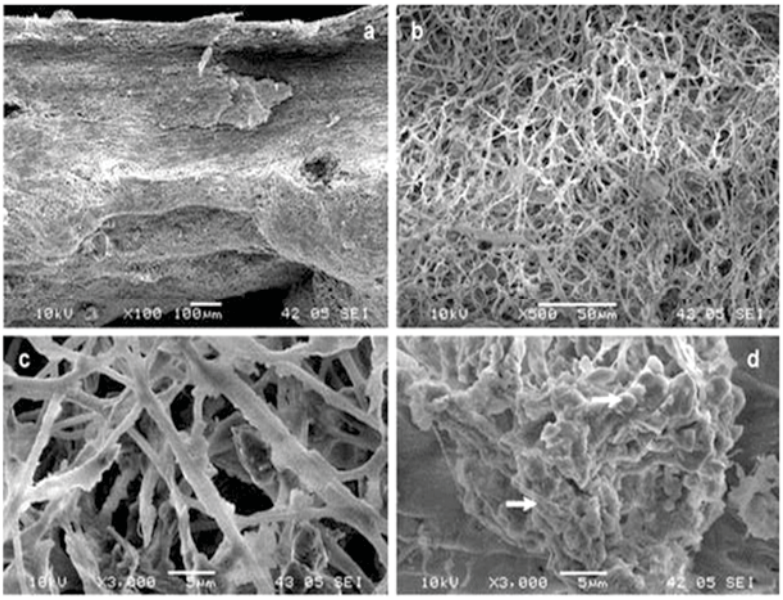

Figure 1. The structure of the Ciocaia microbial mat as observed by SEM. (a) cross-section showing the entire structure; (b-c) middle layer consisting in a tight net of bacterial filaments; (d) inner layer, with an increased number of bacteria (most-likely anaerobic) attached to the substrate.

Bacterial diversity

In order to assess the bacterial diversity in the Ciocaia mat, a 16S rRNA gene clone library (C3b) was constructed and a total of 200 partial sequences (~1000 bp) were obtained. After removing duplicates, the unique sequences were used for OTU identification using the BLAST feature in NCBI (Table 1). Both the rarefaction curve (Fig. 2), and the Chao1 index (26.0) (Yanan *et al*., 2006) showed that clones sampling was performed to saturation.

Bacterial diversity included nine major groups (Fig. 3A) of which Gammaproteobacteria, Betaproteobacteria, Cyanobacteria, Verrucomicrobia and Bacteroidetes accounted for 87% of the total clones in the 16S rRNA gene clone library. We compared OTUs from Ciocaia with OTUs from other microbial mats described in the literature and with nucleotide sequences stored in GenBank (NCBI).

Gammaproteobacteria is the dominant class in the C3b library, totalizing 41% of the sequences obtained (Fig. 3A). The three genera identified were *Ectothiorhodospira*, *Nitrincola* and *Aquimonas* (Table 1, Fig. 4) As far as we are aware, the last two heterotrophic genera were never encountered in association with carbonate deposits or with a geothermal habitat.

Table 1.

Bacterial OTUs detected in C3b library. The closest GenBank matches with accession numbers, percentage of identity and percentage of abundance in the clone library for each OTU are given.

	Phylum/ Class	Closest GenBank match	Accession no.	Ident. %	Abundance%
C3b-G5	Betaproteobacteria	*Azoarcus* sp. CR23	AF011328.1	96	2
C3b-A2	Betaproteobacteria	*Azoarcus* sp. KH32C	AP012304.1	98	2
C3b-B7	Betaproteobacteria	Rhodocyclaceae bact. 5BCVA	DQ343837.1	99	2
C3b-A4	Betaproteobacteria	Beta proteobacterium 2B2	HM587245.1	97	8
C3b-C5	Gammaproteobacteria	Uncult. gamma proteobact. ST5-34	DQ501349.1	98	1
C3b-D2	Gammaproteobacteria	*Ectothiorhodospira* sp. AM4	EU252492.1	99	4
C3b-A1	Gammaproteobacteria	*Nitrincola* sp. E-048	FJ764762.1	99	30
C3b-A3	Gammaproteobacteria	Uncult. *Aquimonas* sp. clone 26	JQ183097.1	99	2
C3b-G8	Gammaproteobacteria	*Ectothiorhodospira shaposhnikovii* strain DSM2111	FR733667.1	99	2
C3b-B11	Deltaproteobacteria	*Desulfovibrio alkalitolerans* strain RT2	NR_043069	99	1
C3b-V2	Cyanobacteria	*Arthrospira platensis* Sp-11	DQ279771.1	99	3
C3b-V1	Cyanobacteria	*Oscillatoria earlei* strain NTAP016	DQ308545.1	96	2
C3b-V4	Cyanobacteria	*Phormidium* sp. 195-A12	EU282429.1	98	5
C3b-B2	Cyanobacteria	*Desertifilum tharense* PD2001/TDC4	FJ158994.1	99	3
C3b-V3	Cyanobacteria	*Leptolyngbya* sp. LEGE 07319	HM217045.1	99	9
C3b-B3	Chloroflexi	Uncult. *Hydrogenophaga* sp. clone XJ64	EF648133.1	89	1
C3b-B4	Chloroflexi	Uncult. Chloroflexi bacterium clone TDNP_Bbc97_242_1_63	FJ516783.1	86	2
C3b-E8	Bacteroidetes	*Flexibacter ruber* IFO 16675	AB078064.1	99	1
C3b-F11	Bacteroidetes	Uncult. *Sphingobacteria* bacterium clone A831	EU283540.1	94	1
C3b-D12	Bacteroidetes	*Belliella pelovolcani* strain CC-SAL-25	EU685336.1	89	1
C3b-C1	Bacteroidetes	Uncult. bact. clone ambient_alkaline-120	GU455103.1	99	7
C3b-H2	Bacteroidetes	Uncult. bacterium clone Pb40	HQ857681.1	96	2
C3b-D7	Verrucomicrobia	Uncult. Verrucomicrobiales clone	FJ516831.1	92	1
C3b-F2	Verrucomicrobia	Uncult. *Verrucomicrobium* sp. clone 78 T12d+oil	FM242437.1	91	5
C3b-E2	Chrysiogenetes	*Desulfurispirillum indicum S5*	NR_074463	99	2
C3b-F4	Firmicutes	Uncult. Firmicutes bacterium clone x1	GQ848202.1	97	1

Interestingly, the C3b_C5 clone, showing a high identity score to an unidentified gammaproteobacterium (Table 1), clustered together in the phylogenetic tree with *Alishewanella jeotgali*, with a bootstrap value of 100 (Table 1; Fig. 4) and until now was not encountered in a thermophilic microbial mat.

In the C3b library, Cyanobacteria, representing 18% of the total clones sequenced (Fig. 3A), comprises five OTUs identified either at species level (*Arthrospira platensis* and *Desertifilum tharense*) or at the genus level (*Oscillatoria* sp., *Phormidium* sp. and *Leptolyngbya* sp.) (Table 1; Fig. 4). Species of *Oscillatoria*, *Phormidium* and *Leptolyngbya* genera are common inhabitants of thermophilic microbial mats and present a worldwide distribution (Bryanskaya *et al.*, 2006; Sompong *et al.*, 2008).

The class Betaproteobacteria encompasses purple nonsulfur bacteria with high metabolic versatility. It represents 16% of the C3b library (Fig. 3A), with clones affiliated to *Azoarcus* sp. and to an unidentified *Rhodocyclaceae* bacterium (Table 1; Fig. 4).

Verrucomicrobia covers 6% of the Ciocaia clone library (Fig. 3A), one OTU being identified at the genus level (*Verrucomicrobium* sp.) (Table 1; Fig. 4). It is a widespread phylum, inhabiting a wide range of habitats (Kanokratana *et al.*, 2004; Bohorquez *et al.*, 2012).

The Bacteroidetes group comprises 6% of the C3b clone library, several 16S rRNA gene sequences presenting a high degree of identity with *Flexibacter* sp., *Belliella* sp. and some uncultured taxa (Table 1; Fig. 4). *Flexibacter* sp. was weakly represented at Ciocaia thermo-mineral spring, with clones that were affiliated to *Flexibacter ruber* (99% sequence identity), described in a hot spring in Yellowstone National Park (USA).

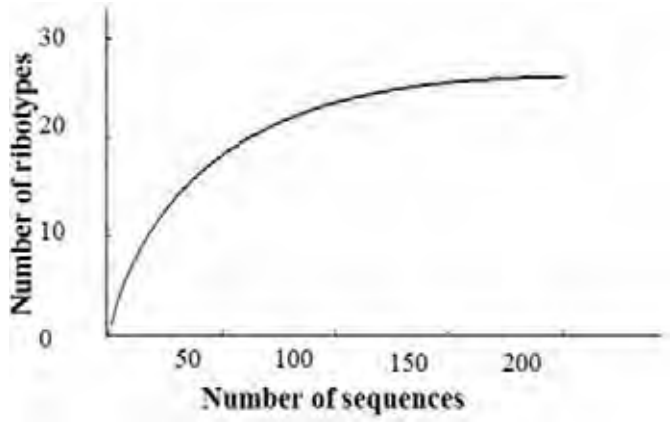

Figure 2. Rarefaction curve for the C2b library. The number of detected OTUs was plotted against the cumulative number of individuals (i.e., clones) analysed.

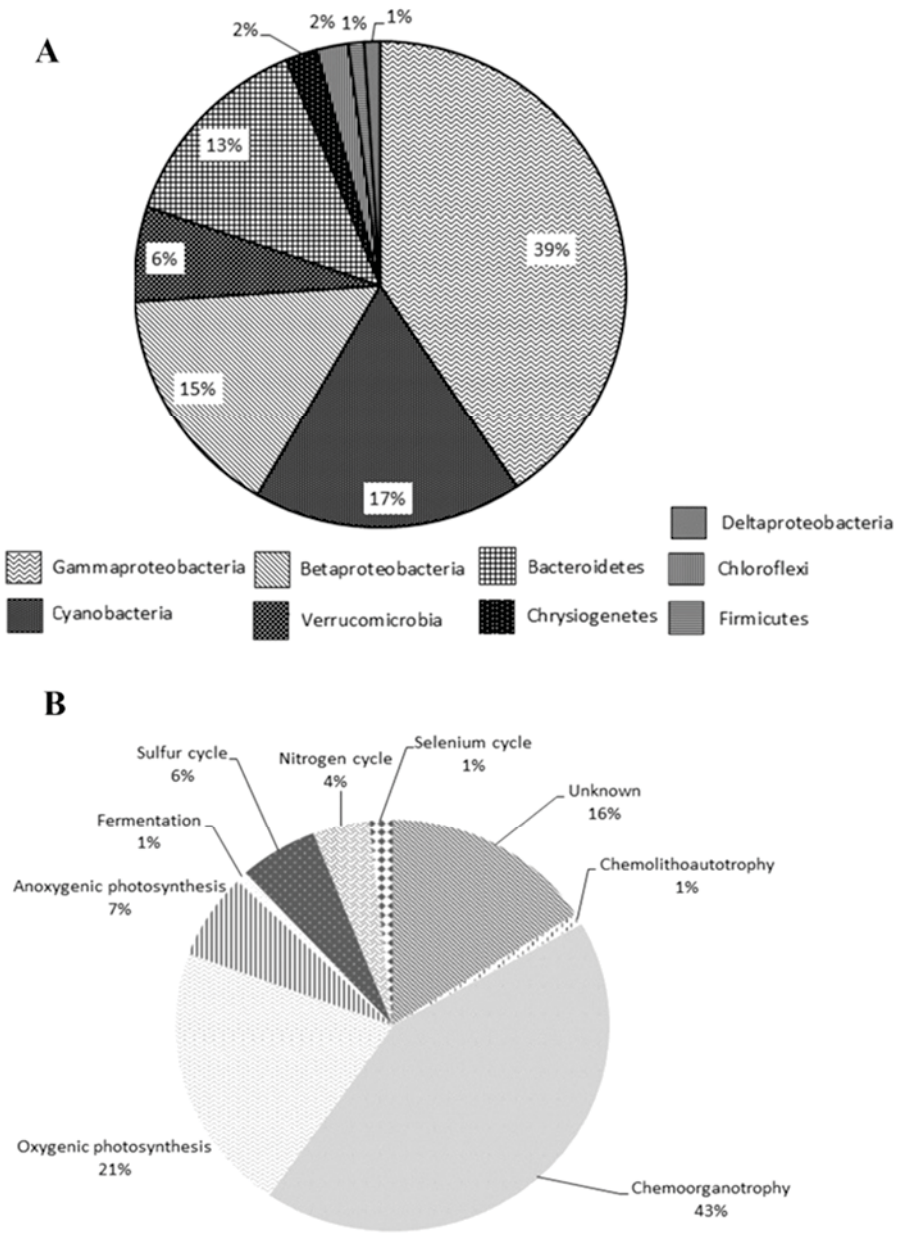

Figure 3. (A) Distribution of major groups in the Ciocaia bacterial clone library; (B) Putative functional groups encountered in the Ciocaia sample.

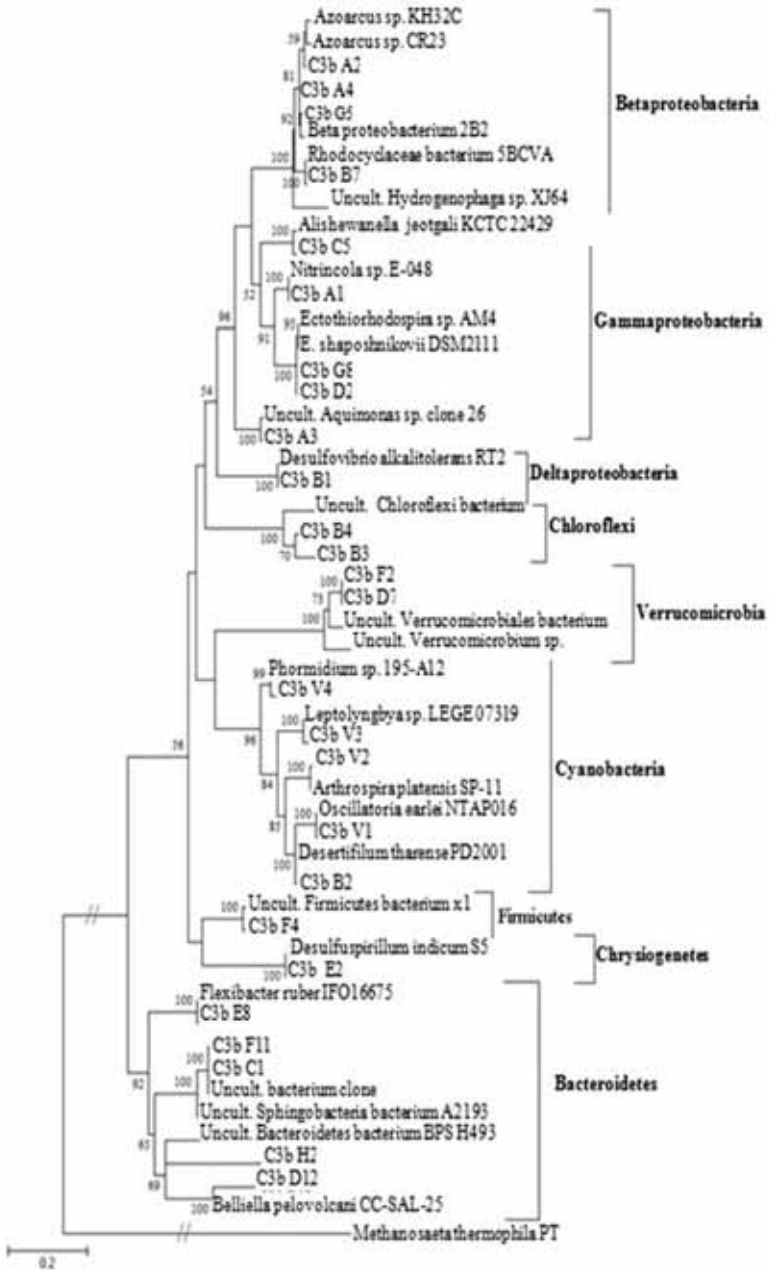

Figure 4. Maximum-likelihood tree (MEGA 5) showing the phylogenetic relationships of bacterial 16S rRNA gene sequences cloned from the microbial mat colonizing the geothermal spring near Ciocaia. Bootstrap values <50% are not shown.

Members of the *Belliella* genus are known to have a preference for carbonate-rich waters (Akhwale *et al.*, 2015). Also, they were previously described as being in close association with *Nitrincola* sp. (Hamamura *et al.*, 2012), one of the most abundant proteobacterial taxon in our sample.

Around 11% of the C3b bacterial clone sequences were affiliated with minor groups, such as Chrysiogenetes, Chloroflexi, Firmicutes, Deltaproteobacteria and some unidentified bacteria (Fig. 3A and 4). The majority of these sequences are closely related to thermophilic OTUs isolated from thermal, alkaline environments (Kanokratana *et al.*, 2004; Bryanskaya *et al.*, 2006; Rauschenbach *et al.*, 2011).

Discussion

Putative functional role of bacterial OTUs within the Ciocaia microbial mat

Phylogenetic analysis performed in this study allowed classification of the majority of the community members at the species/genus level. Thus, a diverse range of putative metabolic pathways can be identified within the mat. Despite the fact that 16S rRNA gene-based analysis is not always completely correlated with similarity of metabolic pathways and caution in attributing functional roles within the microbial community is necessary, a possible scenario regarding the ecological interactions among the observed bacterial groups can be presented (Fig. 3B).

As in many other microbial mats belonging to different environments, cyanobacteria are a dominant group, being the primary producers and the major fraction involved in N_2 fixation (e.g. *Leptolyngbya*) along with *Azoarcus* (Reinhold-Hurek *et al.*, 1993; Charpy *et al.*, 2010). Because the organisms included in this phylum are light dependent, they are distributed at the surface of the carbonate deposit. On account of the death of the primary producers, a compact biomass is accumulated that supports the development of aerobic heterotrophs (e.g. *Belliella, Aquimonas, Verrucomicrobium, Nitrincola*) in the upper layers and anaerobic species in the lower layers (e.g. *Desulfovibrio, Desulfurispirillum*). It is known that Cyanobacteria are a key component of microbial mats and that they are responsible for early lithification of stromatolites, thus linking the studied microbial mat with the formation of the carbonate deposits (Konhauser, 2007).

Specific metabolic bacterial groups have vertical distribution based on the concentration of various gas-phase nutrients such as H_2S, O_2 or CO_2. They tend to be limited, and so, their consumption/production rates may dictate the distribution of various metabolic groups. Usually, under the cyanobacterial layer are found other groups that have photosynthetic or phototrophic nutrition, usually purple sulfur bacteria (e.g., *Ectothiorhodospiraceae*) and green non-sulfur bacteria (e.g., Chloroflexi). Although Cyanobacteria are the most commonly detected microbial group in thermophilic mats, they have been reported to dominate these mats at the functional level together with phototrophic Chloroflexi (Boomer *et al.*, 2000; Portillo *et al.*, 2009). The oxygenic photosynthesis of Cyanobacteria is considered to depend on the sulfide depletion by the anoxygenic *Chloroflexus* sp. (Jørgensen and Nelson, 1988).

Ectothiorhodospira, a genus of purple-sulfur bacteria, are able to perform photosynthesis under anoxic conditions, without O_2 production (Mobberley *et al.*, 2012). Thus, their upper border in the microbial mat is determined by the limit of H_2S diffusion and the lower limit by the light penetration into the mat. Chloroflexi phylum contains genera that are able to perform anoxygenic photosynthesis, but some species were found to exhibits metabolic diversity, growing either as aerobic chemoheterotrophs or as anaerobic photoheterotrophs. Using electrons extracted from H_2 or H_2S, they can fixate CO_2 through the 3-hydroxypropionate pathway instead of Calvin cycle (Konhauser, 2007; Bolhuis and Stal, 2011).

The organic matter synthesized by photosynthetic and chemolithoautotrophic (e.g. *Hydrogenophaga*) microorganisms can be used by various organotrophic (heterotrophic) bacteria in the Ciocaia mat: *Nitrincola* sp., *Aquimonas* sp., *Flexibacter* sp., *Belliella* sp., *Desulfovibrio alkalitolerans*, probably the Firmicutes taxa and *Azoarcus* sp (Reinhold-Hurek *et al.*, 1993; Brettar *et al.*, 2004; Saha *et al.*, 2005). Some heteroorganotrophs, such as *Desulfovibrio alkalitolerans*, are using sulfate, sulfite and thiosulfate as electron acceptors. The presence of autotrophic sulfur-oxidizing bacteria together with heterotrophic sulfate-reducing microbes may imply the coupling of carbon and sulfur cycles (Antony *et al.*, 2013).

An unexpected discovery was the presence of *Desulfurispirillum indicum* in our sample. These organisms are able to utilize selenite, nitrate and arsenate as electron acceptors. It was previously identified in an estuarine canal in Chepau, India (Rauschenbach *et al.*, 2011), but this is the first report of *Desulfurispirillum indicum* in thermal environments or in association with carbonate deposits. The ability of this species to reduce nitrate to ammonium illustrates that nitrogen and carbon cycles could be inter-connected in this microbialite (Antony *et al.*, 2013).

Overall, the Ciocaia mat harbors microbial taxa with diverse types of metabolism. As a possible consequence of the temperature and pH values in the Ciocaia spring, there was no absolute dominance by a single bacterial group. We can assume that there is an ecologically balanced community, with well-defined metabolic niches.

High-temperature and carbonate specific OTUs from Ciocaia sample

Even though the phylogenetic analysis revealed a low diversity of bacterial 16S rRNA gene sequences in the C3b clone library, this situation resembles that of other hot spring mats described in literature (Huang *et al.*, 2011; Pagaling *et al.*, 2012). Certain clone sequences were related to others of low-temperature origin, like soil or marine environments (Fig. 5A), but this situation was also observed in other hot spring biodiversity studies (Song *et al.*, 2009; Huang *et al.*, 2011). Besides contamination with DNA from the surrounding environment, another possible explanation could be that microorganisms with a significant level of 16S rRNA gene sequence similarity may have distinct physiological properties (Jaspers and Overmann, 2004), especially when other features except the temperature (e.g., alkalinity, salinity) create an environment suitable for colonization.

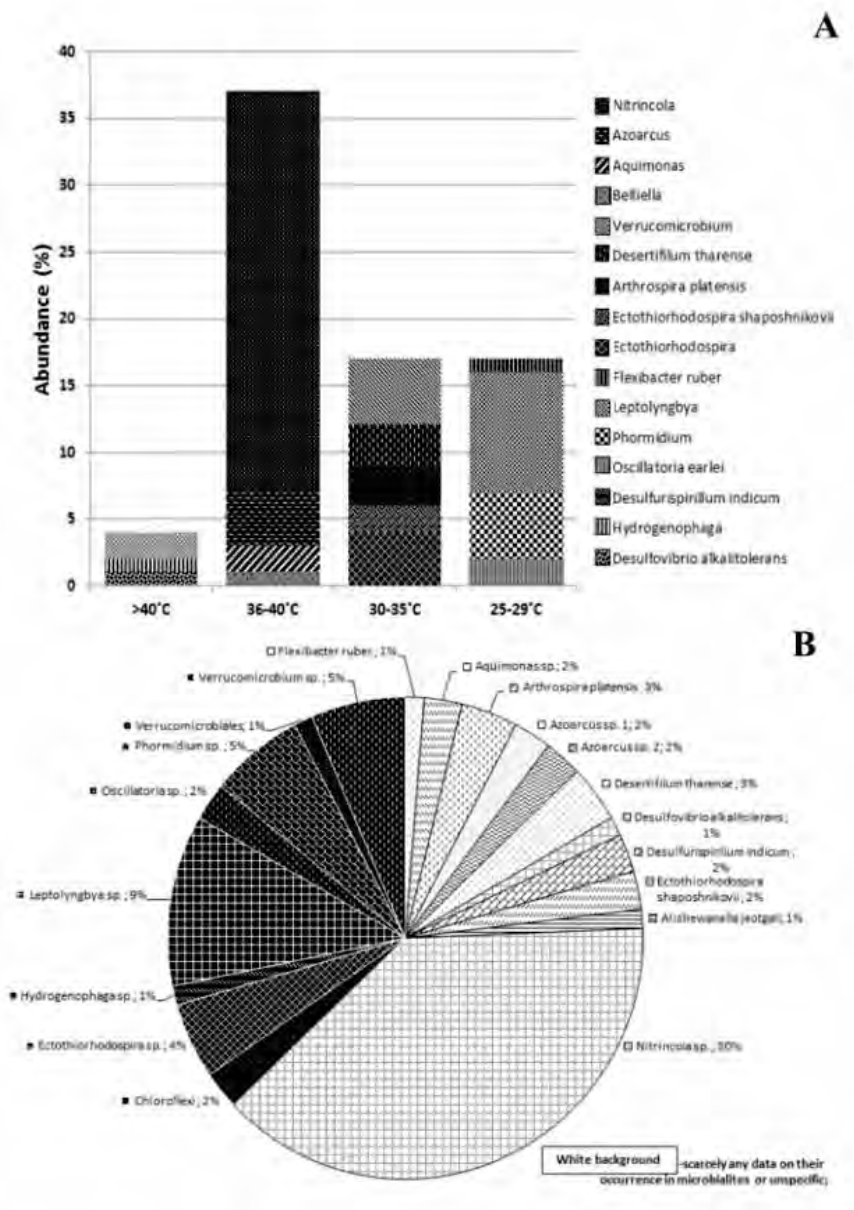

Figure 5. (A) OTUs abundances in relation with their optimum growth temperature. The majority of species have a lower optimum growth temperature than the water temperature which varies between 50 and 85°C; (B) Carbonate specific (black background) and carbonate non-specific (white background) bacterial taxa encountered in Ciocaia microbialite.

The dominant group in the C3b clone library is represented by the gammaproteobacterial OTUs (e.g. *Nitrincola*), a group that was not previously described in literature as being associated with geothermal environments. Nevertheless they have a tendency to colonize saline, alkaline environments (Mwirichia *et al.,* 2011; Hamamura *et al.,* 2012; Antony *et al.,* 2013). Organisms from *Aquimonas* genus were previously isolated from a warm water spring. Because the optimum physico-chemical parameters for the type species, *A. voraii*, are very similar to those in the Ciocaia spring, *Aquimonas* presence in our sample is not surprising (Saha *et al.,* 2005). This genus was found before in a microbial mat from Movile cave, Romania (Chen *et al.,* 2009), but was never encountered in association with carbonate deposits. In the microbial mat from the Ciocaia hot spring habitat, we also encountered the cyanobacteria *Arthrospira platensis.* Even though the harsh conditions that prevail in this environment may cause difficulties for colonization, the increased HCO_3^- concentration in the Ciocaia thermal water may favor the spread of *Arthrospira platensis*, its affinity to high bicarbonate levels and moderately termophilic waters being previously documented (Whitton and Potts, 2000; Fujisawa *et al.,* 2010).

Our study reports for the first time the presence of *Desertifilum tharense* in a hot spring microbial mat. This taxon is a desert cyanobacterium described by Dadheech *et al.* (2012) from an arid area in India. *Microcoleus steenstrupii*, observed in the hot spring mat from Marghita, Romania (Coman *et al.,* 2011), was initially considered a desert cyanobacterium (Garcia-Pichel, 2002), but it was later encountered in other hot spring mats (Boyer *et al.,* 2002; Coman *et al.,* 2011). The fact that 16S rRNA gene sequences belonging to these two cyanobacteria were observed in the C3b clone library does not necessarily imply an active role within the bacterial community. Thus, future culture-dependent studies should be undertaken in order to confirm their functionality in the Ciocaia microbial mat.

As it can be observed in Fig. 5B, the majority of hot spring OTUs could be assigned to carbonate specific or non-specific groups, based on literature data. In contrast to other microbialite communities, the unique character of Ciocaia community is given by the fact that approximately two-thirds of the bacterial community is included in OTUs that were never described in association with carbonate-impregnated structures. Nevertheless, these OTUs are known to have a preference for an alkaline pH that may favour precipitation of carbonate especially in waters with increased HCO_3^- concentration (Saha *et al.,* 2005; Antony *et al.,* 2013).

An alkaline microenvironment can be achieved within the mat most likely through the metabolic activity of different bacterial groups. In mesothermal environments, rich in HCO_3^-, the external pH can increase significantly, as inorganic carbon is consumed by cyanobacteria faster than it can be replaced from the geothermal water (Badger *et al.,* 2006). Sulfate-reducing bacteria (SRB), observed in the C3b clone library in both Deltaproteobacteria and Firmicutes groups, can take part in increasing the environment's alkalinity by generating

carbonate ions through sulfate reduction. Part of the H_2S consumed by anoxygenic photosynthesis may come from the activity of SRB as well. Heterotrophic types, observed in almost all bacterial groups from Ciocaia (e.g., Betaproteobacteria, Bacteroidetes, Chrysiogenetes, Verrucomicrobia), can also increase the environment pH towards alkalinity by decomposing organic residues (Baumgartner *et al.*, 2006; Konhauser, 2007).

The results of this study gave rise to the hypothesis that, in some cases, when a proper ecological niche is created, bacterial diversity can be influenced by abiotic factors (e.g., pH, temperature). Future studies, mainly culture-dependent, should be undertaken, in order to confirm this observation.

Conclusions

The discovery of new modern stromatolites and the characterization of their microbial diversity are very important in order to understand the microbe-mineral relationship in the formation of sedimentary structures. This study was focused on the structure and bacterial diversity investigation of a microbial mat that formed above a man-made geothermal spring from Romania. The mat presented a laminated structure similar to the models described in literature. Twenty-six OTUs were identified, grouped in nine major bacterial groups, Gammaproteobacteria, Cyanobacteria and Betaproteobacteria being dominant. Verrucomicrobia, some Cyanobacteria (*Phormidium, Oscillatoria* and *Leptolyngbya*), Chloroflexi, Firmicutes and Deltaproteobacteria observed in the Ciocaia mat are common inhabitants of these types of environments. *Atrhrospira platensis, Desertifilum tharense* and *Desulfurispirillum indicum* were reported for the first time in association with a geothermal habitat. The representatives of Gammaproteobacteria, Betaproteobacteria, Bacteroidetes and Chrysiogenetes identified are not typical for geothermal habitats, but are known to colonize saline, neutral to alkaline environments. Further cultivation and physiological studies should be undertaken in order to determine whether they actively inhabit the microbial mat around the geothermal spring from Ciocaia and to assess which is their putative functional role within the community. Overall, this study has provided valuable information about the diversity of microorganisms that inhabit the thermal environments from the Western Plain of Romania. In addition, due to the incipient sedimentary structures observed, the geothermal well from Ciocaia is an important site for future studies regarding the results of microbe-mineral interactions on the formation of modern stromatolites.

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